# SCREENING ENDOPHYTIC FUNGAL BROTH FOR L-METHIONINASE ACTIVITY

A

Thesis submitted

in partial fulfilment of the requirement of the degree

Of

**Master of Science**

**In**

**Biotechnology**

By

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# *Certificate*

This is to certify that the thesis entitled ***"Screening of Endophytic fungal broth for L-Methioninase activity"*** being submitted by **Ms Charu Bahl (Roll No-301001007)** in partial fulfillment of the requirements for the award of degree of Master of Science in Biotechnology, Thapar University, Patiala is a bonafide work carried out under the esteemed supervision and conception of Dr. Sanjai Saxena and Dr. Siddhartha Sharma and that no part of this thesis has been submitted for the award of any other degree.

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Date: July 16th, 2012
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This is to certify that the above statement made by the candidate is correct and true to the best of our knowledge.

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Date: 16th July, 2012 *Charu Bahl*

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*Dedicated to my Father....*

*Late Shri Ashwani Kumar Bahl*

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# Abbreviations

| S.no | Abbreviation | Full form |
|---|---|---|
| 1. | IARC | International Agency for Research on Cancer |
| 2. | DNA | Deoxy Ribo Nucleic Acid |
| 3. | mg | Milligrams |
| 4. | ml | Millitres |
| 5. | mm | Milimetres |
| 6. | nm | Nanometer |
| 7. | PDA | Potato dextrose agar |
| 8. | MGA | Methionine glucose agar |
| 9. | GA | Glucose agar |
| 10. | MA | Methionine agar |
| 11. | MTAP | Methlythioadenosine phosphorylase |
| 12. | WA | Water agar |
| 13. | PCA | Potato Carrot agar |
| 14. | CMA | Corn Meal agar |
| 15. | ITS | Internal Transcribed region |
| 16. | Hrs | Hours |
| 17. | rpm | Revolutions per minute |
| 18. | MGB | Methionine glucose broth |

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| | | |
|---|---|---|
| 19. | CFU | Colony forming units |
| 20. | µl | Microlitre |
| 21. | µM | Micromoles |
| 22. | GLA | Grass leaf agar |
| 23. | SNA | Synthetischer nahrtoffarmer agar |
| 24. | EDTA | Ethylenediaminetetraacetic acid |
| 25. | TAE | Tris acetate EDTA |

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# *Executive Summary*

The current anticancer drugs face the problems like tissue specificity, immunogenic response, lower productivity etc. Thus there is a general call for alternative cancer therapeutics which can contribute in increasing the specificity and reducing the side effects of the present chemotherapeutics. This has led to finding novel sources from plants and microbial sources.

Plants shelter a variety of microbes which are classified as epiphytes, pathogens and endophytes. Endophytes are microbes which reside atleast once in their life cycle inside the internal tissue of host plants without indicating their presence and causing any negative effect. These endophytes are believed to be the mines of bioactive compounds. In the present approach, the liquid broth of endophytic fungi obtained from *Aegle marmelos and Cinnamomum sp* was studied for their L-methioninase activity. In all, 50 endophytic fungal isolates obtained from *A. marmelos and Cinnamomum sp* were screened via various pre-screen assays for their potential to produce L-methioninase. 6 endophytic isolates selected from pre screen assays were subjected for production of culture filtrates in Methionine glucose broth which was further tested for L- methioninase activity. Culture code #1088 AMSTITWLS, #5 CZBAWLS, #40 CMLBRT showed an appreciable L- methioninase activity at 37° C in the presence of pyridoxal phosphate. Culture code #1088 AMSTITWLS showed maximum potential to produce L- methioninase. The presence of extracellular enzyme was confirmed by qualitative assay of the crude protein. The protein content was estimated by Lowry's method. The three endophytes possessing maximum potential to produce L- methioninase were further identified using classical and molecular approaches. Microscopic evaluation showed that #1088 AMSTITWLS belongs to *Lasodiplodia theobromea*, #5CZBAWLS belongs to *Fusarium oxysporium* and #40 CMLBRT belongs to *Alternaria* sp. Further for molecular identification, genomic DNA of these three isolates was amplified using universal ITS primers. They showed an amplicon in size between 550-600 bp.

Further protein purification and characterization is warranted along with the molecular taxonomy of the endophytic isolates to develop them as a novel source of L- methioninase producers.

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# Chapter 1

# Introduction

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Cancer is generally defined as a group of diseases characterized by uncontrolled growth and spread of abnormal cells. It can be fatal if it remains uncontrolled. Causes of carcinogenesis are governed by both external and internal factors. The external factors responsible for carcinogenesis are; use of tobacco, chemical, radiation and infectious organism's exposure. The internal factors contributions to cancer development are inherited mutations, hormonal disturbances, immunity imbalance and mutations that occur in metabolism.

As per the estimate of International Agency for Research on Cancer (IARC) there were 12.7 million cases of cancer globally in 2008 and it is expected that the cancer burden would grow up to 21.4 million new cases and 13.2 million deaths due to growth and aging populations. The primary modalities of cancer treatment are surgery, chemotherapy, and radiotherapy; these may be used alone or in combination. There is increasing emphasis worldwide on the development of specialized cancer centers that apply evidence-based multimodal therapies and provide rehabilitation and palliative care.

The most important facet in treatment of cancer is specificity of the treatment. Rational targets for novel cancer therapies is by deregulating the processes which are involved in tumor genesis include regulation of cell cycle progression, angiogenesis and apoptosis. In most cases the deregulation is achieved by using biomolecules as the active agents which include hormones, proteins and nucleic acids. The conventional treatments being used comprise mainly the chemotherapeutics, which have shown the limitations at the advanced stages of cancer and are mostly cytotoxic in nature, so the side effects are immense and moreover the recurrence of the malignant tumors is a very common in the case where chemotherapy is alone used. There are some alternative approaches which are these days taken into consideration. Safe immunological profile, adequate bioavailability or stability is the features which make these biomolecules unsuitable to meet the clinical requirements for their use as drugs.

It has been found that cancer cells are often disrupted in DNA methylation or methionine metabolism. There are different cell lines which have been termed methionine-dependent and include cancer cell lines of very different origins, such as carcinomas of various organs, sarcomas and neurological tumours. The prevalence of methionine dependence in cancer cell lines suggests an important relationship to oncogenic transformation. Enzymes seem to be a promising alternative for anti-tumor/anti-cancer

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therapy. Tumour cells have enhanced requirements for metabolites i.e they are auxotrophic making them susceptible selective targets. High level of L- methionine auxotrophy is observed in certain solid tumours and thus this could be used as a target for cancer therapy. Methioninase or methionine-α-deamino-γ-mercaptomethanelyase or METase is a pyridoxal-L-phosphate dependent enzyme which transforms L-methionine into α ketobutyrate, methanethiol and ammonia. Barring mammals, L-methioninase is ubiquitous to all organisms. L-methioninase was firstly reported for *C. sporogenes* enzyme against Walker carcinoma in rats (Kreis and Hession 1973). L-Methioninase has drawn considerable attention as a therapeutic alternative to combat methionine dependent cancer. It has been isolated, biochemically characterized and purified from prokaryotic sources for studying its effect on different human cancer cell lines. There are very limited studies on exploration of L-methioninase from eukaryotes, particularly fungi and their therapeutic efficacy as compared to bacterial L-methioninase.

Endophytic fungi are a special and important group of microorganisms which reside within the healthy tissue of the plant without providing any obvious clue of their existence and are hypothesized to be providing protection to host plant from the attack of herbivorous insects or vertebrate grazers by production of bioactive compounds (Strobel *et al.,* 2003; Tan and Zou, 2001). Endophytic fungi have been isolated and screened for exploration of an array of bioactive compounds like antimicrobials, antivirals, anti-oxidants and immunosuppressive agents. *Cryptosporiopsis quercina* was isolated as an endophyte from *Tripterigeum wilfordii*, a medicinal plant native to Eurasia and produces a very potent anti-mycotic agent cryptocandin (Strobel *et al*., 1999). Colletotric acid, a metabolite of *Colletotrichum gloeosporioides*, an endophytic fungus in *Artemisia mongolica*, displays antimicrobial activity against bacteria as well as against the fungus *Helminthsporium sativum* (Zou *et al*., 2000). *Taxomyces andreanae* was discovered from *T. brevifolia* and found to produce the billion dollar anti-cancer agent Paclitaxel (Strobel *et al.,* 1993). Pestacin and isopestacin, have been obtained from culture fluids of *Pestalatiopsis microspora*, an endophyte isolated from a combretaceaous plant, *Terminalia morobensis* and displays antimicrobial as well as antioxidant activity (Harper *et al*., 2003). The endophytic fungus *Fusarium subglutinans*, isolated from *T. wilfordii*, produces the immunosuppressive but non-cytotoxic diterpene pyrones subglutinol A and B (Lee *et al*., 1995). *Aspergillus fumigatus* Fresenius, from *Juniperus communis* L. Horstmann plant, collected from the populations at

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the botanical gardens of Rombergpark specifically produces deoxypodophyllotoxin (Kusari *et al*, 2008).

Limited information exists on L methioninase production from fungi especially endophytic fungi. Endophytic fungi thus represent an underexplored biological resource for screening L-methioninase for possible use as a therapeutic agent when compared to prokaryotic L-methioninase.

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# Chapter 2

# Review of Literature

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## 2.1 Cancer: Role of Methionine

Malignant tumors are characterized by a high rate of growth. Tumor cells drain the energy of the host, glucose in particular but amino acids. This has inspired diverse nutritional therapeutics, including dietary amino acid restriction. Methionine is an essential amino acid with at least four major functions. First, methionine takes part in protein synthesis. Second, methionine is a precursor of glutathione, a tripeptide that reduces reactive oxygen species, thereby protecting cells from oxidative stress (Anderson, 1998). Third, methionine is required for the formation of the polyamines spermine amd spermidine, which has far-ranging effects on nuclear and cell division. Fourth, methionine is the major sources of methyl groups for methylation of DNA and other molecules. (Breillout,1990). Fig 1 gives the overview about the different roles played by methionine in metabolism.

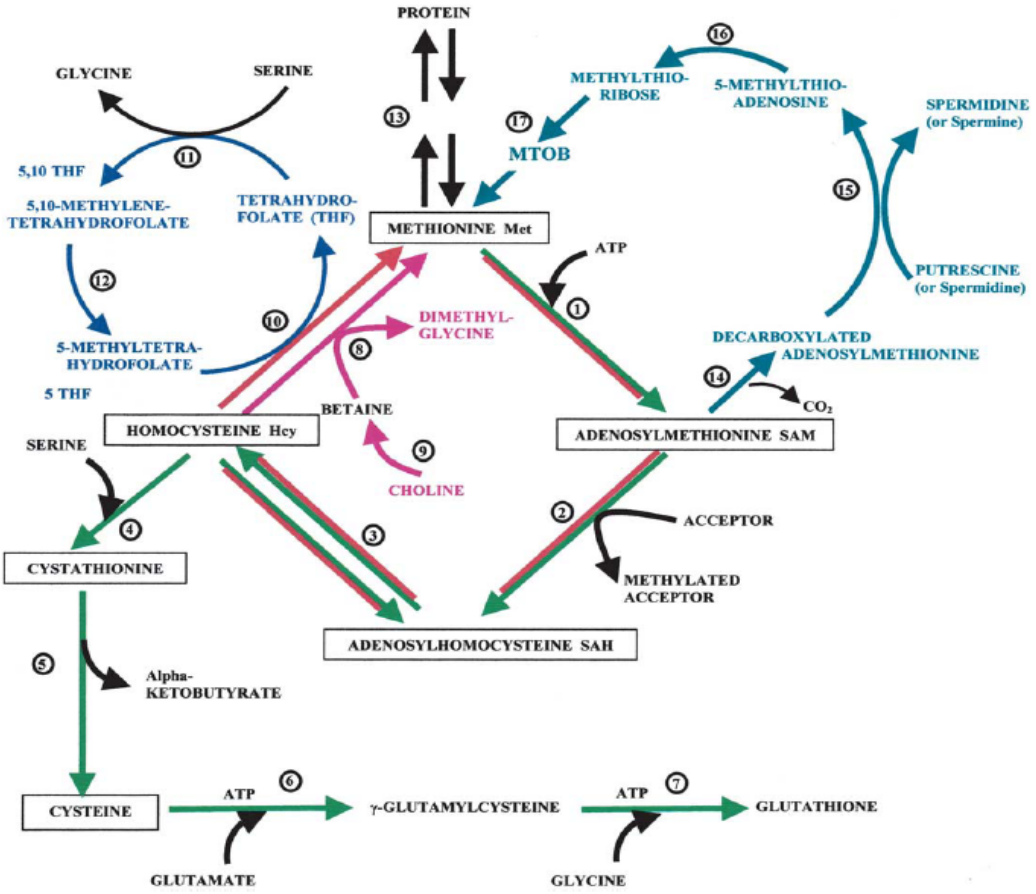


Fig 1: Shows role of methionine in various biochemical pathways (Durando *et al*., 2003).

There are many reports and various studies which revealed that numerous human tumor cells are characterized by their inability to divide when methionine is replaced by its immediate precursor homocysteine, whereas normal cells grow in such a medium. The

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absolute requirement of methionine is known as methionine dependency (Hoffman, 1981). The biochemical basis of methionine dependency is still under investigation, numerous malignant tumors cell lines (breast, lung, colon, kidney, bladder, melanoma, glioblastoma, etc) are methionine dependent (Hoffman *et al*., 1985). Many enzymes and pathways involved in methionine metabolism have been investigated in search of explanation of this metabolic defect. The synthesis of methionine is catalyzed mainly by methionine synthase or by betaine homocysteine methyltranferase and methionine synthase is found in all mammalian tissues, betaine enzyme is found mainly in liver. So, theoretically suggested reason behind this metabolic defect is mainly the functional deficiency of Methionine synthase activity, which can explain the methionine dependency. It was shown that methionine dependent tumor cell lines presented relatively low amounts of methionine synthase (Halpern *et al.,* 1974). Other studies confirmed that the activity of this enzyme could be low in methionine dependent malignant cells (Poirier and Wilson, 1980).

Furthermore, the importance of the enzyme is supported by the finding that a methionine independent reverant cell line had a 31 % greater methionine synthase activity than the methionine dependent cell line from which it was isolated (Judde et *al.,* 1989).

Some of the researchers focus on the importance of a second methionine synthesis pathway so called methionine salvage pathway in explaining methionine dependence (Quas *et al.,* 1996). This pathway may be important in methionine conservation in cells synthesizing large amounts of polyamines. Methylthioadenosine phosphorylase (MTAP) is one of the key enzymes in this salvage pathway. It is abundant in many tissues, including most bone marrow and peripheral blood nucleate cells (Ferro *et al.,* 1986). MTAP is frequently deficient in many kinds of cancer cells and primary tumors including small cell lung cancer (Nobori *et al.,* 1999), leukaemia (Traweek *et al.,* 1998) and melanoma.

However, a recent study indicates that although mehionine dependency and MTAP deletion both frequently occur together, MTAP deletion is not responsible for the methionine dependent growth phenotype, as transfection with MTAP cDNA does not restore the growth of these lines in Methionine deprived and homocysteine supplemented media (Tang et *al.,* 2000).

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Another hypothesis is that methionine dependency would result not from an enzymatic deficiency but rather than increased requirement of methionine (Bergstrom *et al.,* 1987). The amount of methionine required by the tumor cells is much higher than that required by the normal cell, this may be due to the increased protein synthesis and enhanced transmethylation reactions (Hoffman *et al.,* 1985). This ensures that multiple biochemical reactions necessary for the fast growth of tumor cells and have notably action on DNA. In tumors, several growth inhibitors and pro-apoptotic genes are transcriptionally silenced as a result of hypermethylation of DNA (Zingg and Jones, 1997).

**2.2 Methioninase: In anti-cancer therapeutics**

Unlike normal cells methionine dependency has been reported as a metabolic defect in various cancer cells. Thus, L methioninase (E.C.4.4.1.11) which is a pyridoxal 5”-phosphate dependent enzyme and acts on l-methionine carrying out its direct α, γ-elimination to produce α-ketobutyrate, methanethiol and ammonia (Tanaka *et al.*, 1977).

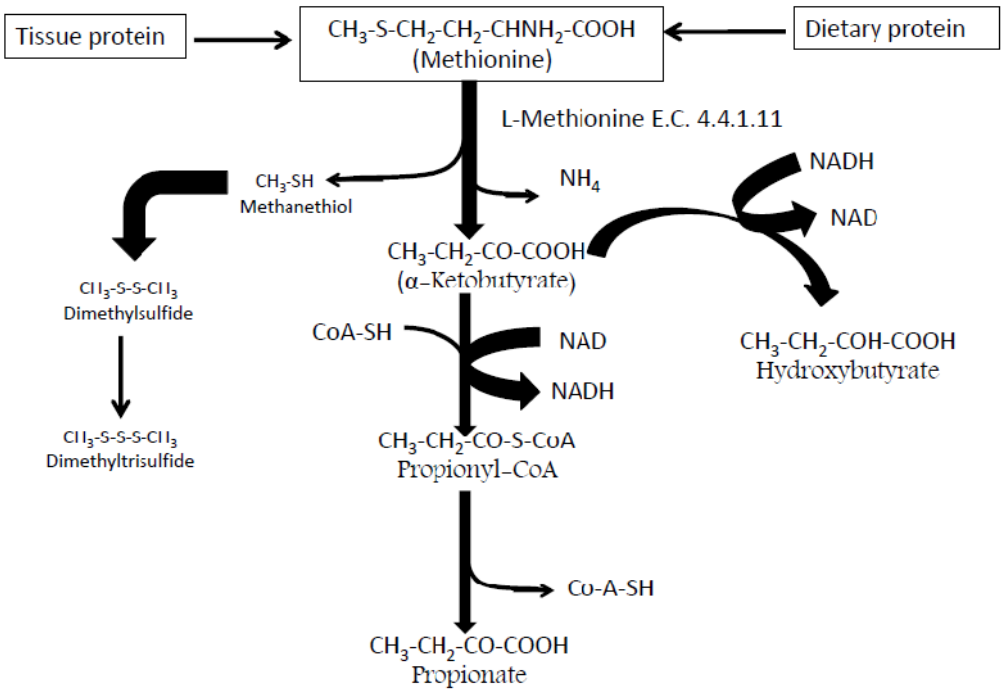


Fig 2 Shows the enzyme reaction when L-Methioninase acts on methionine (El-Sayed, 2010).

Under the condition of methionine depletion, which can be achieved through the action of l-methioninase in cancer cells leads to arrest of cells in the late –S/G2 phase of cell cycle and hence the cells cannot divide further (Hoffman *et al.*, 1980). The various

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chemotherapeutic drugs used against the different types of cancer cell lines, pose a great problem of elevated side effects that may be attributed to their low specificity towards the target tissue and the interference with the normal metabolic pathways leading to great harm to the human body (Devita *et al*., 1993). Thus, the search for novel anti tumor agents, having high selectivity towards the target tissue is one of the main challenges. So, in this regard enzymotherapy such as using L-methioninase is the most effective strategy recently. Several therapeutic approaches were designed for triggering the methionine dependency of tumor cells starvation of tumor cells using methionine free diets is one of the most important therapy, but this method of achieving the objective of methionine depletion, is not efficient due to various economical and technical considerations. (Goeski *et al*., 1992; Tan *et al*., 1996). Thus, L-methionine has received affordable attention as a therapeutic agent against various types of methionine-dependent tumors (Weisendander and Nisman 1953).

The antitumor efficiency of the enzyme was firstly reported for *Clostridium sporogenes* L-methioninase against Walker carcinoma in rats (Kreis and Hession ,1973). There are various studies which were being carried out focusing on its purification and characterization from different microbial sources. The activity of L-methioninase has been reported against several types of cancers including breast, kidney, colon, lung, and prostate cell lines (Tan *et al*., 1996, 1998).

But the therapeutic response in case of L-Methioninase is accompanied with high immunogenicity especially with multiple dosing, which is a serious problem (Tan *et al*., 1996; Sun *et al.*, 2003). There have been several strategies used in order to overcome the above mentioned problem, and to increase the pharmacological potentiality and therapeutic efficiency of the enzyme. One of them is the immobilization of L-methioninase on polyethylene glycol which is an effective trail for reducing its immunogenicity, increasing the plasma half life time and resistant to the proteolytic cleavage *in vivo* (Tan *et al.*, 1996; Takakura *et al*., 2006). Second important alteration is the use of nitrogenous base analogues such as 5-fluorouracil, in order to produce synergistic effect with the L-methioninase which displayed a higher cytotoxicity for cancer cells (Yoshioka *et al*., 1998). Similarly combination of L-methioninase with intercalants drugs, as doxorubicin, displayed a significant effect against human lung carcinoma H460, with no activity using the enzyme or doxorubicin, separately (Gupta *et al*., 2003). Another combination which has been used is L methioninase

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with cisplatin an alkylating agent, gave a higher activity against human breast tumor (Hoshiya *et al*., 1997).

One more new strategy is using gene therapy by transduction of the bacterial gene into the cancer cells done via retroviral vectors which is the most developed strategy used recently for treating many types of cancers recently (Miki *et al*., 2001; Gupta *et al*., 2003; Yamamoto *et al*., 2003) the efficacy of gene therapy for treatment of methionine dependent tumors evolved from depleting intracellular and extracellular methionine in addition to their selectivity in action. But it is fairly associated with side effects and low clinical efficiency; in addition, some tumor cells may release antiapoptotic mitochondrial protein bcl-2 that counteract the introduced methioninase gene (Yamamoto *et al*., 2003)

Unfortunately, the therapeutic response of this enzyme is usually associated with high immunogenicity, short life time, low substrate specificity, and hazardous effects to the kidney and liver as the result of ammonia toxicity. However, L methioninase from eukaryotes may be characterized by their lower immunogenicity due to structural competence of the eukaryotic enzyme with the human immune system, as reported frequently for many bacterial enzymes replaced by the corresponding eukaryotes (Kusakabe *et al*., 1980).

L-methioninase is found to be produced by almost most of the organisms, including bacteria, fungi, protozoa, and plants except mammals. The various L-methioninase producing microbes reported from the previous studies are given in the table no.1 and it can be concluded from the table that the bacterial sources have been explored to a much great extent by the scientists, when compared to the eukaryotic sources. Taking into consideration the nature of the enzyme, the bacterial L-methioninase is essentially an intracellular enzyme (Tanaka *et al*., 1976) and the fungal L-methioninase can be both intra as well as extracellular ( Herrera and Starkey 1969a, El-Sayed 2009). Because of the fact that L- methioninase is most frequently extracellular in case of fungi, fungi can be the potent producers of this enzyme. As it was reported for other fungal enzymes, the extracellular yield is about four times higher than the intracellular one (Pandey *et al*., 1999; El-Sayed 2008)

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| Microbial source | Microbial species | Reference |
|---|---|---|
| | *Achromobacter strakeyi* | Ruiz Herrera and Starkey (1970) |
| ***Bacteria*** | *Aeromonas sp* | Nakayama *et al*. (1984) |
| | *Arthobacter sp* | Bonnarme *et al*. (2000) |
| | *Bacillus subtilis* | Hullo *et al*. (2007) |
| | *Bervibacterium linens* | Amarita *et al*. (2004) |
| | *Citrobacter freundii* | Manukhov *et al*. (2005)/ |
| | *Crotalus intermedius* | Faleev *et al*. (1996) |
| | *Clostridium sp* | Weisendanger and Nisman (1953) |
| | *Corynebacterium glutamicum* | Bonnarme *et al*. (2000) |
| | *Escherichia coli* | Ohigasi *et al*. (1951) |
| | *Klebsiella aerogenes* | Sciflein and Lawerence (2001) |
| | *Micrococcus luteus* | Ohigasi *et al*. (1951) |
| | *Pseudomonas putida* | Ito *et al*. (1976) |
| | *P.ovalis* | Tanaka *et al*. (1976) |
| | *Proteus rottgeri* | Chen *et al*. (1971) |
| | *Staphylococcus equorum* | Bonnarme *et al*. (2000) |
| | *Yarrwia lipolytica* | Bondar *et al*. (2005) |
| ***Fungi*** | *Aspergillus flavipes* | Khalaf and El-Sayed (2009) |
| | *A.flavus* | Khalaf and El-Sayed (2009) |
| | *A.carneus* | Khalaf and El-Sayed (2009) |
| | *A.niger* | Khalaf and El-Sayed (2009) |
| | *A.parasiticus* | Khalaf and El-Sayed (2009) |
| | *A.subolivacoues* | Khalaf and El-Sayed (2009) |
| | *A.tamarii* | Han *et al*. (2002) |
| | *Aspergillus sp., RS-la* | Ruiz Herrera and Starkey (1969a) |
| | *Cladosporium cladosporioides* | Abu-Scidah and Youssef (2000) |
| | *C.oxysporum* | Khalaf and El-Sayed (2009) |
| | *Debaromyces hansenii* | Bonnarme *et al*. (2001) |
| | *Fusarium nivale* | Khalaf and El-Sayed (2009) |
| | *Fusarium oxysporum* | Khalaf and El-Sayed (2009) |
| | *F.solnai* | Khalaf and El-Sayed (2009) |
| | *Geotrichum candidum* | Ruiz-Herrera and Starkey (1969a, b) |
| | *Humicola fuscoatra* | Faleev *et al* . (1996) |
| | *Kkuveromyces lactis* | Bondar *et al*. (2005) |
| | *Microsporum gypseum* | Stahl *et al* . (1949) |
| | *Oidium lactis* | Akobe (1936) |
| | *Penicillium caseicolum* | Tsugo and Matsuika (1962) |

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| | | |
|---|---|---|
| | *Penicillium digitatum* | Khalaf and El-Sayed (2009) |
| | *P.citrinum* | Khalaf and El-Sayed (2009) |
| | *P.notatum* | Khalaf and El-Sayed (2009) |
| | *Saccharomyces cerevisiae* | Bonnarme *et al*. (2001) |
| | *Schizophyllum commune* | Challenger (1959) |
| | *Scopulariopsis brevicaulis* | Challenger (1959) |
| | *Trichoderma koningii* | Khalaf and El-Sayed (2009) |
| ***Protozoa*** | *Entamoeba histolytica* | Tokoro *et al*. (2003) |
| | *Porphyromonas gingivalis* | Yoshimura *et al*. (2000) |
| | *Trichomonas vaginalis* | Lockwood and Coombs (1991) |

Table.no.1. List of L-methioninase producing microbes.

According to the wide application of L-methioninase, the search for low expense culture conditions for overproduction of this enzyme is sustainable. Among the different microbes, fungi seem to be the potent organisms for production of enzymes from solid substrates. Unlike bacteria, solid-state fermentation is a selective media for the enzyme production and metabolic activities of fungi (Pandey *et al.*, 1999; El-Sayed, 2008). Solid state fermentation using agro-industrial wastes for production of L-methioninase by fungi seems to be the potent strategy for this purpose. Therefore it is believed that the extensive biochemical and pharmacokinetic characterization of this enzyme from fungi and especially endophytic fungi may significantly contribute in the development of this enzyme based therapy from both therapeutic and economical point of view.

### 2.3. Endophytic Fungi: Resource of anti-cancer compounds

Endophtyic microorganisms are a significant reservoir of genetic diversity, and an important source for the discovery of novel bioactive secondary metabolites. Endophytes are a rich source of natural products displaying a broad spectrum of biological activities (Strobel *et al*., 2004; Tan *et al*., 2001) and the phytochemistry of endophytic microbes continues to increase in significance. A single endophytic strain will produce multiple bioactive (Strobel *et al*., 1993). The reported natural products from endophytic fungus include antibiotics, antipathogens, immunosupressants, anticancer compounds, antioxidant agents and other biologically active substances.

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The endophytic fungi isolated from various plants are a great source of various anticancer agents, Paclitaxel and some of its derivatives represent the first major group of anticancer agents that is produced by the endophytes. Paclitaxel, a highly functional diterpenoid, is found in each of the world's yew (Taxus) species (Suffness, 1995) This compound is the world's first billion dollar anticancer drug. Its presence on the yew tree paved the way towards exploring the endophytes residing in that tree. After several years of efforts, a novel paclitaxel producing endophytic fungus, *T.andreanae ,* was discovered in *T.brevifolia* (Hess *et al*., 1993) . One of the most commonly isolated endophytic fungi from yew tree is *Pestalotiopsis* spp. (Strobel *et al*., 1996). *P.microspora* is the most frequent one. Various other isolates of *P. microspora* isolated from bald cypress in South Carolina were shown to produce paclitaxel (Ford *et al*., 1996). This was the first evidence which showed that paclitaxel producing endophytic fungi were not indigenous to *Taxus spp.*, the same fungus residing in the different plant can produce paclitaxel. Also, a rubiaceous plant, *Maguireothamus speciosus*, yielded a novel fungus, *Seimatoantlerium tepuiense,* that produces paclitaxel. (Strobel *et al*., 1999) the ecological and physiological explanation for the wide distribution of fungi that make paclitaxel seems to be related to the fact that paclitaxel is a fungicide and the organisms with the most sensitivity to it are plant pathogens such as *Pythium spp*. and *Phytophthora spp*. (Young *et al.,*1992). Thus endophytes may be producing paclitaxel to protect their respective host plant from degradation and disease caused by these pathogens.

Torreyanic acid, a selectively cytotoxic quinine dimmer (anticancer agent), was isolated from a *P. microspora* strain. This strain was originally obtained as an endophyte associated with the endangered tree *T. taxifolia* (Florida torreya) (Lee *et al*., 1996). Torreyanic acid was tested in several cancer cell lines, and it was found that it is highly potent and causes cell death by apoptosis.

The alkaloids are also commonly found in endophytic fungi. Such endophytic fungal genera as *xylaria, Phoma, Hypoxoylon, Chalara* are representative producers of a large group of substances known as Cytochalasins. Many of these compounds possess antitumor and antibiotic properties but due to cellular toxicity they have not been developed into pharmaceuticals. Three novel cytochalsians have been reported from a *Rhinocladiella* as an endophyte on *Tripterygium wilfordii* having antitumor activity (Wagenaar *et al*., 2000).

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Lapachol (naphthoquinone) was found to be produced by two of the endophytic fungi *Aspergillus niger* and *Alternaria alternata* isolated from different parts of *Tabebuia argentea* which is an anticancer compound , this investigation reveals that the metabolites produced by a variety of endophytic fungi can be a potential source of novel natural antimicrobials, antioxidants and anticancer agents (Sadananda *et al*., 2011).

Another anticancer agent which has been reported from endophytic fungi *Colleotrichum sp*. is Asparginase enzyme which has the potential to act as a chemothrepuetic agent in human cancer therapy. The fungus was isolated from Thai medicinal plant. (Theantana *et al*., 2009)

### 2.4 Endophytic fungi: Screening L- Methioninase producers

The screening procedures for L-methioninase producing fungi have been reported where the Methionine –glucose medium have been used for their isolation, this media gives the necessary minimal nutrition required and provides methionine as carbon source which if used by fungi as growth substrate will allow its growth. This media allows the growth of even those fungi which can use glucose and grow, therefore to further screen the obligate methionine degraders, 100% methionine basal agar where methionine is the only sole source of carbon, nitrogen and sulphur (Herrera and Starkey, 1969).

#### 2.4.1 Screening procedures for methionilytic activity

The preliminary screening procedure used for finding out the endophytic fungi producing Asparaginase enzyme, incorporated 0.009% Phenol red in the modified Czapek dox media used for screening. Phenol red shows the basic pH change that occurs on the dissimilation of asparagine, a pink zone is seen (Theantana *et al*., 2007). Similarly this technique can be used to study the dissimilation of methionine by L-Methioninase which results in the fall in pH due to the formation of the α-keto butyric acid and hence a pH change will lead to change in color from red to yellow, therefore this method can be implied for the preliminary screening in the present work.

#### 2.4.2 Liquid culture for enzyme production

The fermentation is a metabolic process in many microorganisms and involves oxido-reduction reactions resulting in the breakdown of complex organic compounds into simpler by products and energy; the microbes carry out this metabolic action by the release of

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extracellular enzymes. Culture broths produced on Methionine glucose media using *Aspergillus flavipes* have shown good methioninlytic activity under submerged conditions (El-Sayed and Khalaf, 2009).

### 2.4.3 Qualitative Assay for testing methonilyitc activity

The qualitative assay of the culture filtrate can be carried out by agar well diffusion technique, in which the wells are punctured on the agar containing the substrate and a indicator and the well is loaded with the culture filtrate to be tested. This method has been widely used in the qualitative estimation of enzymes as in the case of L-Asparginase isolated from bacterial cultures having implication is the treatment of Acute Lymphoblastic Leukemia (Jain *et al*., 2012).

### 2.4.4 Spectrophotometric assays

The screening for methioninase producers has been carried out using various spectrophotometric assays, where the determination of products formed after L-methioninase acts on methionine which are ammonia, α-ketobutyric acid and methanethiol. Estimation of ammonia is carried out by Direct Nesslerization method (Thompson and Morrison, 1951), this method was used for the quantitative estimation of L-methioninase produced from *Aspergillus flavipes* (El-Sayed and Khalaf, 2009). Estimation of Keto butyric acid was done by following the method given by Esaki and Soda (1987). The other method is to estimate the amount of Methanethiol formed which is done by the method given by Laasko and Nurmikko (1976), this was used for enzyme activity studies for *Aspergillus flavipes* (El-Sayed, 2010).

### 2.4.5 Direct Nesslerization method -

The process of Nesslerization has been used for determining the amount of ammonia, the assay is highly sensitive. Nessler's reagent (potassium mercuric iodide) readily reacts with ammonia in alkaline conditions to form colloidal dimercuric ammonium iodide. The product is yellow to brown coloured depending on the concentration of ammonia. Hence, the amount of ammonia can be readily calculated spectrophotometrically. The chemical reaction taking place during the ammonia determination is as follows

$$NH_4^+ OH + 2(KI)_2HgI_2 + KOH^- \longrightarrow NH_2HgI_3 + 5KI + 2H_2O$$

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Thus, it is a method which can easily determine micrograms of ammonia, and has been readily used in determination of ammonia in various chemical and biological processes. (Thompson and Morrison, 1951). This assay has been used in the quantitative estimation of L-methioninase and studying the enzyme activity in the case of *Aspergillius flavipes* (Khalaf and El-Sayed, 2009) and *Trichomonas vaginalis* (Coombs and Lockwood, 1991).

### 2.4.6 Precipitation of crude protein

The protein purification is a step wise process where procedure is followed in order to isolate a single type of protein from a mixture of proteins. Protein purification is vital for the characterization of the function, structure and interactions of the protein of interest. The starting material is usually a biological tissue or a microbial culture. The various steps in the purification process may free the protein from a matrix that confines it, separate the protein and non-protein parts of the mixture, and finally separate the desired protein from all other proteins. Separation of one protein from all others is typically the most laborious aspect of protein purification. Separation steps may exploit differences in (for example) protein size, physio-chemical properties, binding affinity and biological activity. There are several methods for protein purification, Ammonium sulphate precipitation and cold acetone precipitation. In bulk protein purification, a common first step to isolate proteins is precipitation with ammonium sulphate $(NH_4)_2SO_4$. This is performed by adding increasing amounts of ammonium sulphate and collecting the different fractions of precipitate protein. Ammonium sulphate can be removed by dialysis. The hydrophobic groups on the protein get exposed to the atmosphere and it attracts other protein hydrophobic groups and gets aggregated. Protein precipitated will be large enough to be visible. One advantage of this method is that it can be performed inexpensively with very large volumes. This method was used for purifying the l-methioninase protein from *Aspergillus flavipes* (El-Sayed, 2010).

The cold acetone precipitation is another commonly used method. Proteins are insoluble in acetone (particularly at low temperature) whilst many small molecules which could interfere with the downstream protein work are soluble. By precipitating proteins in this solvent, the buffer contaminants are removed and proteins get concentrated into the pellet which can be redissolved in the required buffer. The method has been used readily for isolating the protein during enzyme purification and characterization of enzymes from different microbial sources such as production of xyloglucanolytic enzymes by *Trichoderma*

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*viride*,*Paecilomyces farinosus*, *Wardomyces inflatus*, and *Pleurotus ostreatus* (Tribak *et al*., 2002). Thermostable alkaline-active keratinolytic proteinase was isolated from *Chrysosporium keratinophilum* using acetone precipitation. (Dozie *et al.*, 1994).

### 2.4.7 Identification of Endophytic fungi

The identification of the endophytic fungi which are the potential producers of L-Methioninase by various morphological and molecular techniques. Morphological taxonomy has to be carried by microscopic study of the fungal structures such as mycelia, fruiting bodies, spores are very important features as fungal taxonomy is traditionally based on comparative morphological features (Zhang *et al*., 2008). Various optimization of growth conditions have been used in case of the fungi which are non-sporulating in the culture as , they cannot be identified by the conventional techniques otherwise so different media such as Potato dextrose agar(PDA), Water agar (WA), Potato Carrot agar (PCA), Corn meal agar (CMA) have been used in which the fungi sporulate. (Guo *et al*., 2000)

In contrast the molecular techniques exhibit more sensitivity and specificity for identifying microorganisms and can be used to classify the microbes on the diverse hierarchical taxonomic levels (Sette *et al*., 2006). Most of the endophytic fungi are being identified and detected by the comparative analysis of ribosomal DNA sequences especially ITS region. Harney *et al*. (1997) identified arbuscular mycorhizzal fungi from *Artemesia californica* using the ITS (internal transcribed site) region. The 5.85 S ribosomal gene flanking region ITS 1 amd ITS 4 have also been used extensively to identify the fungi.

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# Chapter 3

# Aim of the study

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**Aim of the present study**

To screen endophytic fungal broths for L-methioninase activity.

**Objectives of the present study include the following**

- Production of the liquid culture broth for qualitative screening preliminary screening for L-Methioninase activity.
- Quantitative Estimation of L-methioninase activity.
- Identification of endophytic fungi having potential of producing L-methioninase.

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# Chapter 4

# Materials & Methods

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**4.1. Sub culturing and maintenance of pure cultures**

This involved preparation of Potato Dextrose Agar (PDA) plates, sub culturing of the endophytic fungal cultures on PDA plates and long term preservation of pure isolates

**4.1.1. Preparation of Potato Dextrose Agar (PDA) Plates**

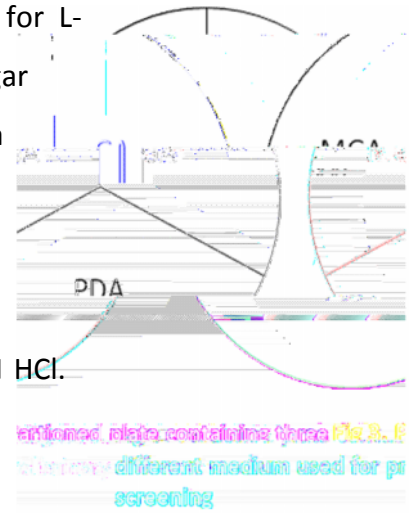
39.0 g of PDA (Hi Media) was dispensed in liter lukewarm double distilled water and stirred thoroughly. This was then dispensed in 250 ml Erlenmeyer Flasks and autoclaved at 121°C, 15 psi for 15 minutes. Glass Petri plates were sterilized at 121°C, 15 psi for 20 minutes. Then under sterile conditions 25 ml of the autoclaved PDA was dispensed in sterile 90 mm Petri plates and allowed to solidify at room temperature. The plates were stored in incubator at the temperature 26 ± 2°C until further use.

**4.1.2. Sub culturing of the test endophytic cultures**

50 endophytic fungi isolated from *Aegle marmelos, Cinnamomum malabaricum, Cinnamomum zeylanicium* and *Cinnamomum camphora* were aseptically subcultured on sterile PDA plates and incubated at 26°C for 7-10 days under 12 hrs of photoperiod. The pure cultures thus obtained were transferred and maintained on PDA slants at 28°C. Sub culturing was done after regular intervals of time. The endophytic cultures were preserved on PDA slants with 15 % glycerol for long term storage at 28°C.

**4.2 Preliminary screening**

The preliminary screening of the endophytic fungal cultures for L-methioninase production was done on Methionine Glucose Agar media (Herrera and Starkey, 1969) and glucose agar media supplemented with 0.009% phenol red as an indicator. Methionine and phenol red were filter sterilized using nitrocellulose membrane syringe filters having pore size 0.22µM. The pH of the media was adjusted at 7 using 1N NaOH and 1N HCl. Endophytic fungal isolates were inoculated in each of the quadrate containing three different media i.e. MGA, GA and PDA. The plates were incubated at 28°C and the observations were recorded after 48 hours for presence of yellow zone formed due to the formation α-ketobutyric acid. in case



different medium used for pr
screening

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methionine is being metabolized by the change in color of the plate from red to yellow due to presence of phenol red indicator which is red in basic condition and turn yellow under acidic condition. The isolates showing yellow zone were selected for primary screening.

| S.no. | Components | Concentration (g/l) |
|---|---|---|
| 1. | L-Methionine | 0.5 |
| 2. | Glucose | 1 |
| 3. | $KH_2PO_4$ | 1 |
| 4. | $K_2HPO_4$ | 1 |
| 5. | $MgCl_2.6H_2O$ | 0.5 |
| 6. | $CaCl_2.2H_2O$ | 0.1 |
| 7. | $FeCl_3.6H_2O$ | 0.02 |
| 8. | $ZnCl_2$ | 0.02 |
| 9. | Agar | 20 |

Table no 2. Chemical composition of Methionine Glucose Agar (MGA) medium.

**4.3. Preliminary screening**

The endophytic cultures that showed color change in preliminary screening were further subjected for primary screening. The primary screening was carried out using basal agar media having Methionine as sole energy source at concentration of 0.5 g/l complemented with and phenol red at final concentration of 0.009%, the pH of the media was adjusted to 7. The MA plates were inoculated with the endophytic fungal cultures that were found positive during preliminary screening. The plates were incubated at 28°C and presence of yellow zone was recorded for 5 to 7 days. The diameter of the zone was recorded (Theantana *et al.,* 2007)

**4.4. Production of culture filtrate**

The endophytic fungal cultures which showed consistent utilization of methionine on the methionine agar plates were selected for the production of culture filtrates. The submerged fermentation was carried out in the Methonine Glucose broth (MGB) (Herrera and Starkey, 1969). Spore suspension was made from 7 day old plate using sterile physiological saline.

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100 ml of MGB broth was inoculated with 1 ml of spore suspension with CFU $10^8$. The fermentation flasks were incubated on orbital shaker at 120 rpm, 26°C for 10 days. (El-Sayed and Khalaf, 2009). After 10 days the filtration was carried out in order to separate the biomass from the broth using Whatman filter paper and further centrifugation at 10,000 rpm for 15 minutes, The supernatant left is the cell free culture filtrate which is kept for further testing.

**4.6. Qualitative estimation**

The potential of culture filtrates to produce L-methioninase was done by the agar well diffusion assay provided with methionine as sole energy source. 4 mm thick Methionine Agar plates (refer table no. 3 for composition) were prepared by pouring 22.5 ml of molten MA into 90 mm sterile petri plates and allowed to solidify. 5 mm wells were punched using sterile cork borer. 30 µl of cell free culture filtrate was loaded in the wells; the plates were sealed and kept in upright position at 37°C for next 48 hours. The plates were observed for yellow zone formation after 48 hours (Jain *et al*., 2012).

**4.7 Protein precipitation**

Protein precipitation was carried out by cold acetone precipitation method. Acetone was stored at -20°C for 24 hours. Cold acetone was added into the sample in the ratio 4:1. The samples were incubated at -20°C for 1hrs. Then the samples were centrifuged at 13,000 g for 10 minutes. The supernatant was discarded and the pellet was air dried inside laminar. The pellet was resuspended in the Citrate Phosphate Buffer (pH=7) and stored at -20°C till further use. (Tribak and Garcı´a-Romera, 2002)

Composition of Citrate phosphate Buffer-

43.6 ml of 0.2M $Na_2HPO_4$ and 6.5 ml of 0.1M Citric acid were mixed and pH was adjusted at 7 and the volume was made up to 100 ml with double distilled water.

**4.8 Protein Estimation**

Protein content was evaluated using Lowry's method. Briefly describing, 0.2 ml of the crude protein solution was pipetted out and 2ml of the analytical reagent [a) 50 ml of the 2% sodium carbonate mixed with 50 ml of 0.1 N NaOH, b) 10 ml 1.56% $CuSO_4$ was mixed with 10 ml of 2.37% sodium potassium tartarate; mix 2 ml of (b) with 100 ml of

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(a)] was added, vortexed and incubated at room temperature for 10 mins. To this solution 0.2 ml of 1N Folin reagent (freshly prepared by diluting 2N with equal volume of water) was added and kept at room temperature for 30 mins. 200 µl of each sample was dispensed into 96 well titre plate and absorbance was taken at 660 nm. All the readings were taken in triplicate. A standard curve of BSA (Himedia) was prepared ($R^2$ =0.98) against which the values of the unknown protein was plotted. Different dilutions of BSA solutions were prepared by mixing stock BSA solution (1 mg/ ml) and water in the test tube as given in the table 3. The final volume in each of the test tubes was 5 ml. The BSA range was 0.05 to 1 mg/ ml. Protein concentration of the unknown samples were thus estimated (Lowry *et al.,* 1951).

| S.no. | BSA (ml) | Water(ml) | Concentration(mg/ml) |
|---|---|---|---|
| 1. | 0.25 | 4.75 | 0.05 |
| 2. | 0.5 | 4.5 | 0.1 |
| 3. | 1 | 4 | 0.2 |
| 4. | 2 | 3 | 0.4 |
| 5. | 3 | 2 | 0.6 |
| 6. | 4 | 1 | 0.8 |

Table 3. Different concentration of BSA during Standard Curve preparation

**4.8 Quantitative estimation**

The quantitative estimation was carried out using the direct Nesslerization method. In this method the amount of ammonia produced is estimated using the Nessler's reagent. The standard curve of ammonia was plotted in order to compare the amount of ammonia produced in the crude protein samples precipitated from the cultures of interest. The standard was prepared by adding 1.179 g of ammonium sulphate in 100 ml of double distilled water. This solution was further diluted by taking 1.4 ml of the solution and making up the volume to 100 ml using double distilled water, this solution contains 1µ mole of $NH_3$ per ml. (main stock)

Further different concentrations of ammonia were formed by diluting it with water. Two stocks of 10 ml were made.

Stock I- 1 µmol/ml (140 µl of 100ml main stock + 9.86 ml water)

Stock II- 2 µmol/ml (280 µl of 100ml main stock + 9.72 ml water)

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| S.no. | Concentration of ammonia | Preparation of ammonia dilutions |
|---|---|---|
| 1. | 0.25 µmol/ml | 750µl of water+ 250µl of Stock I |
| 2. | 0.5 µmol /ml | 500µl water + 500 µl Stock I |
| 3. | 0.75 µmol/ml | 250µl water+ 750µl Stock I |
| 4. | 1.25 µmol/ml | 375µl water+625µl of Stock II |
| 5. | 1.5 µmol/ml | 250µl water+ 750µl of Stock II |
| 6. | 1.75 µmol/ml | 125µl of water + 875µl of Stock II |

Table 4. Represents different concentration of ammonia used during Nesslerization process

To each concentration having the volume 1ml, 0.5 ml Nessler's reagent was added and water was taken as control and absorbance was taken at 450 nm.

The Nesslerization procedure carried out with the samples is as follows-

1ml of 1% Methionine + 0.1 ml of 100µM Pyridoxial Phosphate + 1ml crude enzyme was added in the falcon tube. The mixture was incubated at 37°C for 1 hour. The activity of the enzyme was stopped by adding 0.ml of 1.5 M Tricholroacetic acid. The reaction mixture was centrifuged at 5000 rpm for 5 minutes to precipitate the protein. To this reaction mixture, 0.5 ml Nessler's reagent was added and the absorbance was taken at 450nm. All the readings were taken in triplicates and mean absorbance was used to calculate the enzyme units (El-Sayed and Khalaf, 2009).

### 4.9 Identification of the endophytic fungi producing L-methioninase

Endophytic fungal isolates that were producing L-methioninase was identified using morphotaxonomy (Classical approach) and molecular taxonomy.

#### 4.9.1 Morphotaxonomy

The endophytic fungal isolates which were showing positive results for being the potential producers of L methioninase were examined under the microscope so, as to characterize the isolate on the basis of their microscopic characters and morphology. The cultures were grown on different media namely Potato dextrose Agar (PDA), Corn meal agar (CMA), Water agar (WA), Grass leaf agar (GLA), Synthetischer nahrstoffarmer agar (SNA).Briefly describing, the glass slide was cleaned with alcohol and dried. A drop of water was put on glass slide.

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upon which the mycelial mass that was taken from the tip of the colony using a fine tipped needle was placed along and teased properly. It was then stained with Lactophenol cotton blue (Hi Media). The slide was covered with 18X 10 mm coverslip avoiding the formation of air bubble and mounted with DPX. The slide was microscopically observed at 100X, 400X and 1000X using Nikon Stereozoom microscope and Nikon binocular microscope. The fungi were identified based upon their spore structure and other morphological characteristics.

**4.9.2 DNA Isolation**

The fungal genomic DNA isolation was isolated 3-4 day old culture grown on PDA. 0.5-1 g wet mycelium was grounded into very fine powder by crushing with liquid nitrogen. 660-750 µl of the extraction buffer was added and the biomass was crushed again. The contents were transferred to a 1.5 ml microcentrifuge tube and 10µl of β-mercaptoethanol and 4µl of Proteinase K was added to each tube. The contents were vortexed and incubated at 65°C in water bath for 1 hour, they were mixed after every 15 minutes. After the incubation was over, the microcentrifuge tubes were centrifuged at 10,000 rpm for 15 minutes so as to remove cell debris. Further 6 µl of RNase was added to each tube and incubated at 37°C for 30 minutes. For the removal of protein contents, Equal volume of Phenol: Chloroform (1:1) solution was added to each tube and mixed properly for 15 mins and centrifuged at 12,000 rpm for 10 minutes, this step was repeated three times. Transfer the aqueous layer containing DNA to the fresh microcentrifuge tube carefully avoiding the inclusion of debris and other impurities along with it. Then added 20 µl of 3M sodium acetate and. The content of each microcentrifuge tube was top up with absolute ethanol and incubated at -4° C overnight. Mix the contents by swiftly inverting the tubes so as to observe the white threads of precipitating DNA. On the next day, the microcentrifuge tubes were centrifuged at 12,000 rpm for 10 minutes; the pellet was washed again with 70% ethanol, and centrifuged at 12,000 rpm for 5 minutes. The pellet was air dried and dissolved in 30 µl of Tris EDTA buffer (pH=8).

The qualitative estimation of the DNA isolated was done by Agarose Gel electrophoresis.

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#### 4.9.2.1. Agarose gel electrophoresis

0.8% agarose gel having ethidium bromide (Et Br) at the concentration 0.5 µg /ml was made in 1X Tris Acetate EDTA ( pH=8 ) and casted in the electrophoretic apparatus along with 8 chanbered comb. The gel was allowed to solidify and the comb was carefully removed. Electrophoretic running buffer (1 X TAE) was put into the tank so that the gel is fully immersed into the buffer. The DNA samples were mixed with the 5 X loading dye. The samples were loaded into wells and allowed to run at 50 volts. The gel was observed under UV transilluminator for the presence of DNA. Gel imaging was performed under UV light in Bio- Rad Gel documentation System using Quantity-1-D analysis software.

Quantitative Estimation of the Genomic DNA was done by the Spectrophotometric analysis of the sample. The absorbance of the sample was taken at 260 nm, to determine the concentration of the sample. 1 OD is equivalent to 50µg/ml DNA sample so, accordingly the concentration of the sample is calculated.

$$\text{Concentration (µg/ml)} = \text{O.D}_{260\text{nm}} \text{ X 50 µg/ml X Dilution}$$

The purity of the sample was checked by taking the ratio of its absorbance at 260 nm and 280 nm. The ratio if is less than 1.6 , then there is RNA contamination , if the ratio lies between 1.2-1.6 then DNA sample is considered to be free from RNA and protein . If the ratio is more than 1.8, the DNA might be contaminated with proteins or some other particulate matter.

#### 4.9.3 PCR Amplification

PCR is a rapid process for *in vitro* amplification of desired DNA sequence by using specific primer so as to produce a large amount of desired DNA fragment of defined sequence length. The PCR reaction mixture methodology is as follows. ITS1, 5.8S, ITS2 rDNA sequence was amplified using ITS 1 and ITS 4 primers, synthesized by Integrated DNA Technologies (IDT), USA, in a Thermocycler (My Cycler, Bio-Rad Laboratories, Inc.). PCR reaction was carried out by using the primers ITS1 (5' TCC GTA GGT GAA CCT GCG G 3') AND ITS 4 (5' TCC TCC GCT TAT TGA TAT GC 3') (White *et al*., 1990). Amplification was performed in 25µl reaction mixture containing 1µl of extracted fungal DNA, 10 µM of each primer (ITS1 and ITS4), 2.5mM of dNTP (Bangalore GeNei), 25 mM $MgCl_2$ (Bangalore GeNei), 1.5 U of Taq DNA Polymerase (Bangalore GeNei) in 10 X Taq buffer (Bangalore GeNei). The PCR cycling

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conditions consisted of initial denaturation at 96ºC for 5 min followed by 39 cycles of 95ºC for 45 sec, 60ºC for 45 sec, 72ºC for 45 sec followed by final extension at 72ºC for 5 mins. The PCR products were examined using gel electrophoresis in a 1.5 % agarose gel dissolved in 1X TAE buffer at 40V for 1.30 hr. Gel imaging was performed under UV light in Bio- Rad Gel documentation System using Quantity-1-D analysis software. An approximate 550- 600 bp PCR product was purified by using the Wizard® SV Gel and PCR clean up system kit (Promega, USA).

| S.no | Reagents | Stock concentration | Quantity | Final concentration 25µl |
|---|---|---|---|---|
| **1.** | Sterile double distilled water | _ | 15µl | _ |
| **2.** | Taq buffer | 10 X | 2.5µl | 1X |
| **4.** | dNTPs | 2.5mM | 2.0µl | 0.2mM |
| **5.** | Primers | 10µM | 2.0µl | 0.8 µM |
| **6.** | Taq DNA Polymerase | 3U/µl | 0.5 µl | 1 U |
| **7.** | Template DNA | 25 ng/µl | 1.0 µl | 25ng |

Table 5. Different reagents used during PCR reactions.

The amplification of DNA was done by using the temperature profile given in Table 6

**TEMPERATURE PROFILE**

| STEP | TEMPERATURE | TIME |
|---|---|---|
| STEP I : Initial Denaturation | 96°C | 5 min |
| STEP II : Denaturation | 95°C | 45 sec |
| STEP III : Annealing | 60°C | 45 sec |
| STEP IV: Extension | 72°C | 45 sec |
| STEP V | Step II to Step IV repeated 39 times | |
| STEP VI : Final extension | 72°C | 5min |
| STEP VII: Store | 4°C | ∞ |

Table 6. Showing temperature profile of PCR reaction

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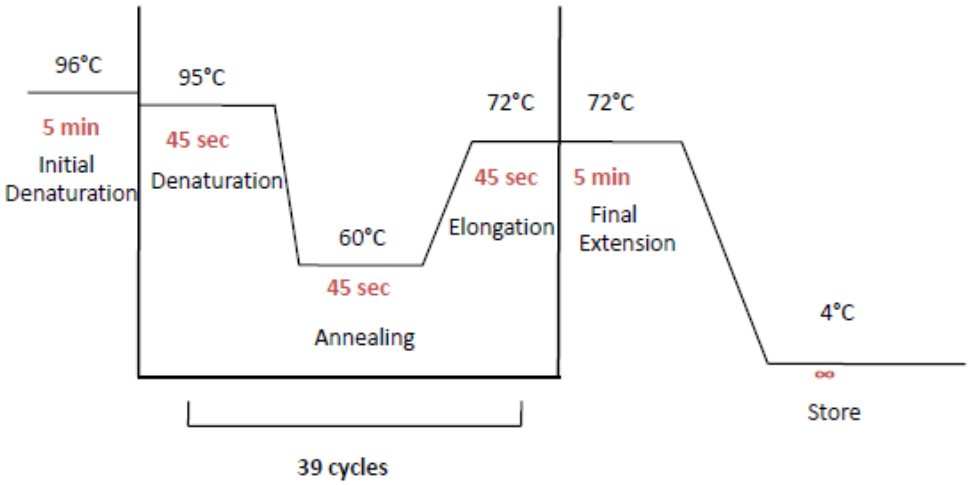


Fig 4. Ray diagram of temperature profile of PCR reaction.

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# Chapter 5

# Results & Discussion

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**5.1 Reculturing**

50 endophytic isolates were maintained in pure form by regular reculturing on Potato dextrose agar at 28° C, the cultures were stored in the PDA slants at 28° C so, that they can be preserved for long term use .The long term storage was carried in the form of PDA slants with 15 % glycerol. Fig.5 shows sub culturing of endophytic isolates and Fig.6 shows the long term storage of endophytic cultures on PDA slants. In the present work, the endophytic isolates from two medicinally important plants in Indian Pharmacopeia namely *Aegle marmelos* and *Cinnamomum* sp belonging to Lauraceae and Rutaceae family collected from biodiversity hot spots of India and the Western Ghats of North east Himalayas were used. These plants have been known to have great medicinal value from the ancient times, hence are expected to have some novel endophytic fungi which can be potential producer of some bioactive metabolite and enzyme that can be used to develop a drug from a natural source. PDA is an energy rich medium on which the fungi can be maintained viable for more than 4-5 months.

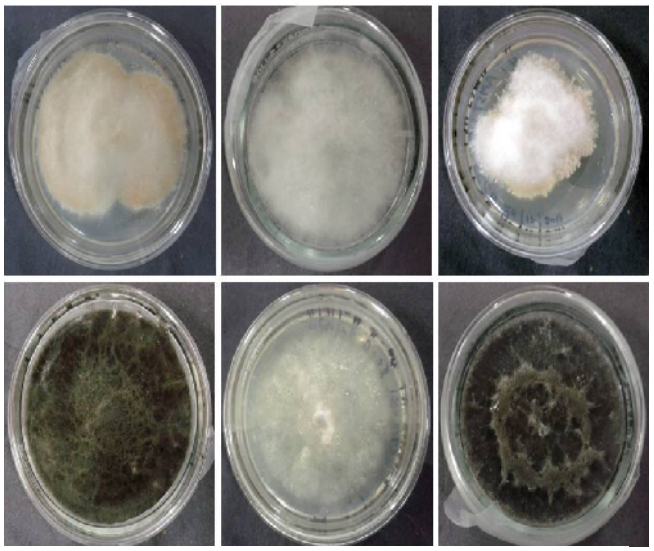
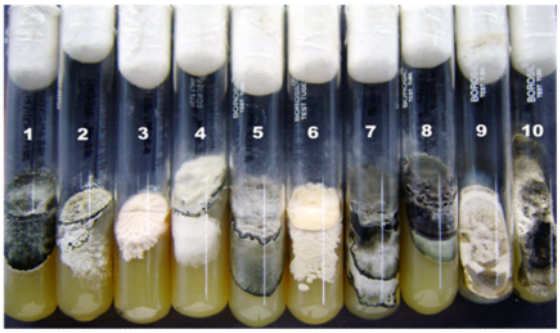


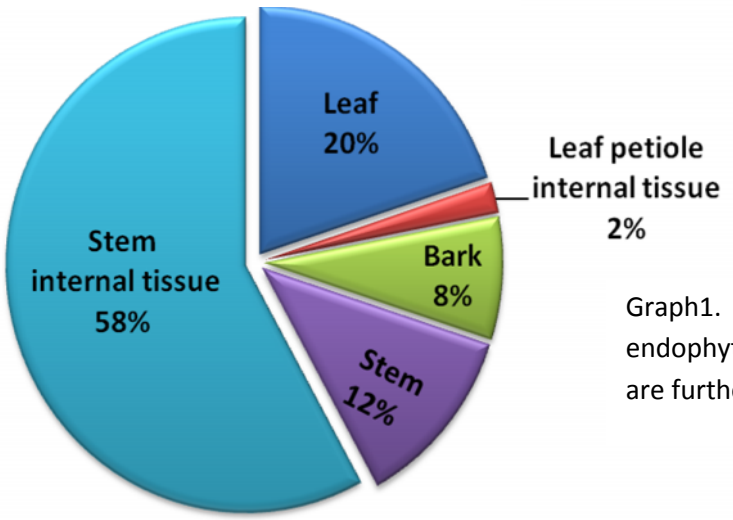
Fig. 5 Pure endophytic fungal isolates on PDA plates




PDA slants of the isolated endophytic fungi

1= # 11 AMBAWLS
2= #10 AMSTITYEL
3= #6 AMLWLS
4= # 1088 AMSTITWLS
5= #1048 AMSTITYEL
6= #9(B) AMSTYEL
7= #1111 AMSTITWLS
8= # 1118 AMSTITWLS
9= #1010 AMSTITYEL
10= # 1013 AMSTITWLS

Fig.6 Preservation of endophytic fungi on PDA Slants for long term storage




Graph1. Pie chart showing distribution of endophytic mycoflora on host plant part that are further used

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**5.2 Preliminary Screening**

All 50 endophytic isolates comprising 26 from *Aegle marmelos* out of which 7 from leaf, 1 from bark, 3 from stem and 15 were from the internal stem tissue, 11 from *Cinnamomum malabaricum* out of which 3 were from leaf, 1 from leaf petiole internal tissue, 3 from bark and 4 were from the internal stem tissue, 7 from *Cinnamomum zeylanicum* out of which 2 were from bark and 6 were from the internal stem tissue of *Cinnamomum camphora* were used. These were subjected for preliminary screening, in which their potential to utilize methionine as a nutrient was screened. Out of the 50 isolates 25 were selected on the basis of their methionilytic activity on MGA after 48, 72, and 96 hours, the yellow zone formation was recorded and growth was compared to PDA colony, in order to find out the effect of Methionine on the colony characteristics. Table 7 represents the data recorded during the preliminary screening on the basis of which the 25 isolates were selected for primary screening to screen the Methionine decomposers and Fig 7 shows the yellow halo formation during preliminary screening. The isolates which the persistent yellow zone formation after 48 hours on MGA and GA were selected. The isolates were allowed to grow on the methionine glucose agar (Herrera and Starkey, 1969),in order to compare the growth pattern on MGA, simultaneously the isolates were grown on Glucose agar , which had the same composition as MGA except it lacked methionine and PDA (as a positive control) to observe for the growth pattern on MGA. MGA and GA were both supplemented with 0.009% phenol red (Theantana *et al*., 2007) which acts as a pH indicator, the acid production takes place in both the cases, methionine utilization produces α- keto butyric acid where as glucose utilization produces organic acids. Herrera and Starkey, in 1969 reported that glucose was decomposed by the fungi in 3 days and deaminated about two-third of the methionine. So in order to find the methionine decomposers the concentration was glucose was reduced to 1 g/l from 10g/l as it was done by Herrera and Starkey,, 1969, where they varied the concentration of glucose from 0.01 to 0.1%.

.

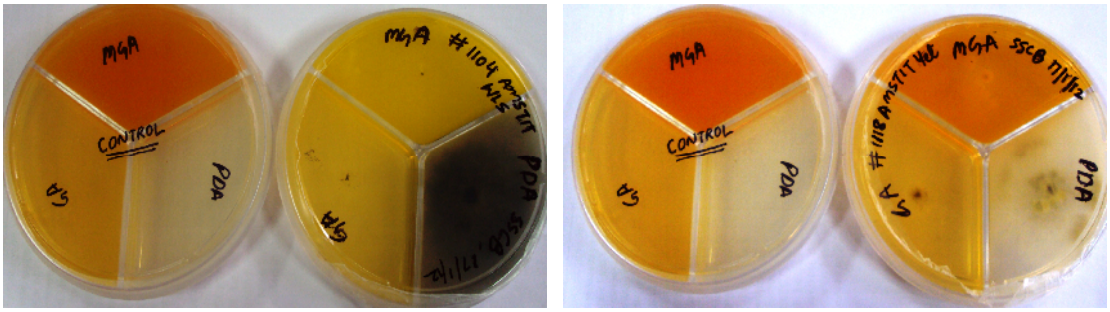


Fig 7. Showing the both positive and negative result in the preliminary screening process where MGA region in the plate 1 is yellow but not in the case of plate 2. PDA and GA media are acting as controls for studying the growth pattern.

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| S. no. | Culture Code | Plant Part | Host Plant | Place of sampling | Activity on MGA (in hours) | | |
|---|---|---|---|---|---|---|---|
| | | | | | 48 | 72 | 96 |
| 1. | #6AMLWLS | Leaf | *Aegle marmelos* | Wayand wild life scantury, Kerela | + | + | + |
| 2. | #16AMLWLS | Leaf | *Aegle marmelos* | Wayand wild life scantury, Kerela | - | - | - |
| 3. | #9AMLBRT | Leaf | *Aegle marmelos* | BRT wild life Scantury, karnataka | + | + | + |
| 4. | #1007AMLBRT | Leaf | *Aegle marmelos* | BRT wild life Scantury, karnataka | -- | -- | -- |
| 5. | #1006AMLBRT | Leaf | *Aegle marmelos* | BRT wild life Scantury, karnataka | -- | -- | -- |
| 6. | #1005AMLBRT | Leaf | *Aegle marmelos* | BRT wild life Scantury, karnataka | ++ | ++ | ++ |
| 7. | #1016AMLBRT | Leaf | *Aegle marmelos* | BRT wild life Scantury, karnataka | ++ | ++ | ++ |
| 8. | #2028AMBARS | Bark | *Aegle marmelos* | Rohini sukhna, West Bengal | -- | -- | -- |
| 9. | #7AMSTYEL | Stem | *Aegle marmelos* | Yelander, Kerela | +++ | +++ | +++ |
| 10. | #9(b)AMSTYEL | Stem | *Aegle marmelos* | Yelander, Kerela | +++ | +++ | +++ |
| 11. | #22AMSTYEL | Stem | *Aegle marmelos* | Yelander, Kerela | ++ | ++ | ++ |
| 12. | #1104AMSTITWLS | stem internal tissue | *Aegle marmelos* | Wayand wild life scantury, Kerela | +++ | +++ | +++ |
| 13. | #1079AMSTITYEL | stem internal tissue | *Aegle marmelos* | Yelander, Kerela | +++ | +++ | +++ |
| 14. | #1088AMSTITWLS | stem internal tissue | *Aegle marmelos* | Wayand wild life scantury, Kerela | +++ | +++ | +++ |
| 15. | #1118AMSTITYEL | stem internal tissue | *Aegle marmelos* | Yelander, Kerela | -- | -- | -- |
| 16. | #1048AMSTITYEL | stem internal tissue | *Aegle marmelos* | Yelander, Kerela | ++ | ++ | ++ |
| 17. | #1013AMSTITYEL | stem internal tissue | *Aegle marmelos* | Yelander, Kerela | | | |

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| | | | | | | | |
|---|---|---|---|---|---|---|---|
| 18. | #1070AMSTITYEL | stem internal tissue | *Aegle marmelos* | Yelander, Kerela | ++ | ++ | ++ |
| 19. | #1069AMSTITYEL | stem internal tissue | *Aegle marmelos* | Yelander, Kerela | -- | -- | -- |
| 20. | #1022AMSTITYEL | stem internal tissue | *Aegle marmelos* | Yelander, Kerela | -- | -- | -- |
| 21. | ##1010AMSTITYEL | stem internal tissue | *Aegle marmelos* | Yelander, Kerela | -- | -- | -- |
| 22. | #20AMSTITWLS | stem internal tissue | *Aegle marmelos* | Wayand wild life scantury, Kerela | -- | -- | -- |
| 23. | #2004AMSTITRS | stem internal tissue | *Aegle marmelos* | Rohini Sukhna, West Bengal | -- | -- | + |
| 24. | #1003AMSTITYEL | stem internal tissue | *Aegle marmelos* | Yelander, Kerela | -- | -- | -- |
| 25. | #1032AMSTITYel | stem internal tissue | *Aegle marmelos* | Yelander, Kerela | +++ | +++ | +++ |
| 26. | #1082AMSTITYel | stem internal tissue | *Aegle marmelos* | Yelander, Kerela | ++ | ++ | ++ |
| 27. | #17CMLNEY | Leaf | *Cinnamomum malabaricum* | *Neyyar, Kerela* | -- | -- | -- |
| 28. | #40CMLBRT | Leaf | *Cinnamomum malabaricum* | BRT wild life Scantury, karnataka | + | ++ | ++ |
| 29. | #29CMLNEY | Leaf | *Cinnamomum malabaricum* | *Neyyar, Kerela* | -- | -- | -- |
| 30. | #31CMLPITNEY | leaf petiole internal tissue | *Cinnamomum malabaricum* | *Neyyar, Kerela* | -- | -- | -- |
| 31. | #19CMBANEY | Bark | *Cinnamomum malabaricum* | *Neyyar, Kerela* | ++ | ++ | ++ |
| 32. | #20CMBANEY | Bark | *Cinnamomum malabaricum* | *Neyyar, Kerela* | +++ | +++ | +++ |
| 33. | #23CMBABRT | Bark | *Cinnamomum malabaricum* | BRT wild life Scantury, karnataka | -- | -- | -- |
| 34. | #1CMSTITBRT | stem internal tissue | *Cinnamomum malabaricum* | BRT wild life Scantury, karnataka | -- | -- | -- |
| 35. | #43CMSTITBRT | stem internal tissue | *Cinnamomum malabaricum* | BRT wild life Scantury, karnataka | -- | + | + |
| 36. | #50CMSTITBRT | stem internal tissue | *Cinnamomum malabaricum* | BRT wild life Scantury, karnataka | +++ | +++ | +++ |
| 37. | #1622CMSTITNEY | stem internal tissue | *Cinnamomum malabaricum* | *Neyyar, Kerela* | | | |

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| | | | | | | | |
|---|---|---|---|---|---|---|---|
| 38. | #5CZBAWLS | Bark | *Cinnamomum zeylanicum* | Wayand wild life scantury, Kerela | + | + | + |
| 39. | #28CZBAG | Bark | *Cinnamomum zeylanicum* | Guwahati, Assam | -- | -- | -- |
| 40. | #23CZSTITG | stem internal tissue | *Cinnamomum zeylanicum* | Guwahati, Assam | + | + | + |
| 41. | #2130CZSTITG | stem internal tissue | *Cinnamomum zeylanicum* | Guwahati, Assam | -- | -- | + |
| 42. | #2116CZSTITG | stem internal tissue | *Cinnamomum zeylanicum* | Guwahati, Assam | -- | -- | -- |
| 43. | #2016CZSTITG | stem internal tissue | *Cinnamomum zeylanicum* | Guwahati, Assam | -- | -- | -- |
| 44. | #2125CZSTITG | stem internal tissue | *Cinnamomum zeylanicum* | Guwahati, Assam | -- | -- | -- |
| 45. | #6610CCSTITD | stem internal tissue | *Cinnamomum camphora* | Tiger hIlls, Darjeeling, WB | -- | -- | -- |
| 46. | #1CCSTITD | stem internal tissue | *Cinnamomum camphora* | Tiger hIlls, Darjeeling, WB | -- | -- | -- |
| 47. | #2CCSTITD | stem internal tissue | *Cinnamomum camphora* | Tiger hIlls, Darjeeling, WB | -- | -- | -- |
| 48. | #36CCSTITD | stem internal tissue | *Cinnamomum camphora* | Tiger hIlls, Darjeeling, WB | -- | + | ++ |
| 49. | #48 CCSTITD | stem internal tissue | *Cinnamomum camphora* | Tiger hIlls, Darjeeling, WB | -- | - | -- |
| 50. | #4CCSTITD | Stem | *Cinnamomum camphora* | Tiger hills, Darjeeling WB | -- | -- | -- |

Table 7. Preliminary screening of endophytic fungal isolates for L-methioninase activity on MGA plates.

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**5.3 Primary Screening**

25 endophytic cultures selected from preliminary screened were screened further for their methioninlytic activity on the Methionine agar plates with phenol red at 0.009% final concentration. The cultures grow slowly as only methionine was the sole carbon, nitrogen, sulphur source. In order to find out the potent methionine degraders, which could utilize it as the sole source of nutrition in the absence of glucose and other minimal salts, they were grown on only Methinone (Herrera and Starkey, 1969) and then the isolates which grew were shifted to MGA.

Out of twenty five, six isolates were found to be potent methionine degraders which showed the consistent yellow zone formation for a week or so. There were some cultures which showed yellow zone formation but the zone started disappearing, these isolates were not selected as they were not obligate methionine decomposers. Fig 8 show the zone formation on the methionine agar plates and all the Zone sizes are recorded in Table 8 and Graph 1 clearly shows the zone size (in mm) comparison for each culture .

During the qualitative estimation of Asparaginase enzyme, out of all the endophytic isolates 82 showed the positive result for the plate assay and 62 showed the pink zone around the colonies and 21 showed the pink zone within the colony. The colonies showing the largest zone were considered to have the maximum potential. The method is easy and asparaginase production can be easily examined by plate examination. It can help in further screening the fungi by spectrophotometric assays (Theantana *et al.,*2009). So, accordingly only those cultures were selected for spectrophotometric assay and protein precipitation which showed consistent zone formation for eg. #1088 AMSTITWLS and #5 CZBAWLS, #40 CMLBRT etc.

| S.no | Culture code | Zone size (mm) | | |
|---|---|---|---|---|
| | | 72 hrs | 96hrs | 144 hrs |
| 1. | #22 AMLBRT | 20 | 90 | 32 |
| 2. | #7AMSTYEL | 30 | 36 | 30 |
| 3. | #1032AMSTITYEL | 5 | 8 | -- |
| 4. | #1082AMSTITYEL | 9 | 9 | -- |
| 5. | #9(b)AMSTITYEL | 16 | 15 | 20 |
| 6. | #1104AMSTITWLS | 25 | 25 | 45 |
| 7. | #1079AMSTITYEL | 24 | 24 | 20 |
| 8. | #1088AMSTITWLS | 40 | 70 | 90 |
| 9. | #1016AMLBRT | 20 | 22 | 23 |
| 10. | #1005AMLBRT | 20 | 26 | -- |

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| | | | | |
|---|---|---|---|---|
| 11. | #50CMSTITBRT | 33 | 37 | 50 |
| 12. | #19CMBANEY | -- | -- | -- |
| 13. | #17CMSTITNEY | -- | -- | -- |
| 14. | #20CMBANEY | 20 | 40 | 50 |
| 15. | #5CZBAWLS | 17 | 35 | 90 |
| 16. | #23CZSTITG | 15 | 25 | -- |
| 17. | #2130CZSTITG | 29 | -- | -- |
| 18. | #40CMLBRT | 14 | 30 | 40 |
| 19. | #1CMSTITBRT | -- | -- | -- |
| 20. | #2125CZTITG | 10 | -- | -- |
| 21. | #29CMLNEY | -- | -- | -- |
| 22. | #43CMSTITBRT | 6.5 | -- | -- |
| 23. | #2028AMBARS | 19.5 | | -- |
| 24. | #2106CZSTITG | 12 | 20 | -- |
| 25. | #36CCTITD | -- | -- | -- |

Table.8. Represents the zone size (mm) after 72, 96, 144 hrs. grown on MA plates .

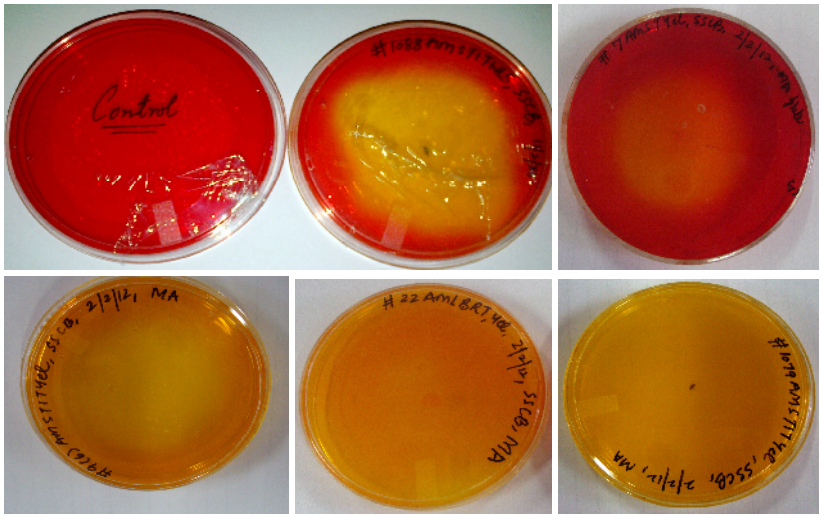
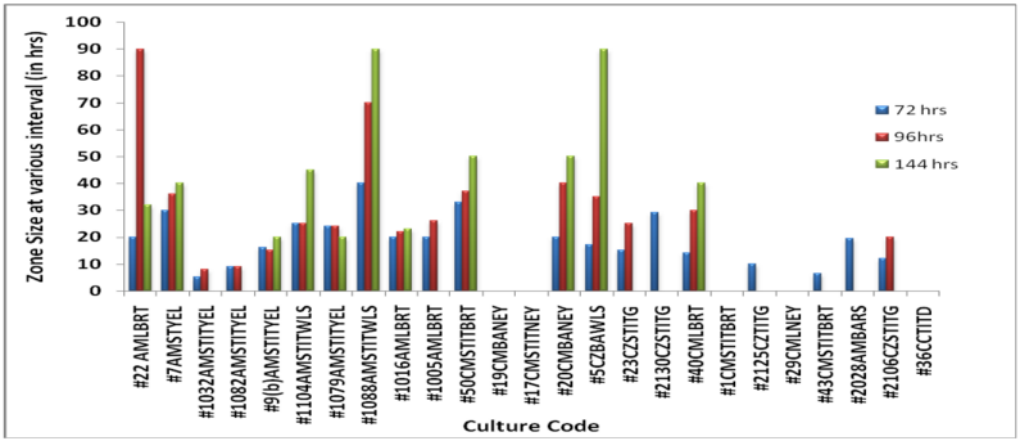



Fig.8 shows the yellow zone formation in case of methionine agar plates.




Graph.2 Showing yellow colored zone size (in mm) after 72 hrs, 96, hrs and 144 hrs of incubation on MA plates.

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### 5.4 Production of Culture filtrates

Six endophytic fungal cultures coded as #1088 AMSTITWLS, #20 CMBANEY, #50 CMSTITBRT, #1104 AMSTITWLS, #5 CZBAWLS, #40 CMLBRT were selected for production of culture filtrates because it showed persistent yellow zone formation. The fungal isolates were screened for their L methioninase productivities using methionine glucose liquid medium. (Herrera and Starkey, 1969) at 28°C for 6 days for 25 ml culture. The biomass produced was calculated in order to trace the growth rate. Table 9 gives the biomass production for all the six cultures.

| S.no. | Culture Code | Biomass Production (g) |
|---|---|---|
| 1. | #1088 AMSTITWLS | 7.45 |
| 2. | #20 CMBANEY | 5.09 |
| 3. | #50 CMSTITBRT | 6.16 |
| 4. | #1104 AMSTITWLS | 3.88 |
| 5. | #5 CZBAWLS | 5.18 |
| 6. | #40 CMLBRT | 5.11 |

Table 9. Showing the biomass production in culture filtrate production.

#1088AMSTITWLS showed the highest amount of biomass production, where as #1104AMSTITWLS showed the least one. The biomass production can be taken as the parameter to denote the extent upto which the cells are using the available nutrients under the given set of conditions of pH, temperature etc. In the case of *Aspergillus flavipes*, the enzyme production showed the dependency upon the initial pH of the production medium, which was optimized at 7 also, it was elucidated that the productivity of L-methioninase by different fungal isolates is directly proportional to the rate of methionine uptake but may not to the biomass of the organism (El-Sayed and Khalaf, 2009).The cell free culture filtrate produced after the centrifugation and filtration were stored at -20°C till put in further use.

### 5.5 Qualitative Estimation of L-methioninase activity by culture filtrates

Agar well diffusion method was used to qualitatively estimate L-methioninase production. The zone sizes were recorded and accordingly the potential of the culture to produce the enzyme. Table no. 10 represents the zone size (in mm) for all six culture filtrates. Zone size is directly proportional to the potential of L-methioninase production.

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| S.no. | Culture code | Mean Zone Size After 48 hrs (mm) | SD |
|---|---|---|---|
| 1. | #5 CZBAWLS | 20 | 0.57 |
| 2. | #40 CMLBRT | 20 | 0 |
| 3. | #1088 AMSTITWLS | 35 | 0 |
| 4. | #20 CMBANEY | 20 | 0.57 |
| 5. | #1104 AMSTIWLS | 0 | 0 |
| 6. | #50 CMSTITBRT | 0 | 0 |

Table 10. Zone formation by culture filtrates on MA plates after 48 hrs.

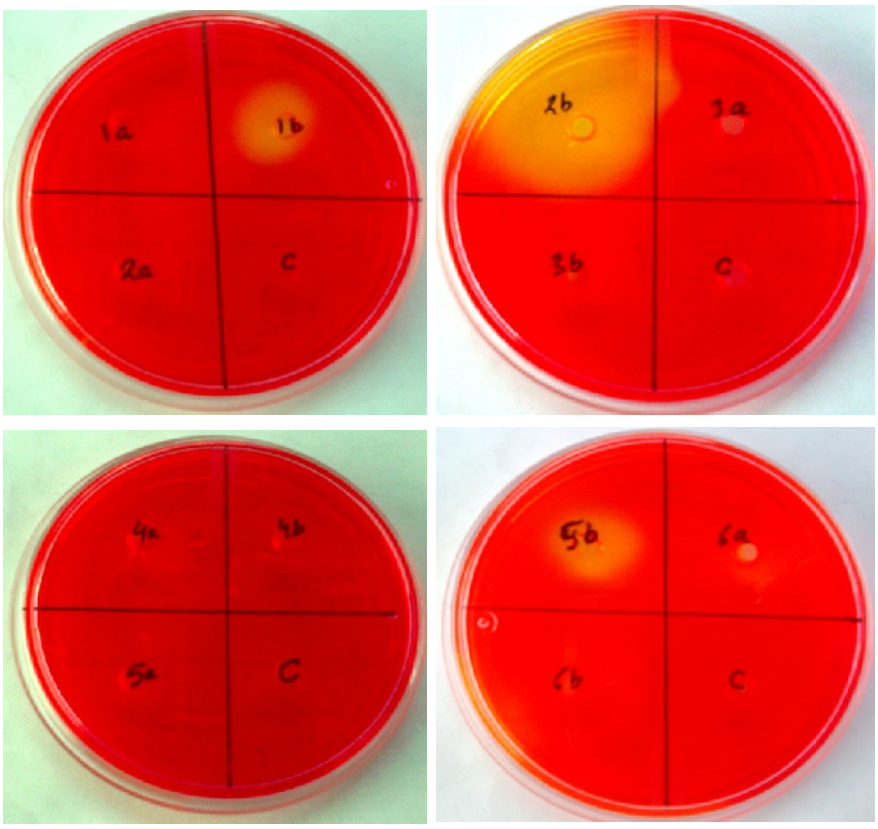


Fig. 9 shows the zone formation of the culture filtrate where “a” stands for intracellular filtrate and “b” stands for extracellular extract. 1 denotes #1088 AMSTITWLS ,2 denotes #20 CMBANEY, 3 denotes #50 CMSTITBRT, 4 denotes #1104 AMSTITWLS, 5 denotes #5 CZBAWLS and 6 denotes #40 CMLBRT.

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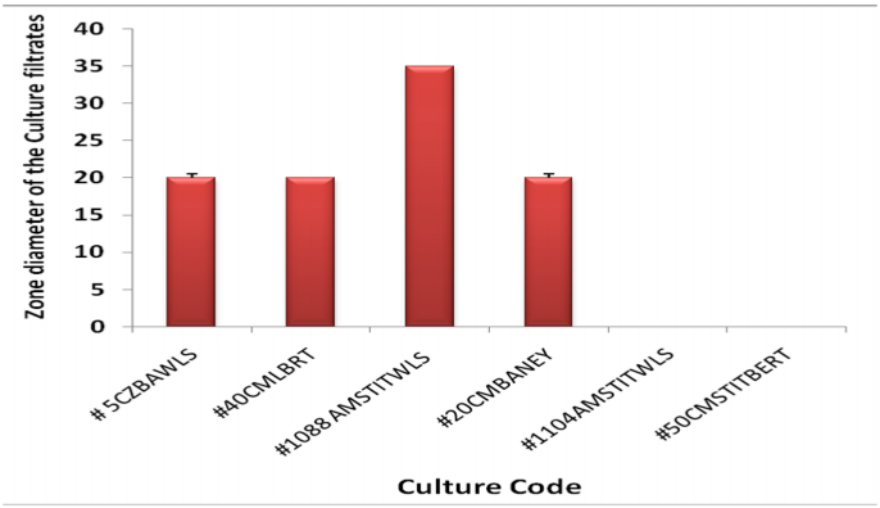
Both extracellular and intracellular enzyme activity of culture filtrates was checked. The intracellular extract did not show activity in any of the endophytic culture, #1088 AMSTITWLS showed the maximum zone size in the extracellular filtrate. But #1104 AMSTITWLS and #50 CMSTITBRT showed a negligible zone. Fig. no 9 shows the zone of activity observed on the methionine agar plates. This technique was also used for testing L-Asparginase production from the *E.coli* cultures isolated from sewage water, in which 6 showed weak activity and 8 showed no activity in the agar well diffusion technique (Jain *et al.*, 2012). Thus the isolates which showed the activity in the culture filtrate on MA plates were considered to be potent producers. Graph 3 shows the zone sizes of the culture filtrates on MA plates.




Graph 3. Showing the zone size of the culture filtrates on MA plates

**5.6 Protein precipitation and estimation**

The protein was precipitated with the cold acetone at low temperature from the culture filtrate and further the protein was estimated by Lowry's method in order to determine the protein yield. Bovine serum albumin (BSA) was used to plot the standard curve for determining the protein concentration in the protein samples precipitated by acetone. The standard curve for BSA is represented by Fig 10 The protein content was found to be maximum in #1104 AMSTITWLS and others all showed comparable amount of protein.

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The amount of protein is necessary for further finding the specific enzyme activity for each culture. The similar protocol was used by El-Sayed and Khalaf (2009) in order to determine the extracellular crude protein using bovine serum albumin as the standard for 21 fungal strains isolated from soil samples.

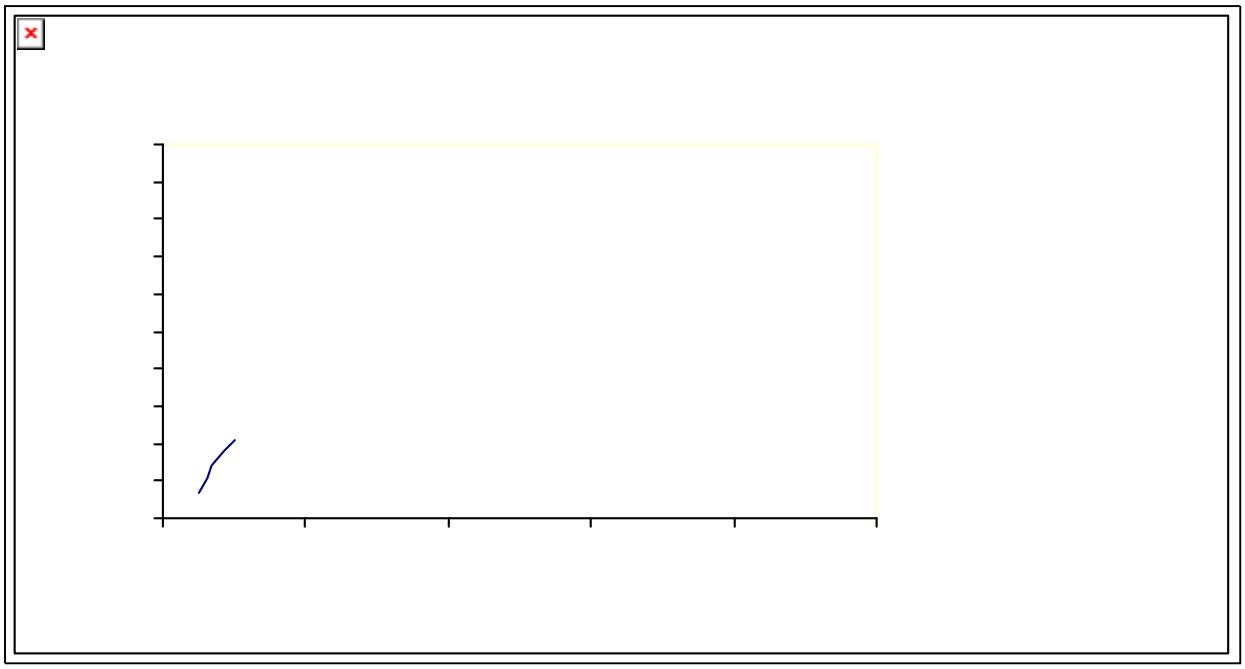

Fig 10 Showing the Standard curve for BSA.

| S.no. | culture code | Absorbance at 660 nm | Protein concentration |
|---|---|---|---|
| 1. | # 5CZBAWLS | 0.1576 ± 0.0011 | 0.72 mg/ml |
| 2. | #40CMLBRT | 0.1473 ± 0.000577 | 0.678 mg/ml |
| 3. | #1088 AMSTITWLS | 0.137 ± 0.001 | 0.62 mg/ml |
| 4. | #20CMBANEY | 0.1583 ± 0.000577 | 0.733 mg/ml |
| 5. | #1104AMSTITYEL | 0.189 ± 0.001 | 0.8865 mg/ml |
| 6. | #50CMSTITBRT | 0.195 ± 0.0005 | 0.91 mg/ml |

Table 11. Showing the protein concentration of the samples by Lowry's method

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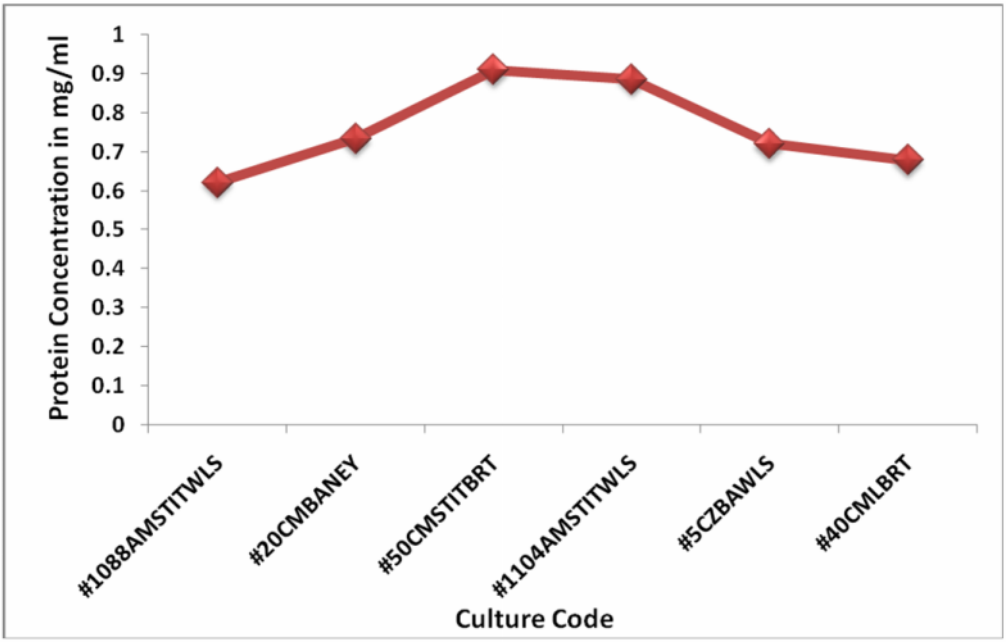
Using the correlation equation, obtained from the standard curve the protein concentration of the samples was determined. Table11 represents the protein concentration estimated in the samples using Lowry's method.




Graph 4. Estimation of crude protein using Lowry's Method.

### 5.7 Quantitative Estimation

The quantitative estimation of the L-methioninase activity was carried out by direct nesslerization method in which the amount of liberated ammonia is estimated. The standard curve for ammonia was plotted using ammonium sulphate and taking the absorbance at 450 nm.

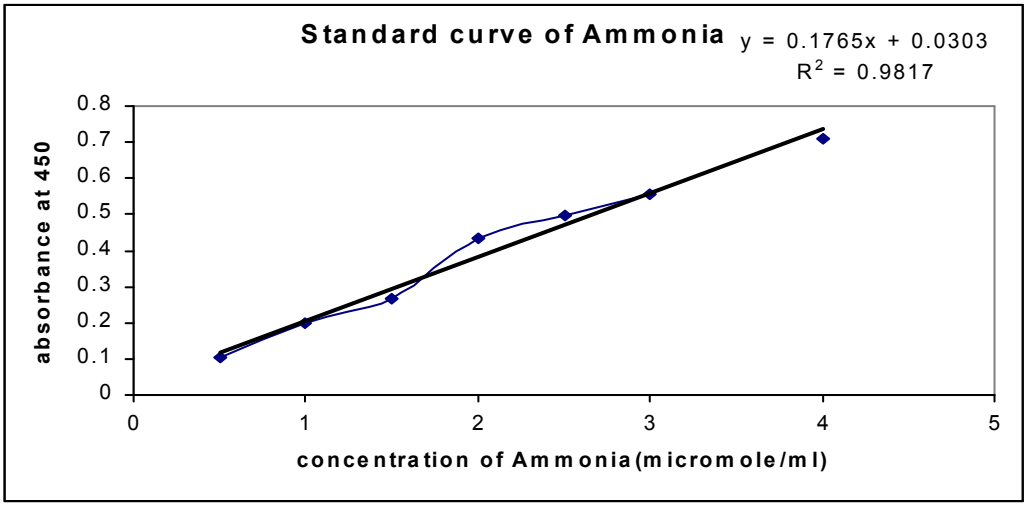



Fig 11. Showing the standard curve of ammonia.

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The correlation equation was used to calculate the amount of ammonia produced by each sample, the enzyme units were calculated as 1U/ml = amount of ammonia produced in micromole/ml.

Further the specific activity (U/ml/hour/mg of crude protein) of each sample was calculated by finding the enzyme units per mg of protein taken in each sample.

$$\textbf{Specific activity} = \frac{\textbf{enzyme units (U/ml)}}{\textbf{Amount of protein X Time of incubation}}$$

| S. no. | Culture code | Absorbance | Protein concentration (mg/ml) | $NH_3$ (micromole per ml) | Specific activity (U/hr/mg of crude protein) |
|---|---|---|---|---|---|
| 1. | #1088AMSTITWLS | 2.84 ± 0.005 | 0.62 | 15.9 | 25.64 |
| 2. | #20CMBANEY | 0.135 ± 0.002 | 0.73 | 0.59 | 0.8 |
| 3. | #50CMSTITBRT | 1.911 ± 0.015 | 0.91 | 10.6 | 11.6 |
| 4. | #1104AMSTITWLS | 1.33 ± 0.031 | 0.886 | 7.36 | 8.3 |
| 5. | #5CZBAWLS | 2.709 ± 0.008 | 0.72 | 15.17 | 21.06 |
| 6. | #40CMLBRT | 2.627 ± 0.012 | 0.678 | 14.71 | 21.6 |

Table 12. Specific methionilytic activity of the crude protein in nesslerisation.

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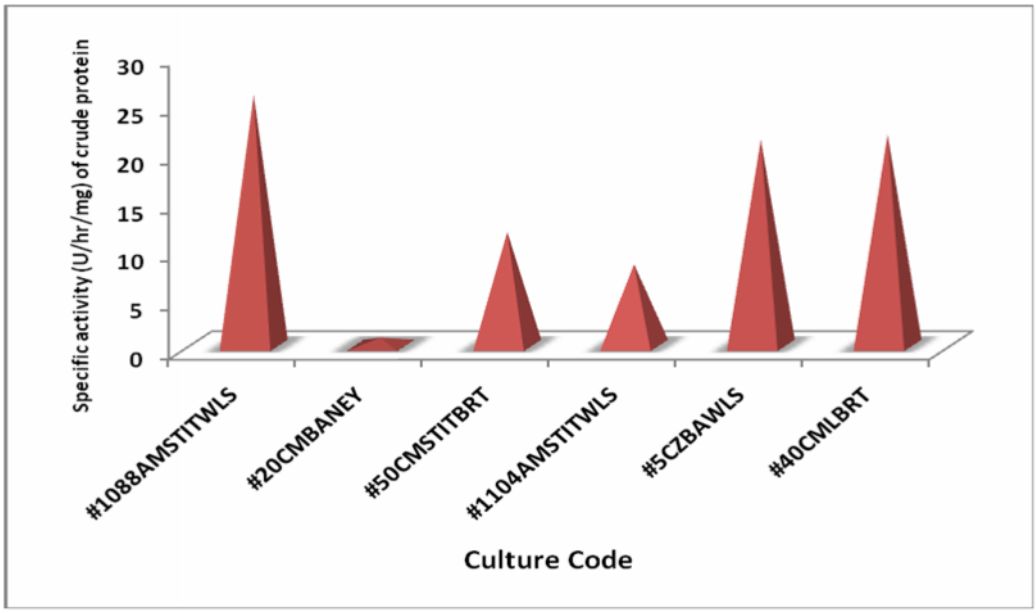


Graph 5. Showing Specific activity (U/hr/mg) of crude protein in quantitative estimation by Nesslerization.

The specific activity was found to be maximum in case of #1088AMSTITWLS and #5 CZBAWLS, #40 CMLBRT showed almost similar potential for producing L-Methioninase, the others showed a comparatively less amount of activity. The reported specific activity in case of *Aspergillus flavipes* was 10.78 U/mg (El-Sayed and Khalaf, 2009) whereas in the present study the specific activity of #1088 AMSTITWLS was found to be almost two fold higher than the earlier reported one.

Optimization of L-methioninase production from *Aspergillus flavipes* have been carried out by the source of isolating this fungus was different types of siols from Sharika province, Egypt, further the purification and characterization of this L-methioninase have been carried out by El-Sayed and Khalaf 2009. Also the same enzyme have been isolated from *Trichomonas* (Coombs and Lockwood, 1991).Therefore, further experimentation is required in order to utilize the potential of the endophytic fungi to produce L-methioninase which is an enzyme gaining therapeutic application.

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**5.8 Morphhotaxonomy**

The endophytes showing the potential to produce L-methioninase were grown on different medium such as PDA, CMA, SNA, Water Agar, Grass leaf agar so that they can be characterized on the basis of their morphology and microscopic characteristics.

**A. #40 CMLBRT**

**1. On PDA**

Colonies are velutinous, grayish green in color from front and brown colored from back, fast growing, elevated with rough margin. Hyphae brown colored, septate, multinucleate, broad and thick walled. They bear simple or branched large conidia which have both transverse and longitudinal septation. These conidia may be observed singly or in acropetal chains and may produce germ tubes. They are ovoid to obclavate, darkly pigmented, smooth or roughened. The end of the conidium nearest to the conidiophore is round while it tapers towards the apex. This gives the typical beak or club-like appearance of the conidia (Barnett and Hunter, 1998). Fig 12(A) shows the colony texture on PDA plate and Fig 12 B shows the mycelium with simple and branched conidiophores on which conidia are borne in acropetal succession on PDA

**2. On CMA**

Colonies are downy, green in color and fast growing covering 90 mm plate in 7 days on incubation at 26°C. Mycelium is septate, brown in color, branched, thick from which simple or branched conidiophores arise. Conidia are produced sympodially from simple, branched, short or elongate conidiophores. Conidia are formed in acropetal succession. Chains of multicelled Conidia are obclavate, sometimes ovoid or ellipsoidal in shape, often with a short conical or cylindrical beak, pale brown, rough walled (Barnett and Hunter, 1998). Fig 12 C shows the Obclavate and ovoid Conidia produced on PDA and Fig 12 D-E shows the pale brown conidia produced on CMA and Conidia formed in chains in acropetal succession.

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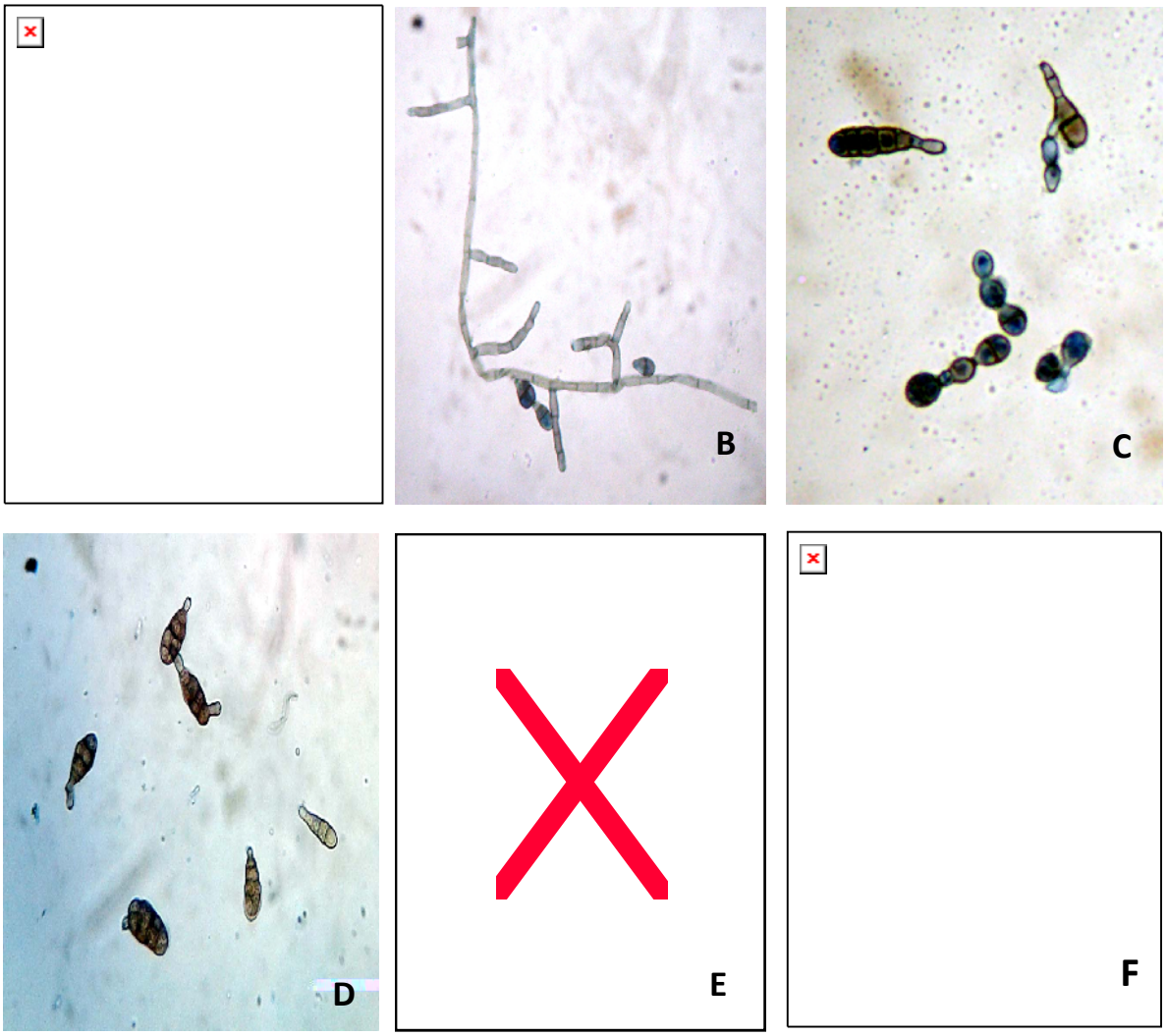


Fig. 12 A: colony texture on PDA, B: Mycelium with simple and branched conidiophores on which conidia are borne in acropetal succession on PDA C: Obclavate and ovoid Conidia produced on PDA. D-E: Pale brown conidia produced on CMA, F: Conidia formed in chains in acropetal succession.

Based on the morphological characters #40 CMLBRT is identified as ***Alternaria sp***

## B. #5 CZBAWLS

### 1. On PDA

Colony was floccose, white to red colored that turns to violet or lavender on sporulation and fast growing. Hyphae are septate and hyaline. Conidiophores are short and. Macroconidia were slightly sickle-shaped, thin-walled, with an attenuated apical cell and a foot-shaped basal cell. They are 3 to 5-septate. Microconidia are abundant, non-septate, ellipsoidal to cylindrical, slightly curved or straight (Barnet and Hunter, 1998).

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**2. On SNA**

Colony was floccose, white colored, flat and fast growing. Hyphae septate, branched giving rise to small and stout conidiophores on which the conidia borne singly. Two types of conidia are present; macroconidia and microconidia. The macroconidia are larger in size, sickle in shape, 3-5 septate with hooked apical cell and foot shaped basal cell. Microconidia are smaller in size, produced abundantly, slightly curved, mostly nonseptate, ellipsoidal in shape. (Booth, 1971)

**3. On GLA**

Colony hyaline, downy and slow growing. Hyphae hyaline, septate, branched giving rise to short conidiophores. Macroconidia are borne directly on the monophialides. Macroconidia are hyaline, 3-5 celled and sickle shaped. Microconidia are also hyaline, smaller in size, comma shaped that are usually non sepatate. Conidia are entrapped inside the clestothecium. (Fuskey,1996)

**4. On WA**

Colony hyaline, downy and slow growing. Hyphae septate, thin, slender branched giving rise to short conidiophores. Macroconidia are borne directly on the monophialides. Macroconidia are less in number, 3-4 septate, sickle shaped. Microconidia are smaller in size, slightly curved or comma shaped that are usually present in pair or in clump. 8 celled clavate shaped ascospores are present. Conidia are entrapped inside the clestothecium.

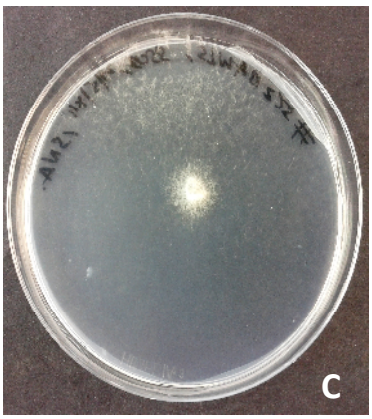


Fig. 13(a) A-B: Colony on PDA (Change of color from White red to violet), C: White colony on SNA medium.

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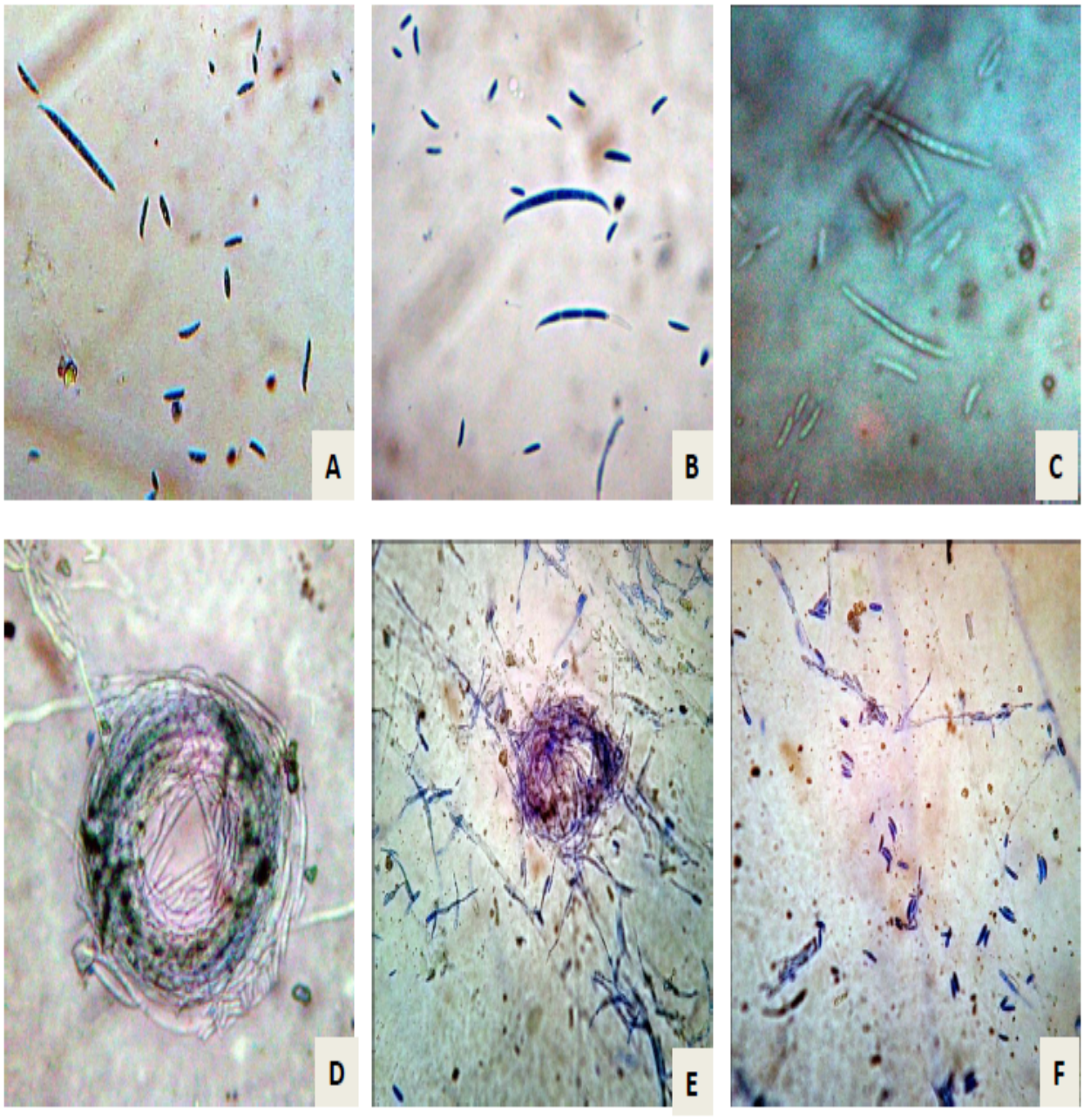


Fig. 13(b) A: Macroconidia and microconidia produced on PDA, B: Macroconidia and microconidia produced on SNA, C: Hyaline Macroconidia and microconidia produced on GLA, D: Clestothecium consisting conidia on GLA, E: Ascospores and ascus developed on water agar, F: Paired and scattered microconidia on water agar.

Based on the morphological characters #5 CZBAWLS is identified as ***Fusarium oxysporium***

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## C. #1088 AMSTITWLS

### 1. On PDA

Colony floccose, grayish green colored from front and dark green or black colored from back side, fast growing, non elevated with formation of some aerial mycelium. Pycnidia are simple or compound. Conidiophores are hyaline, simple, sometimes septate, rarely branched cylindrical, arising from the inner layers of cells lining the pycnidial cavity. Obclavate shaped conidia are present. (Sangeetha *et al.,* 2011)

### 2. On CMA

Colony floccose, grey to green colored, fast growing, with formation of aerial hyphae. Black colored fruiting bodies were also formed. Two types of hyphae are present. The first hyphae is brown colored, septate, broad, thick walled whereas the second hyphae is long, thin, slender and branched. Long, broad and septate conidiophore arises from the mycelium. Two types of conidia were present. Primary conidia were single celled, oval shaped, double walled, multinucleate whereas the mature conidia is golden brown colored, 2 celled, double walled with septa often longitudinally striate. Conidiogenous cells are simple, cylindrical to subobpyriform, holoblastic, Paraphyses when present are cylindrical and sometimes septate (Javadi *et al*., 2010; Damn *et al*., 2007)

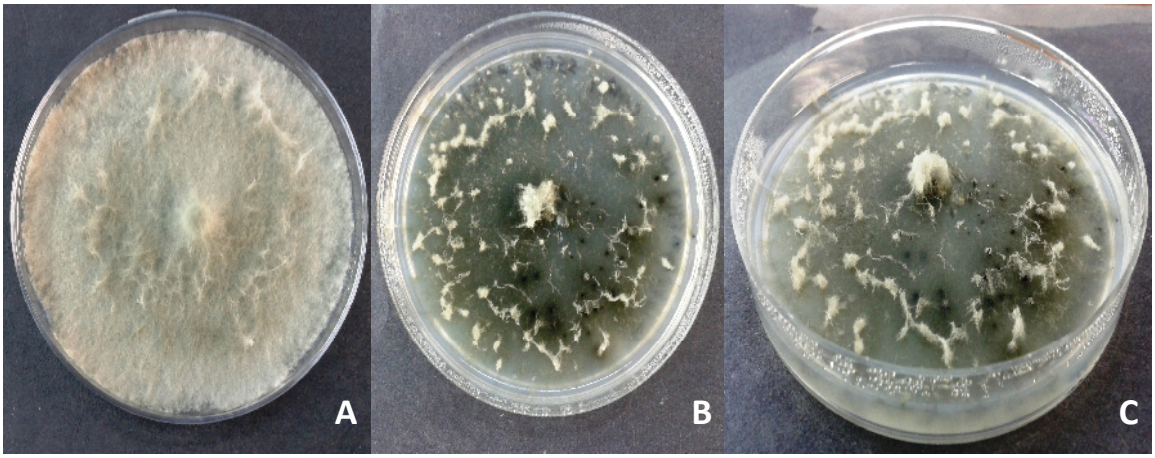


Fig. 14(a) A: Colony of #1088 AMSTITWLS on PDA, B-C: #1088 AMSTITWLS on CMA

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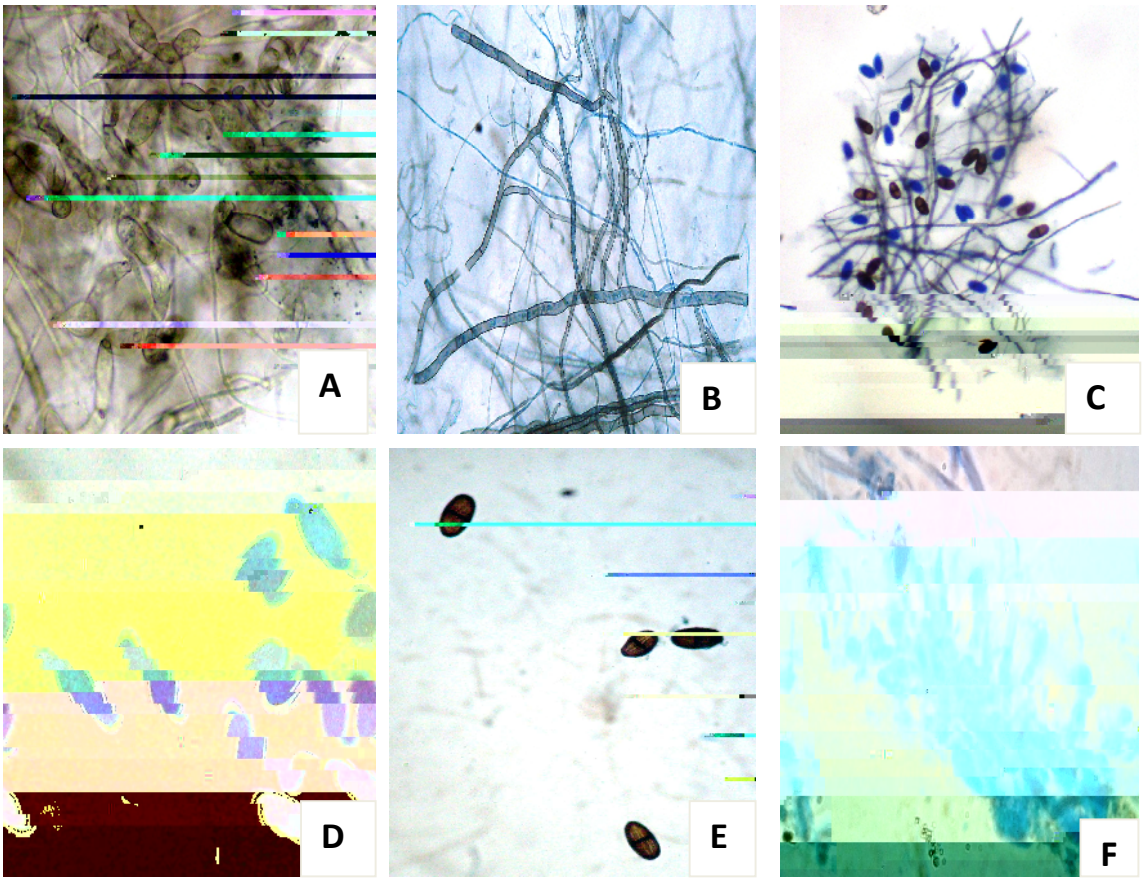


Fig.14(b). A: Pycnidia and obclavate type conidia developed on PDA, B: Hypahal structure on CMA, C: Hypha and conidia developed on CMA, D: Primary immature conidia, E: Mature two-celled dark brown conidia with typical striations of *Lasiodiplodia theobromae,* F: Conidiogenous cells with paraphysis developed on CMA.

Hence based on morphological structures it is identified as ***Lasiodiplodia theobromae***

**5.9 Genomic DNA Isolation and PCR Amplification**

The genomic DNA isolation of the three cultures showing the highest potential for producing L-Methioninase was done. The DNA was qualitatively estimated using agarose gel electrophoresis and the size of the genomic DNA was deciphered by comparing its mobility in the gel with the 1kb DNA ladder (Stepup, Bangalore Genie) which ranges from 1 kb to 10 kb. There was no RNA bands seen, hence the RNase treatment was successful. The size of the genomic DNA was found to be approximately more than 10 kb

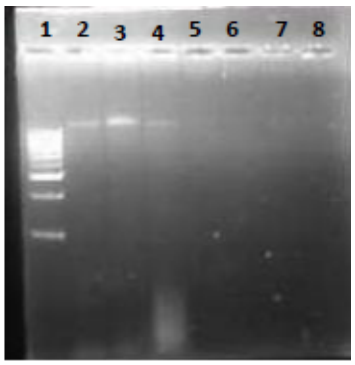


Fig.15 shows 1kb ladder in lane 1, Genomic DNA band of #1088 AMSTITWLS in lane 2, DNA band of #5 CZBAWLS in lane 3 and DNA band of # 40 CMLBRT in lane 4

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The quantitative estimation of DNA was done by taking the absorbance at 260 nm and the amount was quantified as follows

| Culture code | Concentration ( µg/ml) |
|---|---|
| #1088 AMSTITWLS | 25 |
| #5 CZBAWLS | 20 |
| #40 CMLBRT | 20 |

Table 13. Concentration of Genomic DNA of the L methioninase producing endophytic fungi.

The PCR amplification of the genomic DNA was carried out, the amplicons obtained were resolved on 1.5% agarose gel in order to check the size on the basis of the mobility and comparison with the 500 bp ladder. The size of the amplicon was found to be approximately 500 bp to 550 bp. This size can be easily compared to the ITS region, which was amplified in order to characterize the fungi at molecular level. Fig 16 represents the gel showing the PCR products along with the ladder.

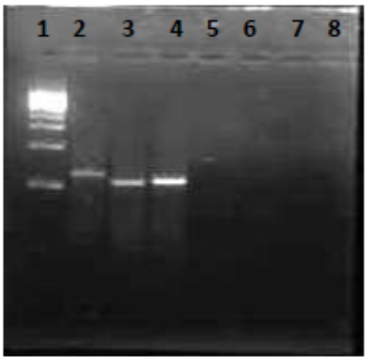


Fig.16 Shows 500 bp ladder in lane 1, PCR product of #1088AMSTITWLS in lane 2, PCR product of #5 CZBAWLS in lane 3 and PCR product of # 40 CMLBRT in lane 4.

Further taxomonic identification of the endophtyic fungi must be carried out by processing the samples for sequencing and establishing the phylogenetic relationship and hence characterizing the fungi upto species level.

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# Chapter 6

# Conclusion

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# *Conclusion*

In the present study, six fungal isolates were reported to possess L-methioninase activity extracellularly. Best activity was found in #1088 AMSTITWLS, #5 CZBAWLS and #40 CMLBRT which were identified as *Lasiodiplodia theobromae*, *Fusarium oxysporium* and *Alternaria sp*. respectively.

Further, studies on protein purification, kinetics, characterization along with phylogenetic placement of the endophytic isolates would open up the possibilities for therapeutic use of the enzyme as an anticancer agent.

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# Chapter 7

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