# SCREENING OF ENDOPHYTIC FUNGI FOR PRODUCTION OF ASPARAGINASE ENZYME

**Dissertation**

Submitted in the partial fulfillment of the requirement for the degree of

**Master of Technology**

in

**Biotechnology**

By

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## Declaration

I hereby declare that the work being presented in the thesis entitled **"Screening of endophytic fungi for the production of asparaginase enzyme"** in the partial fulfillment of requirements for the award of degree of Masters in Technology in Biotechnology, Department of Biotechnology, Thapar University, Patiala is my own laboratory work during the period of July 2013 to June 2014, under the conception and supervision of Dr. Sanjai Saxena, Associate Professor, Department of Biotechnology (DBT), Thapar University, Patiala. I have not submitted the matter embodied in this thesis for the award of any other degree.

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This is to certify that the above statement made by candidate is correct and true to the best of my knowledge.

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## Certificate

This is to certify that the thesis entitled **"Screening of endophytic fungi for the production of asparaginase enzyme"** being submitted by Ms Preetinder Kaur (Roll no. 601204020) in the partial fulfillment of the requirements for the award of degree of Masters of Technology in Biotechnology, Thapar University, Patiala is a bonafide work carried out under the esteemed supervision and conception of Dr. Sanjai Saxena and that no part of this thesis has been submitted for the award of any other degree.

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Preetinder Kaur

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# Abbreviations

| | |
|---|---|
| AA | Asparaginase Agar |
| ALL | Acute Lymphoblastic Leukemia |
| APS | Ammonium persulphate |
| ASN | Asparaginase |
| ATP | Adenosine tri-phosphate |
| AWD | Agar Well Diffusion |
| BSA | Bovine Serum Albumin |
| DTT | Dithiothreitol |
| ECA | *Escherichia coli* Asparaginase |
| FDA | Food and Drug Administration |
| h | hour |
| kDa | kilodalton |
| g | gram |
| L | Litre |
| M | Molar |
| MCD | Modified Czapek Dox |
| min | minute |
| ml | millilitre |
| mm | millimeter |
| MW | Molecular Weight |

| | |
|---|---|
| nm | nanometer |
| O.D. | Optical Density |
| PAGE | Poly-acrylamide Gel Electrophoresis |
| PDA | Potato Dextrose Agar |
| psi | pounds per square inch |
| rpm | revolutions per minute |
| rxn | reaction |
| SDS | Sodium do-decyl Sulphate |
| TEMED | N,N,N',N'-tetramethylethylene |
| µg | microgram |
| µmol | micromole |
| WBC | White Blood Cell |
| V | Volt |

# EXECUTIVE SUMMARY

L-asparaginase (EC 3.5.11. L-asparaginase amidohydrolase) is first enzyme, studied very intensively in human beings with regard to its antitumor potential against tumor of lymphoid precursor, acute lymphoblastic leukemia (ALL). It has profound bearings in medical as well as non-medical fields. The current drugs are suffering from many side effects like immune-suppression, infertility, secondary neoplasm. The immunogenic complications associated with its present microbial sources *Escherichia coli, Erwinia carotovora* limits its medicinal frontier. So there exists a need of switching to novel natural sources to serve as non immunogenic and better production sources of L-asparaginase.

Plants harbor a variety of micro-organisms known as epiphyte, endophytes and pathogenic micro-organisms. Endophytic fungi are reported to be hub of novel bioactive compounds. In the present study, 50 endophytic fungi isolated from medicinally important plants collected from biodiversity hot spots of India are screened for the production of L-asparaginase. The endophytic fungi's potential to produce this enzyme was appraised by screening them on modified Czapek Dox medium (Patil *et al*., 2012) and then secondary screening was carried on with L-asparagine as a sole carbon and nitrogen source and phenol red as pH indicator. Four cultures viz. #5AMSTYEL, #1048AMSTITYEL, #6CCSTITD, #17AMSTYEL selected on the basis of primary and secondary screening were subjected for culture filtrate production which was further tested for L-asparaginase production by agar well diffusion assay. #1048AMSTITYEL and #6CCSTITD exhibited appreciable L-asparaginase activity. #5AMSTYEL showed maximum potential for L-asparaginase production. The crude protein of selected four isolates was quantitatively tested for L-asparaginase activity. #5AMSTYEL holds maximum L-asparaginase activity followed by #1048AMSTITYEL.

Protein estimation was done by Lowry's method (Lowry *et al*., 1951). The selected isolates were then screened for glutaminase contamination. The isolate #5AMSTYEL and #1048AMSTITYEL, isolated from *Aegle marmelos* was not possessing Glutaminase contamination. The crude sample was separated by SDS- PAGE, stained by silver staining showed single band of approximately 60 kDa. Further studies on L-asparaginase purification, characterization, kinetic parameters and anti-tumor assays would untap L-asparaginase anti-tumor potential and open up ways for its large scale production.

# Chapter 1

# Introduction

## 1. INTRODUCTION

L-asparaginase (EC 3.5.11., L-asparaginase aminohyrolase) is an enzyme which catalyzes the conversion of L-asparagine to L-aspartic acid and produces ammonia (a hydrolysis reaction). Asparaginases (ASNs) are naturally expressed enzymes which are produced by microorganisms. This enzyme has been a subject of over 500 publications since a decade as it has clinical and non- clinical applications in the industry. The enzyme is widely distributed and is found in animals, plants as well as microorganisms.

Clinical applications of L-asparaginase: In 1953 Kidd reported the antitumor properties of guninea-pig serum which was subsequently attributed to asparaginase activity (Broome, 1961). Mashburn and Wriston (1964) found that *Escherichia coli* enzyme possessed antitumor activity, which was then found to be *E. coli* asparaginase II, the first L-asparaginase to be used clinically.

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. Complications involved with bacterial preparations are hypersensitivity reactions, toxicity and instability. Moreover, bacterial asparaginases are contaminated with glutaminase, which adds to side effects like hyperglycemia, abnormalities of homeostasis, lipid metabolism and neurological disorders. Table 1 shows the clinically approved forms of L-asparaginases isolated from bacterial sources.

| **Source** | **Marketed by** | **Commercial name** | **Country** |
|---|---|---|---|
| *E.coli* | EUSA Pharma | Kidrolase | United Kingdom |
| | Ovation pharmaceuticals Deerfield, Illinois | Elspar | United States |
| | Bayer AG, Leverkusen | Crasnitin | Germany |
| | Sanofi-Aventis Paris | Leunase | France |
| PEGylated form of Native *E.coli* | Kyowa Hakka, Tokyo Sigma-Tau Pharmaceuticals Inc., Gaitherburg | Oncaspar | Maryland |
| *Erwinia* | EUSA Pharma, Oxford | Erwinase | United Kingdom |

Table 1 Available Clinical forms of L-asparaginase (Kumar and Sobha, 2012)

L-asparaginase extraction from the mammalian cells is difficult and very expensive. So, microorganisms are better alternative, as they are efficient producers. *E.coli* and other gram negative bacteria like *Achromobacteriaceae* and *Vibrio succinogenes* are among the efficient producers, but L-ASN from gram positive bacteria received little attention. In case of *Saccharomyces cerevisiae*, asparaginase II is a periplasmic enzyme. Actinomycetes belonging to the genus *Streptomyces* are able to produce appreciable amount of ASN (Mostafa and Salman, 1979). Asparaginase isolated from purified marine *Chlamydomonas* sp. (Paul, 1982), which was specific for L-asparagine was the first enzyme to be purified from a microalgae. L-ASN of algal origin, possessed limited antitumor activity, when tested in vivo. The problems with existing bacterial preparations demand for switching to better sources.

Endophytic fungi came into picture recently. Fungal endophytes are group of diverse, polyphyletic microorganisms which are an integral part of the plant micro biome, that internally infect and reside within plants without causing any noticeable infections and live in mutualistic association with plants for part of their life (Schulz *et al*., 2002; Kusari *et al*.,2012). In the evolutionary time scale endophytes are thought to be pathogens or parasites with the extended periods of latency and reduced virulence (Saikkonam *et al*., 2004).

The rationale behind studying endophytic fungi is due to the fact that they are potential source of unexplored novel drugs. Fungal endophytes have been recognized as repository of novel secondary metabolites (Strobel *et al*., 2003). *Aspergillus, Penicillium and Fusarium* spp. have been reported to produce L-asparaginase. (Patil *et al*., 2012). Asparaginase derived from *Mucor* sp. and *Aspergillius terreus,* isolated from decomposing vegetable substrate (Ali, 1994) was reported to be non toxic and possessed myelo-suppressive and immunosuppressive activity.

L-ASN derived from endophytic fungi have many benefits over existing L-ASN preparations. The first and most important benefit is fungal L-asparaginases are non-immunogenic as they are phylogenetically related and post-translational modifications are present in their system, being the eukaryotic microbes and residing inside the plant body so the stability issues are also tackled. Moreover, the L-ASN from fungal sources is not always accompanied with glutaminase activity as present in L-ASN derived from bacterial sources. The presence of extracellular L-ASN in fungi also paved the ways into depth investigations of this enzyme among the various genera of fungi (Nagarethinam *et al*., 2012). There are only a 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 of L-asparaginase and also the basis of the current study.

# Chapter 2

# Review of Literature

### 2.1. L-Asparagine

Asparagine is a non-essential, neutral amino acid, which is genetically coded and is synthesized from Aspartate by amidation in the presence of asparagine synthetase. It is involved in metabolic control of cell functions in nerve and brain tissue. Being a food additive it is allowed for direct consumption by humans. In asparagine catabolism, asparaginases play an important role.

### 2.2. Role of L-asparagine in normal and tumor cells

L-asparagine is the non-essential, uncharged polar amino acid in the normal human cells but for tumor cells it is essential amino acid required for protein synthesis. The biosynthetic pathway of L-asparagine involves oxaloacetate conversions by the enzyme transaminase to aspartate and then transfer of amino group from glutamate to oxaloacetate producing alpha- ketoglutarate and aspartate.

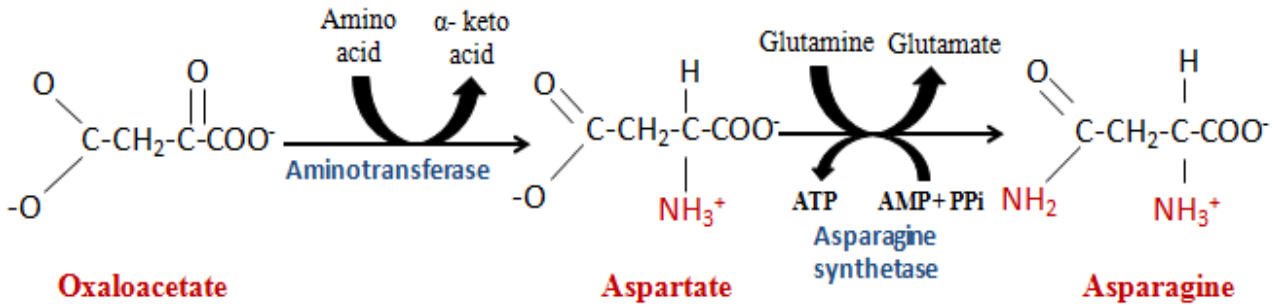



Figure 1 Showing biosynthetic pathway of L-Asparagine

In healthy cells, the enzyme asparatate synthetase is located on the chromosome no. 7q 21.3 (Andrulis *et al*., 1990), responsible for the conversion of aspartate to asparagine using ATP. Whereas tumor cells entirely depend on serum levels of L-asparagine for their proliferation as asparagine synthetase is absent in the genome of tumor cells. L-asparaginase selectively depletes the serum asparagine, which devoid the tumor cells of their only source and leads to their arrest in G1 phase which ultimately ends up with their apoptosis (Kumar and Sobha, 2012; Offman *et al*., 2013)

### 2.3 L-Asparaginase as an anti-tumor agent

History of L-asparaginase dates back to 1922 when Clementi reported the presence of L-ASN in

the blood of guinea pig. Kidd, in 1953 reported that it inhibits the growth of lymphosarcoma in mice. Neuman and McCoy showed the dependency of cell line derived from Walker carcinosarcoma on L-asparaginase. The evidence of antitumor activity of this enzyme was further supported by Broome in 1961. Then it was later published that this enzyme is necessary for the growth of neoplastic cells.

In 1964 Mashburn and Wriston produced L-ASN from the microbial sources. L-ASN is first enzyme, studied very intensively in human beings with regard to its antitumor activity. Then, it was shown that the growth of normal cells was independent of L-asparagine, making L-ASN more specific tumoricidal activity. In 1967, Oettgen demonstrated its efficacy in the treatment of leukemia. It was soon discovered that bacterial, *E coli* produced two types of L-ASN i.e. L-asparaginase I which is cytoplasmic in nature and asparaginase II, periplasmic form. ECAI has lower affinity for L-asparagine making it inefficient against tumor so the amido-hydrolytic activity is only associated the ECAII. FDA then approved L-asparaginase for the treatment of acute lymphoblastic leukemia in the year 1978. Then the preliminary efforts to determine the primary structure were carried out and in 1990, sequence of this enzyme was confirmed. Crystallographic structure of L-ASN was published in 1988 by Ammon *et al.* Current research is focus on the oncological importance of this enzyme and the transcription factors which are targeted by ECAII.

ALL (Acute Lymphoblastic Leukemia) cancer of WBCs is characterized by the excessive progression and multiplication of lymphocytes. The treatment of ALL includes the use of steroids, radiation therapies, stem cells and bone marrow transplants and chemotherapy, which is most successful among other treatments. The drugs like prednisolone, dexamethasone, vincristine, etoposide, cyclophosphamide are also used for ALL treatment. These varieties of drugs are associated with many side effects like immune-suppression, infertility, secondary neoplasm, vomiting, etc. So, need of better chemotherapeutic agents with no or minimum side effects are the need of time.

### 2.4 Natural sources of L-asparaginase

Nature has always kept his basket full of novel bioactive compounds. Natural products include products derived from natural sources like plants, animals, micro-organisms (bacteria, fungi) and marine sources.

| Natural Source | Organism name | References |
|---|---|---|
| **Bacteria** | *Acinetobacter calcoaceticus* | Joner (1976) |
| | *Bacillus* sp. | Mohapatra (1995) |
| | *Citrobacter* sp. | Bascomb (1975) |
| | *Corynebacterium glutamicum* | Mesas (1990) |
| | *Escherichia coli* | Netrval (1977) |
| | *Enterobacter aerogenes* | Mukherjee (2000) |
| | *E. cloaceae* | Nawaz (1998) |
| | *Erwinia aroideae* | Tiwari and Dua (1996) |
| | *E. carotovora* | Maladkar (1993) |
| | *E. chrysanthemi* | Moola (1994) |
| | *Helicobacter pyroli* | Stark (1997) |
| | *Klebsiella pneumonia* | Reddy and Reddy (1990) |
| | *Mycobacterium phlei* | Pasterzak and Szymona (1976) |
| | *Pseudomonas ovalis* | Badr and Foda (1976) |
| | *Serratia marcescens* | Rowly and Wriston (1967) |
| | *Staphylococcus* sp. | Mikucki (1977) |
| | *S. aureus* | Rozaiska and Mikucki (1992) |
| | *Streptococcus albus* | Reddy and Reddy (1990) |
| | *Tetrahymena pyriformis* | Tsirka and Kiriakidis (1990) |
| | *Thermus thermophilus* | Pritsa and Kyriakidis (2001) |
| | *Vibrio succinogenes* | Disteasio (1976) |
| **Yeast** | *Candida utilis* | Kil (1995) |
| | *C. guilliermondii* | Stepanyan and Davtyan (1988) |
| | *Pichia polymorpha* | Foda (1980) |
| | *Saccharomyces cerevisiae* | Bon (1997) |
| **Actinomycetes** | *Nocardia* sp. | DeJong (1972) |
| | *S. albidoflavus* | DeJong (1972) |
| | *Streptomyces gulbargensis* | Mostafa and Salama (1979) |
| | *Streptomyces karnatakaensis* | Mostafa (1979a) |
| | *S. venezuelae* | Mostafa (1979b) |

| | | |
|---|---|---|
| | *S. collinus* | Mostafa and Salama (1979) |
| | *Thermoactinomyces vulgaris* | Mostafa and Ali (1983) |
| **Fungi** | *Aspergillus nidulans* | Drainas and Drainas (1985) |
| | *A. niger* | Ali (1994) |
| | *A. terreus* | Ali (1994) |
| | *Cylidrocapron obtusisporum* | Raha (1990) |
| | *Mucor* sp. | Mohapatra (1997) |
| | *Penicillium* sp. | Mohapatra (1997) |
| **Algae** | *Chlamydomonas* sp. | Paul (1982) |

Table 2 Major L-asparaginase producing microorganisms (Savitri and Azmi, 2003)

The crystal structure of ECA (*E. coli* Asparaginase) is a tetramer which contains two threonine residues in its active site. This threonine residues act as primary nucleophiles and both are necessary for activity, and neither kinetic nor structural data has yet been able to show among both threonine residues which is the most likely primary nucleophile.

**2.5 Endophytic fungi as a source of asparaginase**

Endophytic fungi are the microorganisms which reside inside the plant tissues without giving rise to any disease symptoms (EBG *et al*., 2008). Interactions between endophytic fungi and plants include balanced antagonism, mutualism, latent type, commensalism and virulent and pathogenicity (Hallmann *et al*., 1997). These interactions of endophytic fungi with its host leads to the production of battery of secondary metabolites like alkaloids, terpenoids, steroids, quinines, phenols, peptides, phenolic acids and flavanoids which are of great therapeutic and medicinal value against numerous diseases. It has been observed that most of fungal endophytes belong to ascomycete family.

The absorptive mode of nutrition in fungi has emanated in the secretion of enzymes that carry out the most useful conversions (Suryanarayanan *et al*., 2013). The enzymes which are produced by endophytic fungi are chitin deacetylase, chintinase, chitosanase, alkaline protease, acidic protease, tannase, laccase, β-glucosidase and many more (Suryanarayanan *et al*., 2012). Asparaginase is also one among these which catalyzes the amide bond in L-asparagine to produce aspartic acid and ammonia. This hydrolysis in medicinal industry is used to treat ALL

and also other neolpastic forms like Hodgkins disease, acute chronic leukemia, acute myelocytic leukemia, acute myelomonocytic leukemia, melanosarcoma treatment and lymphosarcoma. Moreover, L-asparaginases, which are used in baking industry to reduce the acrylamide formation. Commercially available L-asparaginase currently used in food industry includes the brand names like Preventase produced from *Aspergillus niger* by DSM company, Acrylaway from *Aspergillus oryzae* by Novozyme (Lapmak *et a*l., 2010).

L-ASN from sources such as *Penicillium camemberti* (Dox 1909), *Aspergillus niger* (Bach,1928; Schmalfuss and Mothes, 1930), *Brucella abortus* (Altenbern and Housewright, 1954), *Mycobacterium avium* (Tsuji,1957) and *Rhodopseudomonas capsulatum* (Tchan and Kobayashi 1971) have not been tested yet for their anti-leukemia activities (Imada *et al*., 1973).

The screening of L-asparaginase producing fungal endophytes have been reported on the modified Czapek Dox media with L-asparagine as nitrogen source (Jain *et al*., 2012). Endophytic fungi from Thai medicinal plants were evaluated on MCD media for their ability to produce asparaginase (Theantana *et al*., 2007). Asparaginase producers were also assessed on only L-asparagine-agar media, where L-asparagine acts as a sole carbon and nitrogen source.
L-ASN production on the agar plates lead to the coloring of plates from red at pH 7.0 to pink. The pink color indicates towards the amido-hydrolytic activity of the L-ASN which involves the conversion of L-asparagine into L-aspartic acid and ammonia. Accumulation of ammonia is responsible for the pH rise which turns, phenol red from red to pink and thus acts as preliminary procedures for L-ASN activity. (Khamna *et al*., 2009; Thirunavakkarasu *et al*., 2011; Patil *et al*., 2012).

**2.6 Shake flask culture filtrate activity**

Fermentation is an anaerobic process which is carried out in the presence of nutrients and metabolites, which are required for growth and reproduction of microorganisms. It is a metabolic process which involves the breakdown of complex organic compounds into simpler products with the release of energy, which is further used to carry out many activities. This oxido-reduction process is mostly extracellular and involves the enzymes which are released by microorganisms. Maximum yield of L-asparaginase from *Aspergillus niger* was obtained using by-products of agro wastes incorporated with organic salts in submerged fermentation process (Zia *et al*., 2013).

### 2.7 Qualitative procedures for assessing amide-hydrolytic activity

The culture filtrates obtained after filtration were used for assessing the L-ASN activity qualitatively by agar plug well diffusion assay. Wells were punctured in the agar plates incorporated with L-asparagine substrate and phenol red as a dye indicator. Culture broth to be tested was loaded in the wells and incubated at 37°C. This assay procedure was used as a qualitative estimation of L-ASN isolated from bacterial cultures which are implicated in the treatment of ALL (Jain *et al*., 2012).

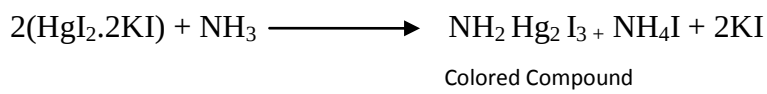
### 2.8 Secondary screening of asparaginase producing endophytic fungi

Spectrophotometric analysis: The quantitative estimation of L-ASN producers is done spectro-photometrically by quantifying the by-products i.e. ammonia and L-aspartic acid released after the catalysis of L-asparagine. Ammonia produced can be estimated by Berthelot reaction in which ammonia liberated subsequently reacts with phenol nitroprusside and alkaline hypochlorite which results in blue color at 600 nm. Nesslerization done using nessler's reagent, which is Potassium tetraiodomercurate (II) which is a method for ammonia detection (Meister, 1955). An automated technicon method is also there for determination of L-ASN activity by quantifying ammonia released. Paper electrophoresis and Chromatography are used to analyze the L-aspartic acid. Conversion of [$14_C$] L-asparagine to [$14_C$] L-aspartic acid is followed by the rapid chromatography on the ion exchange paper (Cedar *et al*., 1967).

### 2.9 Direct nesslerization

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. Further, the activity of enzyme is determined by protocol given by Imada *et al* with slight modifications. Activity of L-asparaginase isolated from ripened fruit of *Capsium frutescence* was assessed using direct nesslerization technique (Audipudi *et al*., 2014). L –asparaginase activity isolated from *Bipolaris* sp. BR438 isolated from brown rice was determined by direct nesslerization technique and expressed in U/ml (Lapmak *et al*., 2009).

Reaction involved in nesslerization:




**2.10 Protein precipitation**

Protein purification is a multistep procedure serving as an isolation, recovery and concentration process of desired product. Precipitation involves the conversion of the soluble solutes into insoluble solids, which can be subsequently separated from the liquid by physical methods like filtration or centrifugation. Protein precipitation methods classified broadly into two groups one, in which protein solubility is reduced and precipitation is done by changing some physio-chemical property of the solvent such as pH (isoelectric precipitation), dielectric constant (organic solvent mediated precipitation), ionic strength (salting out) and water availability (precipitation by non-ionic polymers). Another group is, in which protein precipitation is induced by a direct interaction between the protein and a precipitating agent. Two major types of precipitation carried out are ammonium sulphate precipitation and acetone precipitation.

L-ASN from *Cornyebacterium glutamicum* has been purified 98-fold by protamine sulphate precipitation, DEAE-Sephacryl anion exchange, ammonium sulphate precipitation and Sephacryl S-200 gel filtration (Kumar and Sobha, 2012).

Ammonium sulphate precipitation is a classic step to fractionate proteins in the solvent with respect to the ionic strength. Major advantage of this technique is that it easily causes the precipitation of protein and is non-denaturing to the structure of proteins. Another advantage of ammonium sulphate is at saturation it has sufficient high morality, that causes the precipitation of most of proteins and it doesn't have large heat of solution. Moreover, its concentrated solutions are generally bacteriostatic and protect most of proteins from denaturation. Concentration of ammonium sulphate salt required for precipitation varies from protein to protein and is determined empirically. Typically, it is used in series of steps performed at 2 to 8°C and is added in increments to concentration of 20% of saturation, gently stirring added drop wise and then allowed to dissolve and equilibrate. The percentage is increased to 50% then 80% till the protein of interest is salted out. *Mycobacterium phlei*, asparaginase was precipitated by ammonium sulphate.

Acetone precipitation is a technique with a purpose to concentrate the sample and remove small interfering species such as salts and detergents, for downstream applications. When the protein of interest is small i.e. (<low microgram), then it is necessary to add carrier protein, such as insulin (Wessel and Flugge, 1984). Intracellular L-asparaginase produced by cells of *Erwinia carotovora* was extracted (yield 60 %) by treating the culture filtrate with acetone (Maladkar , 1993).

Addition of water miscible solvent i.e. acetone to an aqueous extract of protein brings about the precipitation of protein due to a combination of factors like reduction in water activity and the solvating power of water for a charged hydrophobic protein molecule. Decreased solubility of protein leads to aggregation and precipitation due to electrostatic and dipolar interactions between opposite charged region of protein molecule.

**2.11 Estimation of extracellular proteins**

Extracellular protein content in crude enzyme and culture broth can be determined using Lowry's method (Lowry *et al*., 1951) and Bradford's method using Bovine serum albumin as the standard curve.

In lowry's proteins react with the Folin ciocalteau reagent to give a colored complex. The phenolic group of amino acids like tyrosine and tryptophan residues in a protein will produce a blue purple color complex, at wavelength of 660 nm, with Folin ciocalteau reagent which is sodium tungstate molybdate and phosphate. The intensity of the color formed depends on the amount of these aromatic amino acids. Mostly BSA is used to prepare standard protein, because of its low cost, high purity and ready availability.

Bradford assay is another method for protein determination involving the bonding of Coomassie Brilliant Blue G-250 dye to proteins (Bradford, 1976). The dye used in assay exists in 3 forms; cationic i.e. red, neutral is green and anionic blue in color (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 maxima 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.

### 2.12 Detection of proteins in SDS-PAGE by silver staining

Electrophoresis is used for analytical separation, identification and purification technique of bio molecules. Electrophoresis is defined as the migration of charged particles in an electric field under aqueous conditions. Mobility of a molecule in a separation gel medium depends on its net charge, size and shape.

SDS-PAGE: Protein electrophoresis is most commonly carried out in polyacrylamide gels under denaturing conditions in the presence of sodium do-decylsulphate (SDS). SDS molecules interact with the proteins in a constant ratio of approx. 1.4 mg of SDS per mg of protein. At this ratio, the charge density/unit length of proteins is equal so all the proteins are separated solely due to differences in their size. SDS-PAGE is used for characterizing protein mixtures, maintaining homogeneity of proteins and molecular weight identification. Proteins molecules ranging from 5 to 300 kDa as well as extremely basic or acidic proteins can be resolved and visualized using this technique. Molecular weight determination of L-asparaginase from *Penicillium brevicompactum* NRC 829 was done by preparing 12% separating gel and 5%stacking gel. The log molecular weight of different standard molecular weight marker proteins was plotted against their relative mobility in the gel for 2 hours (Elshafei *et al.*, 2012). The purity of asparaginase obtained from *E.coli* VRY-15 was checked by running SDS-PAGE using slab gel of 7% acrylamide in a Tris-HCl buffer containing 0.1 % SDS and gel was stained with 0.025 Coomassie brilliant blue, R-250 and then destained (Jain *et al.*, 2012).

### 2.13 Screening of positive L-asparaginase producers for L-glutaminase contamination

It is observed that L-asparaginase isolated from various microbial sources especially bacteria also produce glutaminase up to 10% (Campbell and Mashburn, 1969). This contamination has many adverse side effects leading to allergic responses and anaphylaxis in patients (Muller and Boos, 1998). L-glutaminase can be derived from animal and plant tissues, but glutaminase isolated from microbial sources, like bacteria, fungi and yeast fulfill the industrial demands (Sajitha *et al.*, 2013).

Fungal isolates from seaweed and medicines plants were screened for glutaminase free L-asparaginase production using Czapek Dox agar plate's method using glutamine instead of asparagine and 0.009% phenol red as dye indicator (Thangavel *et al* ., 2013). L-asparaginase and glutaminase activities were found together in a large proportion of *pseudomonads*. Two

organisms belonging to the family *Moniliaceae* showed glutaminase activity together with L-asparaginase. Whereas, few ascomycetous fungi formed L-asparaginase or L-glutaminase activity. Further, studies revealed that stains of *Candida scottii* and *Cryptococcus albidus*, related to basidiomycetes showed only L-glutaminase activity. Glutaminase activity can be determined by direct nesserlization using L-glutamine at place of L-asparagine and Tris HCl buffer, 7.2 pH for actinomycetes, yeast and fungi measuring extinction at 450 nm (Imada *et al*., 1973).

Sources of L-glutaminase synthesis are *Escherichia coli*, *Pseudomonas* sp., *Acinetobacter* sp.*, Bacillus* sp.*, Hansenula, Cryptococcus, Candida, Aspergillus oryzae* and *Beuveria bassiana* (Sabu, 2003). The use of inhibitors for biosynthesis of glutamine and asparagine in combination with L-asparaginase leads to increase in the therapeutic efficiency of L –asparaginase enzyme.

# Chapter 3

# Aim of study

# 3. AIM OF STUDY

The present study is subjected towards screening of fungal endophytes for the production of L-asparaginase.

So the objectives of current study involve:

- Screening and selection of asparaginase producing endophytic fungi
- Quantification of L-asparaginase enzyme activity
- Assay to check for glutaminase contamination

# Chapter 4

# Materials and Methods

### 4.1 Preservation and maintenance of Endophytic Fungi

Potato Dextrose Agar (PDA) was used for recovering the fungal isolates from stock cultures. Then, maintaining them time to time as pure isolates by their sub-culturing and finally preservation of pure cultures in PDA slants (Agarwal and Hasija, 1986).

#### 4.1.1 Preparation of Potato Dextrose Agar (PDA) plates

39.0 g of PDA (Hi Media) was weighed and dissolved in 1L double distilled water and stirred to mix properly. After that it was transferred to 250 ml Erlenmeyer flasks and autoclaved at 121 °C, 15 psi for 15 min. Aseptically, 25 ml of the media was poured on to 90 mm pre-sterilized petri plates and allowed to solidify.

#### 4.1.2 Sub-culturing and preservation of pure culture

50 endophytic fungi were aseptically sub cultured onto PDA plates and incubated at 26±2 °C for 7-10 days till the profuse fungal growth was seen. The loop full of the metabolically active culture was aseptically inoculated on to PDA slants containing 10% glycerol for long term preservation.

### 4.2 Preliminary screening for L-asparaginase producers

Primary screening of 50 endophytic fungi was done for L-asparaginase production by using modified protocol as previously described by Patil *et al*., 2012. For this assay, L-asparagine and phenol red were filtered sterilized using filter assembly fitted with 0.22 µm nitrocellulose membrane.

In order to check for L-asparaginase producers, all the 50 endophytic fungi were screened on Modified Czapek Dox (MCD) agar pH 7.0. The composition of the MCD agar media is shown in Table 3. MCD media was prepared by mixing all the components well except agar and pH was adjusted with 1N HCL to 7.0, then agar was mixed and media was autoclaved at 121°C, 15 psi for 15 min . After that media was allowed to cool down, filter sterilized L-asparagine and phenol red was added into it and mixed properly. Plating was done and plates were kept overnight at 28°C.The next day, 5 mm plug of 7 days old culture was inoculated on MCD agar plates. The plates were then kept at 28±2°C for 5-7 days and were monitored for formation of pink zone after every 24 hours for 7 days.

| S. No. | Components | Concentration(g/l) |
|---|---|---|
| 1 | L-asparagine | 10 |
| 2 | Glucose | 1% |
| 3 | Sodium nitrate | 2 |
| 4 | Ferrous sulphate | 0.01 |
| 5 | Magnesium sulphate | 0.5 |
| 6 | Potassium chloride | 0.5 |
| 7 | Di-potassium phosphate | 1.0 |
| 8 | Agar | 15 |

Table 3 Composition of Modified Czapek Dox (MCD) Agar

**4.3 Secondary screening for L-asparaginase producers**

The cultures selected on the basis of preliminary testing were further screened on L-asparagine-agar plates containing 0.009% phenol red. L-asparagine acts as sole carbon and nitrogen source. All the positive fungal isolates were grown on L- asparagine-agar plates for 7 days at 28°C. The color change from red to pink was observed and diameter of zone was measured (Theantana *et al*., 2007).

**4.4 Shake flask culture filtrate activity**

The selected endophytic fungi on the basis of secondary screening were subjected for culture filtrate production in Modified Czapek Dox (MCD) medium. 5 mm mycelial plug of 7 day old culture were inoculated in 25 ml pre-sterilized MCD broth in Erlenmeyer flask under aseptic conditions and were incubated in shaker incubator at 120 rpm, 28°C for 7 -10 days (Imada *et al*., 1973). After the incubation is over, the fungal mycelium was separated from broth through filtration using Whatman filter paper No.1 followed by centrifugation at 12,000 rpm for 15 min to get cell free culture filtrate. The cell free culture filtrate was then used for further qualitative testing by Agar well Diffusion assay.

### 4.4 Qualitative test by Agar well diffusion (AWD) assay

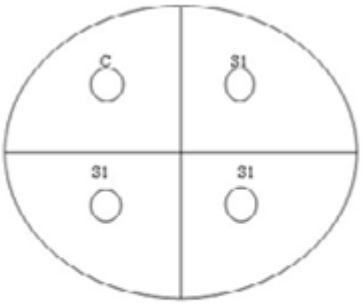


Figure 2 Petriplate showing AWD assay, where S1: Test sample and C1: Control

Agar well diffusion assay is a modified version of Ditch Plate Assay; this technique was initially designed by Heatley in 1944. Culture filtrates of selected isolates were qualitatively screened for L-asparaginase production using plate assay. L-asparaginase agar plates containing phenol red were prepared in similar way as previously described. The plate was divided into four quadrants and 5 mm well were made in each quadrant using sterilized cork borer. 30 µl of culture broth was dispensed in 3 wells and in fourth well un-inoculated media was added which served as control. The plates were incubated at 37°C for 48 h. After the incubation was over, the plates were observed for the formation of pink halo formation around the wells. The zone diameter was recorded in triplicate, mean and standard deviation was calculated.

### 4.5 Precipitation of extracellular L-asparaginase from culture filtrate

The extracellular protein precipitation was carried out by cold acetone precipitation method (Wessel and Flugge, 1984). Acetone was kept at -80°C prior to precipitation. Cold acetone was added to the culture filtrate of selected sample in ratio of 4:1 and it was incubated at -20°C overnight. The next day, the samples were centrifuged at 13,000 rpm for 15 minutes so as to precipitate the total protein. Supernatant was discarded and pellet obtained was weighed and dissolved in 1 ml of 0.5 M phosphate buffer (pH 7.0) and stored at -80°C till further use

### 4.6 Protein estimation

Total protein content was estimated by using Lowry's Method (Lowry *et al*., 1951). First of all the standard curve of Bovine serum albumin (BSA) was made. Different dilutions of BSA ranging from 0.05 to 1 mg/ml were prepared by adding adequate volume of BSA stock solution (1 mg/ml) and double distilled water in test tubes as shown in the Table 4. To estimate the protein content of unknown precipitated protein sample, 0.2 ml of protein sample was added followed by addition of 2 ml of Analytical reagent [Analytical reagent was prepared by mixing 2 ml of (b) with 100 ml of (a) where (a) is 50 ml of 2% sodium carbonate mixed with 50 ml of 0.1N NaOH solution and b) is 10 ml of 1.56% copper sulphate solution mixed with 10 ml of

2.37% sodium potassium tartarate solution. The solutions were mixed well and incubated for 10 minutes at room temperature. After that 0.2 ml of Folin Ciocalteau solution was added to each test tube and incubated for 30 min. Absorbance was measured at 660 nm. All the reading were noted in triplicate. The Absorbance versus concentration graph was used to deduce the protein content of unknown protein sample.

| **BSA (ml)** | **Water (ml)** | **Sample conc. (mg/ml)** | **Alk. $CuSO_4$ (ml)** | **Lowry reagent (ml)** |
|---|---|---|---|---|
| 0.25 | 4.75 | 0.05 | 2 | 0.2 |
| 0.5 | 4.5 | 0.1 | 2 | 0.2 |
| 1 | 4 | 0.2 | 2 | 0.2 |
| 2 | 3 | 0.4 | 2 | 0.2 |
| 3 | 2 | 0.6 | 2 | 0.2 |
| 4 | 1 | 0.8 | 2 | 0.2 |
| 5 | 0 | 1.0 | 2 | 0.2 |

Table 4 representing the concentrations of BSA used for preparation of standard curve

**4.7 Quantification of L-asparaginase activity**

The quantification was carried out by using direct nesslerization to find the activity of the L-asparaginase (Imada *et al*., 1973). First of all, the standard curve of ammonia was constructed in order to estimate the amount of ammonia liberated by protein sample of interest.

Standard curve of ammonia

1.179 g of ammonium sulphate was dissolved in 100 ml of double distilled water. The main stock (1µmole/ml) was prepared by pipetting out 1.4 ml of above solution and volume was made up to 100 ml with double distilled water.

From main stock, two stocks viz. Stock I and Stock II were prepared.

Stock I (1µmole/ml): 140 µl of main stock and 9.86 ml $dH_2O$

Stock II (2 µmole/ml): 280 µl of main stock and 9.72 ml $dH_2O$

From these stock solutions different concentration of ammonia were prepared as shown in Table 5.

| S. No. | Conc. of $NH_3$ (µmol/ml) | Preparation of ammonia dilutions |
|---|---|---|
| 1 | 0.25 | 750 µl of $dH_2O$ + 250 µl of Stock I |
| 2 | 0.5 | 500 µl of $dH_2O$ + 500 µl of Stock I |
| 3 | 0.75 | 350 µl of $dH_2O$ + 750 µl of Stock I |
| 4 | 1.25 | 375 µl of $dH_2O$ + 625 µl of Stock II |
| 5 | 1.5 | 250 µl of $dH_2O$ + 750 µl of Stock II |
| 6 | 1.75 | 125 µl of $dH_2O$ + 875 µl of Stock II |

Table 5 Showing different concentrations of ammonia used for Direct Nesslerization

To each concentration, 1ml of Nessler's reagent was added and Absorbance was measured at 450 nm. Standard curve was plotted between Absorbance and concentration of ammonia used.

### 4.7.1 Determination of L-ASN activity

To the test tube, 0.1 ml of L-asparagine (40 mM), 1 ml of crude protein sample of interest, 0.5 ml of 0.5 M phosphate buffer (pH 7.0) and 0.4 ml of $dH_2O$ were added. L-asparagine was dissolved by shaking very gently. Enzyme sample was handled very carefully and kept in the ice. This mixture was incubated at 37°C for 30 minutes. After incubation is over, 0.5ml of 1.5M trichloroacetic acid (TCA) was added to stop the reaction. Centrifugation was done at 10,000 rpm for 5 min. The pellet obtained was discarded. To the 0.1 ml of supernatant solution, 3.7 ml of distilled water was added. To this solution, 1 ml of Nessler's reagent was added, vortexed and then incubated for 10 min. Blank was prepared containing all the other reagents except enzyme. Absorbance was measured at 450 nm. All the readings were noted in triplicate.

Then, the amount of ammonia liberated from protein sample of interest is calculated from Standard curve of ammonia in units/ ml using the formula:

$$\text{Activity of enzyme (units/ml)} = \frac{(\mu\text{mol of } NH_3 \text{ liberated}) \times (\text{initial volume of mixture in ml})}{(\text{Vol. of enzyme mixture Used in final rxn in ml}) \times (\text{Incubation time}) \times (\text{Vol. of enzyme used})}$$

Where,

Initial volume of enzyme mixture (ml) = 2.5,

Volume of enzyme mixture used in final reaction (ml) = 0.1,

Incubation time (minutes) = 30, Volume of enzyme used (ml) = 1

One International unit of enzyme is defined as the amount of enzyme needed to liberate one micromole of ammonia from L-asparaginase under the conditions of assay (pH = 7.0; 37°C).

### 4.8 Detection of proteins by silver staining for SDS-PAGE protein samples

SDS-PAGE is used for characterizing proteins and for molecular weight identification. SDS-PAGE system includes: a tank, lid with power cables, electrode assembly, and cell buffer dam, casting strands, casting frames, combs and glass plates. It is carried under denaturing conditions as SDS is used to give uniform negative charge to the protein.

5 ml stacking gel 5%, (pH 6.8) and 10 ml separating gel 12% was prepared as given below:

| **Reagents used** | **Volume (ml)** | |
|---|---|---|
| | **Stacking gel (5%)** | **Separating gel (12%)** |
| Double distilled water | 2.975 | 3.2 |
| 0.5M Tris-HCl (pH 6.8) | 1.25 | -- |
| 1.5M Tris HCl (pH=8.8) | -- | 2.6 |
| 10% (w/v) SDS | 0.05 | 0.1 |
| Acrylamide/Bis-acrylamide | 0.67 | 5 |
| 10% (w/v) APS | 0.05 | 10 µl |
| TEMED | 0.005 | 10 µl |

Table 6 Compositions of stacking gel and separating gel

#### 4.8.1 SDS-PAGE protocol

Separating gel was prepared as mentioned above and casting frames were set on the casting stands. The gel solution was prepared as described above in a separate small beaker. The solution was swirled gently and dispensed into the gap between the glass plates. Top of the separating gel was overlaid with saturated butanol. Gel was then kept for 20-30 minutes in order to gelate. After gelation, the butanol was drained off and top of the gel was rinsed with double distilled water. Stacking gel was prepared and dispensed above separating gel and comb was inserted without trapping air under the teeth. It was kept undisturbed for 20-30 min. After gelation of stacking gel was completed, comb was removed. Glass plates were taken out of the casting frame. Running

buffer was poured into the inner chamber and it was kept pouring after overflow until the buffer surface reaches the required level in the outer chamber. The sample was prepared by mixing 20 µl of the sample, 10 µl of 1% bromophenol blue dye with glycerol (20% w/w). In case of Protein weight marker, 10 µl of it was mixed with 20 µl of dye. After that the samples were boiled for 3-4 min and 20µl of each sample was loaded into the wells Protein marker (29-200 kDa) was loaded into the last lane. The top was covered and connected to the anodes. An appropriate volt for 12% separating gel 1 hour for 120 V was set. When samples reached the bottom the gel was removed and kept in destain solution I for further staining procedure.

**4.8.2 Detecting Proteins with Silver Stain**

For protein detection first of all, the destaining solutions were prepared. Silver nitrate solution was prepared freshly and covered properly in order to prevent from light.

| **Solutions** | **Composition** |
|---|---|
| Destain Solution I | 40% Methanol +7% Acetic acid |
| Destain II | 5% Methanol +7% Acetic acid |
| Cross-Linking Solution | 10% Glutaldehyde |
| DTT Solution | 5mg/l |
| Silver Nitrate Solution | 0.1% w/v i.e. 0.1g in 100ml $dH_2O$ |
| Developing Solution | 3% Sodium carbonate+0.019% Formaldehyde |
| Stop Solution | 2.3M of Sodium Citrate.$2H_2O$ |

Table 7 Representing the composition of destaining solutions used in silver staining

**4.8.2.1 Procedure of Silver Staining**

The gel was agitated in 100 ml of destain I solution and kept overnight. Destain solution I was then drained off and the gel was agitated in 100 ml of destain II solution for 30 minutes. After rinsing with Destain II solution, the gel was agitated in 100 ml of cross linking solution for 30 minutes. Cross linking solution was removed and the gel was washed for several times in double distilled water for the period of 2 h. Then gel was agitated in DTT solution for 30 minutes, solution was drained well after 30 minutes from gel. Next, the gel was agitated in 100 ml of silver nitrate solution for 30 minutes in dark. After the incubation was over the staining tray was kept under running de-ionized water and swirled for few seconds. Water was drained off and 50

ml of developing solution was added, swirled briefly and solution was discarded. This step was repeated again. 100 ml of developing solution was added and shaking was done slowly.

When the bands became slightly slighter then the desired staining level, the developing solution was removed and the gel was quickly rinsed with water and De-stain solution II was added to cover the gel. Next, stop solution was added. Then the gel was washed several times in De-stain II solution and rinsed with water. Then the gel was visualized in the white light.

### 4.9 Assay to check for L-glutaminase activity

Fungal isolates showing L-asparaginase activity were checked qualitatively for glutaminase presence. Endophytic fungi with confirmed L-ASN activity were selected and 5 mm plug of each culture was inoculated on MCD plates. These plates contained L-glutamine and 0.009% phenol red. Uninoculated MCD plate served as control. Plates were then incubated for 7 days and observed for pink zone formation within and around the colonies after every 24 h (Audipudi *et al*., 2014).

| **Constituents** | **Concentration(g/l)** |
|---|---|
| Glucose | 1% |
| L-glutamine | 10 |
| $KH_2PO_4$ | 1.52 |
| KCl | 0.52 |
| $MgSO_4.7H_2O$ | 0.52 |

Table 8 Composition of (Modified Czapek Dox) MCD for glutaminase assay

# Chapter 5

# Results and Discussions

### 5.1 Re-culturing

50 Endophytic fungi used in the present study were regularly sub-cultured on PDA plates and maintained at 28˚C. For long term preservation, all the isolates under study were transferred to PDA slants containing 10% glycerol and maintained at 28˚C.

Figure 3: showing pure endophytic cultures on PDA plate.

In the present study, endophytic fungi were isolates of the medicinally important plant belonging to family Rutaceae (*Aegle marmelos*), Lauraceae (*Cinnamomum malabaricum, Cinnamomum zeylanicium, Cinnamomum camphora*), Theaceae (*Camellia sinensis*), Taxaceae (*Taxus baccata),* Apocyanaceae (*Catharanthus roseus, Tabernaemontana divaricata)* and *Euphorbiaceae (Jatropha curcas)* collected from the biodiversity hot spots of India.

Out of the 50 isolates, 18 were isolated from *Aegle marmelos*- 8 were from stem, 6 from stem internal tissue, 3 from leaves, 1 was from bark; 6 endophytic fungi were isolated from stem internal tissue of *Cinnamomum camphora*; 7 endophytic fungi were isolated from *Cinnamomum malabaricum*- 2 were from bark, 5 from stem; 5 were from *Catharantus roseus*- 1 from leaf and 4 from stem; one endophyte was isolated from bark of *Camellia sinensis*, 3 endophytic fungi were isolated from *Cinnamomum zeylanicium*- 2 were from bark, 1 from stem internal tissue; 2 from leaf of *Jatropha* sp; 6 endophytic fungi from bark of *Taxus bacata*; 1 endophyte was isolated from stem of *Tabernaemontana divaricata* and 1 from the bark of *Raulwofia serpentina.* The endophytic fungi associated with theses medicinally important plants are reported to be potential producer of many bioactive metabolites and enzymes that can be used as potential drugs for number of diseases.

### 5.2 Qualitative screening assay for L-asparaginase

#### 5.2.1 Preliminary screening for L-asparaginase producers

50 endophytic cultures were subjected for preliminary screening, in order to assess their potential to utilize L-asparagine as a nitrogen source. The isolates when grown on modified Czapek Dox agar medium with phenol red as a pH indicator were selected on the basis of their ability to convert red plates to pink under alkaline conditions. The changed color indicated the accumulation of ammonia which resulted as L-asparagine converted to L-aspartic acid.

Table 9 represents the list of fungal endophytes along with their host plant, the part of host plant from where they are collected, place of sampling and their ability as L-ASN producers or L-ASN non producers.

Of the 50 cultures, 39 were found to be positive for extracellular asparaginase production. Whereas, 20 isolates among 39 positive cultures exhibited promising L-asparaginase production and rest were very slow producers being active after 7-10 days. The maximum positive isolates were isolated from *Aegle marmelos* (AM). #16AMLWLS found to exhibit highest ASN activity, as pink color intensity was found to be maximum (Figure 4) followed by #1048AMSTITYEL, #1088AMSTITWLS, #61AMLWLS, #5AMSTYEL, #17AMSTYEL; but on the other hand, #13CMSTNEY, #6610CMSTITBRT, #25AMSTWLS, #6CCSTITD, #36CMSTNEY, #21TBBALM showed pink zone formation on 4$^{th}$ day. #2CCSTITD was the slowest L-asparaginase producer among all the selected positive isolates.

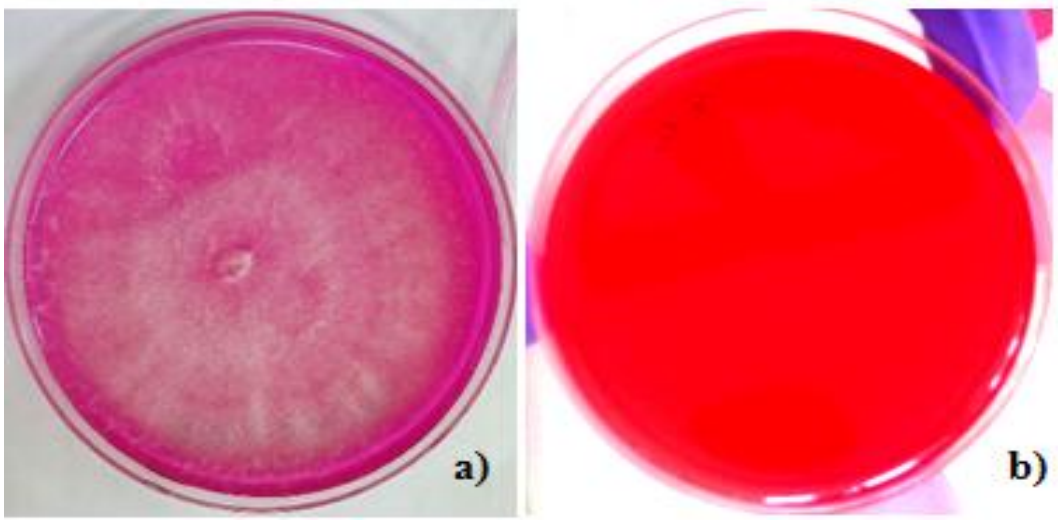



Figure 4 (a) #16AMLWLS showing highest pink color intensity (b) Control plate

Few culture isolates shown pink color within as well as around the fungal colony (Figure 5). Whereas, fungal endophyte coded as #1118AMSTITYEL produced yellow color around their colony, this drift towards yellow color may be due to the sudden decrease in the pH which turns the MCD agar plate to yellow.

| S.No | Culture Code | Plant Part | Host Plant | Place of Sampling | Enzyme Activity after 5 days |
|---|---|---|---|---|---|
| 1. | #1AMSTYEL | Stem | *Aegle marmelos* | Yelandur, Kerala | +++ |
| 2. | #3AMSTYEL | Stem | *Aegle marmelos* | Yelandur, Kerala | ++ |
| 3. | #5AMSTYEL | Stem | *Aegle marmelos* | Yelandur, Kerala | ++++ |
| 4. | #6AMLWLS | Leaf | *Aegle marmelos* | Wayand Wildlife Scantuary, Kerala | ++ |
| 5. | #9AMSTYEL | Stem | *Aegle marmelos* | Yelandur, Kerala | +++ |
| 6. | #11AMBALWLS | Bark | *Aegle marmelos* | Wayand Wildlife Scantuary, Kerala | - |
| 7. | #11AMSTYEL | Stem | *Aegle marmelos* | Yelandur, Kerala | - |
| 8. | #16AMLWLS | Leaf | *Aegle marmelos* | Wayand Wildlife Scantuary, Kerala | +++++ |
| 9. | #17AMSTYEL | Stem | *Aegle marmelos* | Yelandur, Kerala | ++++ |
| 10. | #23(b)AMSTYEL | Stem | *Aegle marmelos* | Yelandur, Kerala | + |
| 11. | #25AMSTWLS | Stem | *Aegle marmelos* | Wayand Wildlife Scantuary, Kerala | +++ |
| 12. | #53AMSTITWLS | Stem internal Tissue | *Aegle marmelos* | Wayand Wildlife Scantuary, Kerala | +++ |
| 13. | #61AMLWLS | Leaf | *Aegle marmelos* | Wayand Wildlife Scantuary, Kerala | ++ |
| 14. | #1013AMSTITYEL | Stem internal tissue | *Aegle marmelos* | Yelandur, Kerala | - |
| 15. | #1032AMSTITD | Stem internal tissue | *Aegle marmelos* | Tiger hills, Darjeeling West Bengal | - |
| 16. | #1048AMSTITYEL | Stem internal tissue | *Aegle marmelos* | Yelandur, Kerala | ++++ |
| 17. | #1088AMSTITWLS | Stem internal tissue | *Aegle marmelos* | Wayand Wildlife Scantuary, Kerala | ++++ |
| 18. | #1118AMSTITYEL | Stem internal Tissue | *Aegle marmelos* | Yelandur, Kerala | - |
| 19. | #1CCSTITD | Stem internal Tissue | *Cinnamomum camphora* | Tiger hills, Darjeeling West Bengal | - |
| 20. | #2CCSTITD | Stem internal tissue | *Cinnamomum camphora* | Tiger hills, Darjeeling West Bengal | ± |
| 21. | #6CCSTITD | Stem internal tissue | *Cinnamomum camphora* | Tiger hills, Darjeeling West Bengal | ±±± |
| 22. | #6(b)CCSTITD | Stem internal tissue | *Cinnamomum camphora* | Tiger hills, Darjeeling West Bengal | - |

| | | | | | |
|---|---|---|---|---|---|
| 23. | #1639CCSTITD | Stem internal tissue | *Cinnamomum camphora* | Tiger hills, Darjeeling West Bengal | - |
| 24. | #6610CCSTITD | Stem internal Tissue | *Cinnamomum camphora* | Tiger hills, Darjeeling West Bengal | ±± |
| 25. | ##1CMSTNEY | Stem | *Cinnamomum malabaricum* | Neyyar, Kerala | + |
| 26. | #4CMBABRT | Bark | *Cinnamomum malabaricum* | BRT Wildlife Scantury, Karnataka | - |
| 27. | #4CMSTNEY | Stem | *Cinnamomum malabaricum* | Neyyar, Kerala | ± |
| 28. | #13CMSTNEY | Stem | *Cinnamomum malabaricum* | Neyyar, Kerala | +++ |
| 29. | #20CMBANEY | Bark | *Cinnamomum malabaricum* | Neyyar, Kerala | + |
| 30. | #28CMSTNEY | Stem | *Cinnamomum malabaricum* | Neyyar, Kerala | + |
| 31. | #36CMSTNEY | Stem | *Cinnamomum malabaricum* | Neyyar, Kerala | ++++ |
| 32. | #2CRSTBRT | Stem | *Catharanthus roseus* | BRT Wildlife Scantury, Karnataka | ++ |
| 33. | #16CRLPAL | Leaf | *Catharanthus roseus* | Palampur | ++ |
| 34. | #17CRSTBRT | Stem | *Catharanthus roseus* | BRT Wildlife Scantuary, Karnataka | +++ |
| 35. | #42CRSTBRT | Stem | *Catharanthus roseus* | BRT Wildlife Scantury, Karnataka | ++ |
| 36. | #43CRSTBRT | Stem | *Catharanthus roseus* | BRT Wildlife Scantury, Karnataka | + |
| 37. | #2CSBAOT | Bark | *Cimmelia sinensis* | Ooty, Tamil Nadu | + |
| 38. | #5CZBAWLS | Bark | *Cinnamomum zeylanicum* | Wayand,Wildlife Scantury, Kerala | + |
| 39. | #23CZSTITG | Stem internal Tissue | *Cinnamomum zeylanicum* | Guwahati, Assam | + |
| 40. | #31CZBAG | Bark | *Cinnamomum zeylanicum* | Guwahati, Assam | ++ |
| 41. | #4JTLSVNP | Leaf | *Jatropha curcas* | Silent Valley National Park | - |
| 42. | #97JTLSVNP | Leaf | *Jatropha curcas* | Silent Valley National Park | ++ |
| 43. | #12RSBANEY | Bark | *Raulwolfia serpentina* | Neyyar, Kerala | ++ |
| 44. | #11TBBALM | Bark | *Taxus baccata* | Almora, Uttarakhand | + |
| 45. | #21TBBALM | Bark | *Taxus baccata* | Almora, Uttarakhand | + |
| 46. | #28TBBALM | Bark | *Taxus baccata* | Almora, Uttarakhand | + |

| | | | | | |
|---|---|---|---|---|---|
| 47. | #30TBBALM | Bark | *Taxus baccata* | Almora, Uttarakhand | + |
| 48. | #61TBBALM | Bark | *Taxus baccata* | Almora, Uttarakhand | + |
| 49. | #120TBBALM | Bark | *Taxus baccata* | Almora, Uttarakhand | - |
| 50. | #2(a)TMDSTYEL | Stem | *Tabernaemonta divaricata* | Yelandur, Kerala | ++ |

Note: poor activity (+); average activity (++); Good activity (+++), Very Good activity(++++); Excellent activity(+++++),color within colony (±); No activity (-)

Table 9 Screening of L-asparaginase producers on MCD agar plates

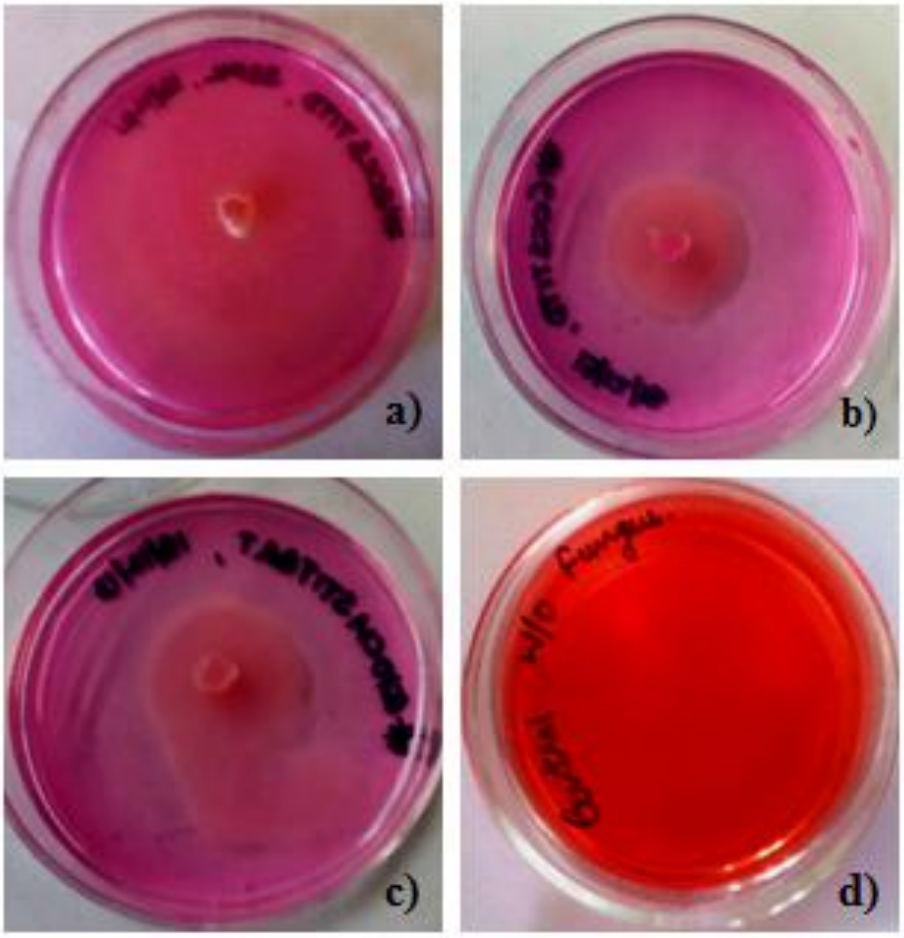



Figure 5 Petriplates showing positive results for L-asparaginase (a) #2CCSTITD, (b) #6CCSTITD, (c) #6610CMSTITBRT (d) Control plate (without fungus)

### 5.2.2 Secondary screening for asparaginase producers

Twenty cultures that came up with positive L-asparaginase production were further evaluated for L-asparaginase production using only L-asparagine agar (AA) plates with phenol red as dye indicator for 5 days in order to remove the false positive producers. The pink zone formation around and within fungal colony indicates the increase in pH from neutral to alkaline, due to accumulation of ammonia in the medium (Theantana *et al* ., 2007).

| Culture Code | Zone Dia.(mm) Mean ± SD |
|---|---|
| #5AMSTYEL | 90 ± 0 |
| #17AMSTYEL | 73 ± 0.008 |
| #1048AMSTITYEL | 49 ± 0.03 |
| #6CCSTITD | 48.5 ± 0.78 |

Table 10: Showing zone diameter after 48 h of culture filtrates producing L-ASN

On the basis of secondary screening, it was found 5 out of 20 cultures were true potential producers of L-asparaginase. The zone diameter was noted in triplicate after every 24 hours. The zone of 90 mm was seen around fungal colony of #5AMSTYEL followed by 73 mm zone of #17AMSTYEL. #6CCSTITD showed the zone diameter of 48.5mm.

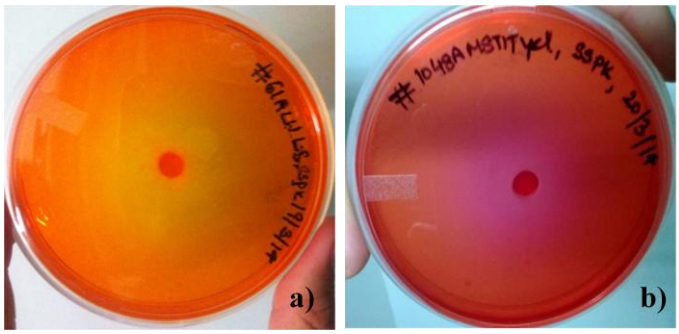



Figure 6 (a) ASN production within colony (left), (b) zone diameter of ASN produced after 48 hours (right)

Isolate #61AMLWLS showed pink color only within colony and diameter of pink colored zone which was equivalent to 0.5 to 0.85 cm i.e. 5 mm to 8.5 mm only (Figure 6). So, this culture was not carried for further studies.

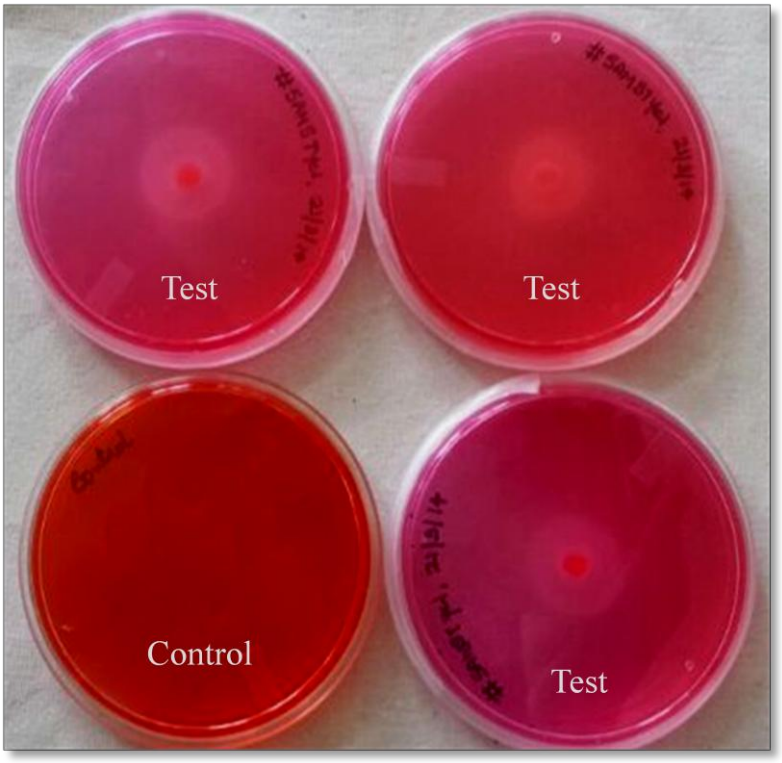



Figure 7: Pink color indicates enzyme production by #5AMSTYEL as compared to control plate

**5.3. Shake flask culture filtrate activity**

The four cultures viz. #5AMSTYEL, #1048AMSTITYEL, #17AMSTYEL and #6CCSTITD

were selected after secondary screening for further confirmation of L-asparaginase production. These four cultures were subjected for production of secondary metabolites in L-asparagine- glucose medium so as to evaluate amido-hydrolytic activities of L-asparaginase. #17AMSTYEL produced highest biomass followed by #1048AMSTITYEL and least amount of biomass was produced by #6CCSTITD (Table 11). Cultures with highest biomass exhibited promising asparaginase production in the preliminary and secondary qualitative screening assays. The selected culture filtrates were further evaluated for L-asparaginase activity using agar well diffusion assay

| Culture Code | Wt. of Biomass (g) |
|---|---|
| #17AMSTYEL | 7.5 |
| #1048AMSTITYEL | 6 |
| #5AMSTYEL | 4.5 |
| #6CCSTITD | 3.5 |

Table 11 Showing biomass produced by culture filtrates

### 5.4 Agar Well Diffusion (AWD) Assay

The culture filtrate of four selected endophytic fungi was evaluated for L-asparaginase production by agar well diffusion assay. #5AMSTYEL exhibited maximum zone of 2.44 cm followed by #1048AMSTITYEL with zone diameter of 2.41cm. Control well containing un-inoculated broth did not formed any zone thus eliminating the risk of false results (Figure 8).

| Culture Code | Zone Diameter (cm) |
|---|---|
| #5AMSTYEL | 2.44 ± 0.03 |
| #1048AMSTITYEL | 2.41 ± 0.065 |
| #17AMSTYEL | 2.29 ± 0.011 |
| #6CCSTITD | 2.1 ± 0 |

Table 12 Showing the zone Diameter (in cm) produced by selected fungal culture filtrates after 48 h.

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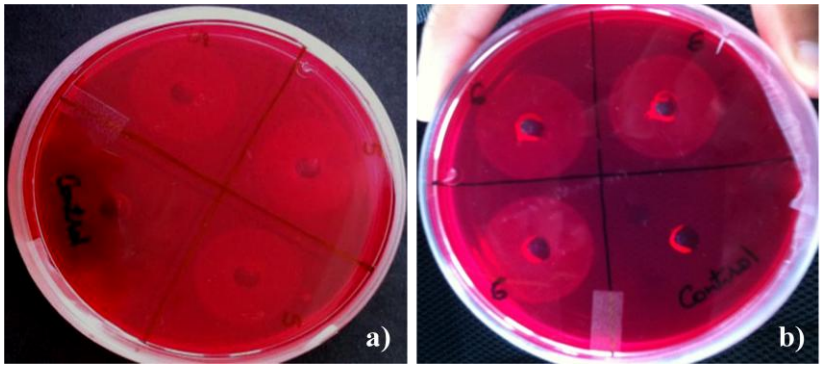



Figure 8 Agar well diffusion assay. (a) zone formed by #5AMSTYEL (b) #1048AMSTITYEL after 48 hours.

### 5.5 Protein precipitation and estimation

Crude protein was amply recovered from cell free filtrate of selected isolates by cold acetone precipitation method. The total protein content obtained per ml of culture filtrate was shown in Table 13. The maximum protein content was observed to be present in #17AMSTYEL i.e. 3.175 mg / ml.

| Culture Code | OD @660nm | Protein amount (mg/ml) |
|---|---|---|
| #17AMSTYEL | 0.153 | 3.175 |
| #5AMSTYEL | 0.138 | 2.97 |
| #1048AMSTITYEL | 0.131 | 2.87 |
| #6CCSTITD | 0.115 | 2.66 |

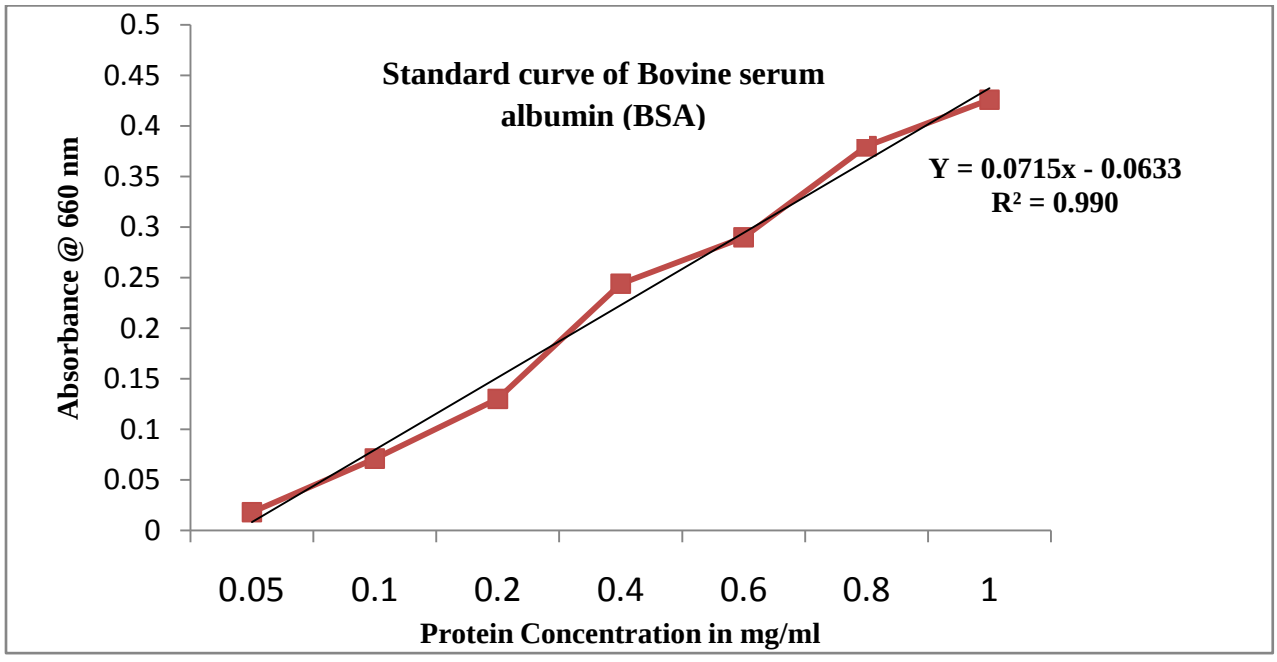
Table 13 Showing protein content of selected endophytic fungi

The protein estimation of all the fungal isolates was done by Lowry's method as the amount of protein obtained is important for further findings. Amount of protein in test samples was calculated by using the correlation equation obtained by BSA standard curve (Graph 1). The following equation was obtained:

$$Y = 0.0715x - 0.0633$$

$$x = \frac{Y + 0.0633}{0.0715}$$

Where, Y= Absorbance at 660 nm, x= Concentration of BSA (mg/ml).




Graph 1: Standard cure for Bovine serum albumin (BSA)

### 5.6 Quantitative estimation of asparaginase by nesslerization

The activity of L-asparaginase was evaluated when the accumulation of ammonia resulted in the conversion of L-asparagine to L-aspartic acid as a result pink color appeared, which was next evaluated for L-ASN production in the liquid conditions by direct nesslerization (Imada *et al*., 1973).

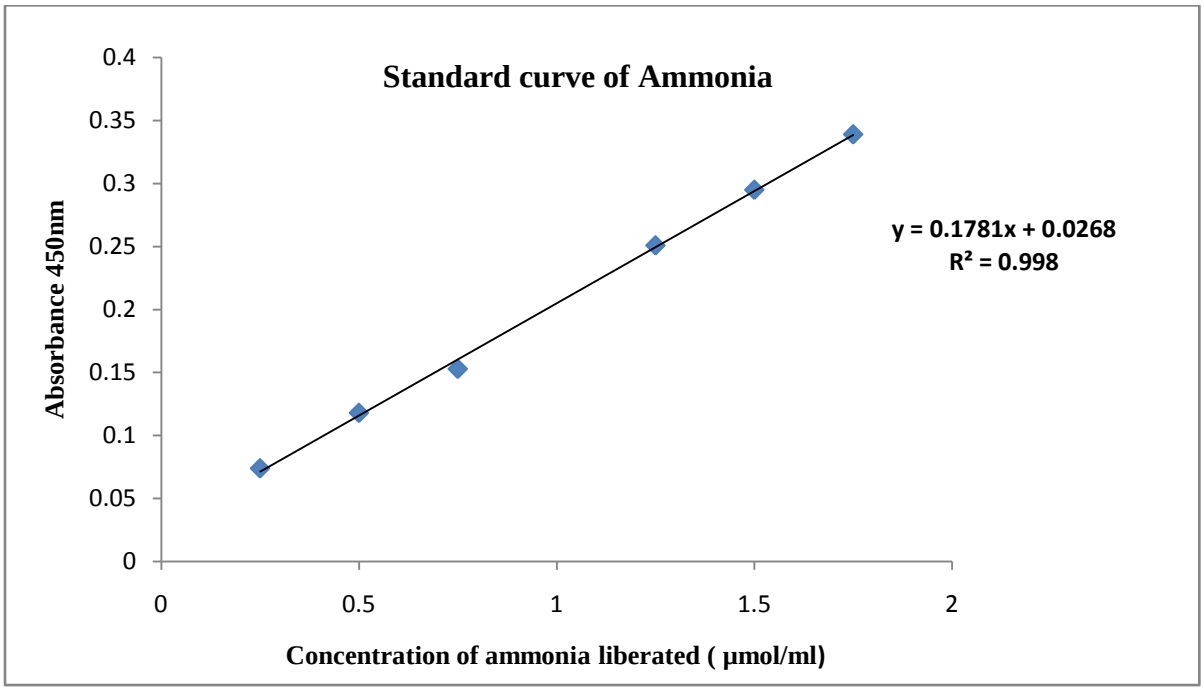
To find the activity of L-ASN by quantifying the amount of ammonia released, Standard curve of Ammonia was constructed viz. equation obtained was Y= 0.1781x + 0.0268 with $R^2$ =0.998, (Graph 2). Then, from this graph the ammonia released in the test sample was calculated using following equation:

$$Y = 0.1781x + 0.0268$$

$$x = \frac{Y - 0.0268}{0.1781}$$

Where, Y = Absorbance at 450 nm, x = concentration of ammonia liberated (µmol/ml)

Finally, activity of the L-ASN from test fungal isolates was calculated in (Units/ml) using the formula as mentioned previously.




Graph 2 Showing standard curve of Ammonia

The crude protein of #5AMSTYEL possessed maximum activity of 5.882 Units/mins/ml followed by #1048AMSTITYEL with activity of 5.486 Units/mins/ml , which is approximately 3 times the activity of #17AMSTYEL. The lowest activity was observed in #6CCSTITD (0.043 Units/mins/ml).

| Culture Code | Absorbance ±S.D. | Protein Conc (mg/ml) | U/mins/ml of crude protein |
|---|---|---|---|
| #5AMSTYEL | 1.284±0.0045 | 2.97 | 5.882 |
| #1048AMSTITYEL | 1.20±0.023 | 2.87 | 5.486 |
| #17AMSTYEL | 0.34±0.135 | 3.175 | 1.465 |
| #6CCSTITD | 0.036±0.00602 | 2.66 | 0.043 |

Table14 Shows the activity of the L-ASN of test samples in Units/mins/ml of crude protein.

**5.7 Detection of proteins in SDS by Silver Staining**

SDS-PAGE of protein sample of #5AMSTYEL gives a single band with molecular weight of approximately 60 kDa. L-asparaginase isolated from *Escherichia coli* is a tetramer with molecular weight of 134 kDa. The molecular weight by SDS –PAGE was detected in case of L-ASN purified from *Pseudomonas stutzein MB-405* (Manna *et al*., 1995 ), *Thermus thermophilus* (Prista and Kyridio *et al* ., 2001). *Pseudomonas aeruginosa* after SDS-PAGE separation revealed a peptide chain with molecular weight of 160 kDA (El-Bessoumy *et al*., 2004). L-ASN from *Penicillium* sp. showed single peptide chain of ~66.00 kDa (Patro and Gupta *et al*., 2012).

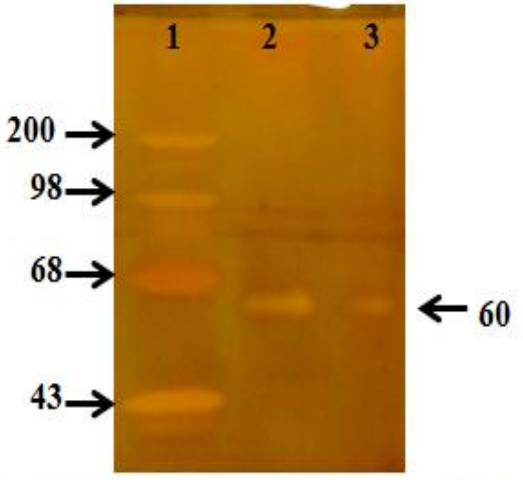



Figure 9 showing SDS-PAGE gel. Lane 1: Protein molecular weight marker (29-200 kDa),Lane 2 &3: #5AMSTYEL

### 5.8 Assay to Check for L-glutaminase activity in L-asparaginase producers

The selected four cultures exhibiting L-asparaginase activity were tested for L-Glutaminase production on MCD agar plates with L-Glutamine as nitrogen source and phenol red (0.009%) pH 6.2. Two culture isolates, #6CCSTITD and #17AMSTYEL showed Glutaminase presence, as confirmed due to the color change in the MCD agar plates. The pink color produced in the MCD plates was due to the alteration in the pH of medium, as L-Glutaminase has amido-hydrolytic activity which leads to the breakdown of amide bond in L-Glutamine with the liberation of ammonia. Ammonia accumulation, in the medium leads to alteration of the pH of phenol red from acidic to alkaline. But, #5AMSTYEL and #1048AMSTITYEL did not show any color change confirming that glutaminase contamination is not there.

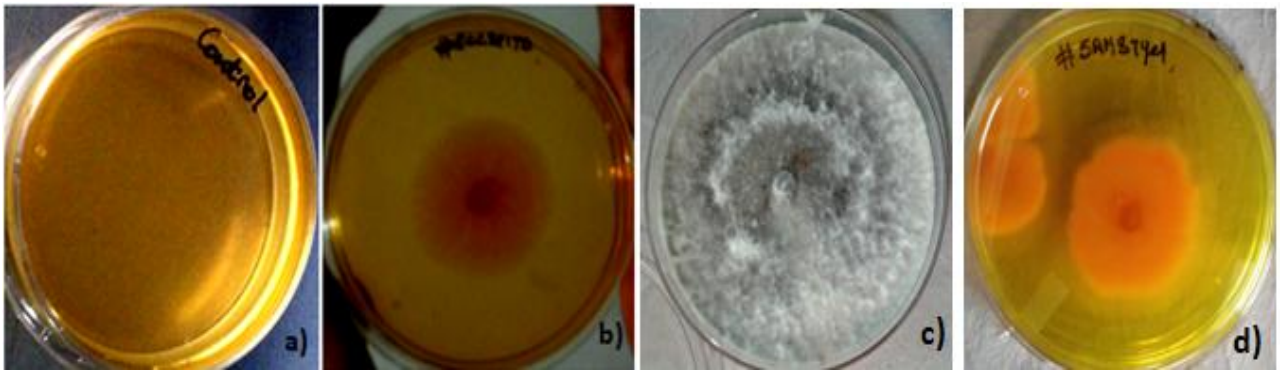



Figure 10 Glutaminase plate assay. (a) Control plate without culture isolate, (b) glutaminase contamination in #6CCSTITD (the colony turned pink), (c) & (d) #1048AMSTITYEL and #5AMSTYEL showed glutaminase absence as there is no change in color of the colony.

# Chapter 6

# Conclusion

The current study describes that beside bacteria, actinomycetes, algae and other known eukaryotic sources, endophytic fungi are the micro-organisms which can be proved to be better source of L-asparaginase with lower immunogenic response.

Out of 50 endophytic fungi from different medicinal plants of Indian pharmacopeia, #5AMSTYEL (isolated from *Aegle marmelos*) exhibited maximum potential of L-asparaginase production followed by #1048AMSTITYEL by both qualitative and quantitative assays with no glutaminase contamination as faced by current bacterial L-asparaginase preparations.

Further profound enzyme kinetics studies, purification and testing of its anti-tumor potential is desired to compare its efficiency with existing commercial L-asparaginase producers.

# Chapter 7

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