ISOLATION, IDENTIFICATION AND BIOACTIVE PROPERTIES OF ENDOPHYTIC MICROORGANISMS FROM

Polianthes tuberose AND *Antirrhinum majus*

DISSERTATION REPORT

Submitted in partial fulfilment of the

Requirement for the award of the

Degree of

Master of Science

In

(Biotechnology)

By

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Under the guidance of

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CERTIFICATE

This is to certify that the Dissertation Report titled "**ISOLATION, IDENTIFICATION AND BIOACTIVE PROPERTIES OF ENDOPHYTIC MICROORGANISMS FROM *Polianthes tuberose* AND *Antirrhinum majus***" that is being submitted by "AMANDEEP KAUR" is in partial fulfilment of the requirements for the award of MASTERS IN SCIENCE DEGREE, is a record of bonafide work done under my guidance. The contents of this Dissertation Report, in full or in parts, have neither been taken from any other source nor have been submitted to any other Institute or University for award of any degree.

The Dissertation Report is fit for submission and the partial fulfilment of the conditions for the award of Masters in Science.

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DECLARATION

I, Amandeep Kaur hereby declare that the project that the project report entitled "**ISOLATION, IDENTIFICATION AND BIOACTIVE PROPERTIES OF ENDOPHYTIC MICROORGANISMS FROM *Polianthes tuberose* AND *Antirrhinum majus***." Submitted for the partial fulfilment of the degree of M.Sc. Biotechnology (Hons.) is the record of work carried out by me under the supervision of "Dr. Reena Singh Chopra", Assistant Professor, Lovely Professional University, Phagwara, Punjab.

I further declare that the material taken from other sources has been duly acknowledged in this report.

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TERMINOLOGY

DNA	Deoxyribose Nucleic Acid
PCR	Polymerase Chain Reaction
A.majus	*Antirrhinum majus*
P.tuberose	*Polianthes tuberose*
D.W	Distilled Water
AML	*Antirrhinum majus* leaf

ABSTRACT

Endophytes are those microorganisms which are found in plants as they live in a mutual relationship with them. Endophytes can have potential traits which can be helpful for plants. *Antirrhinum majus* and *Polianthes tuberose* plants were collected and cultured under laboratory conditions for isolating endophytes from them. Surface sterilization of plant was firstly done then different parts of both the plants i.e. roots and leaves were cultured onto the water agar for growing endophytes from them later on were sub cultured onto PDA. Various enzyme assays were done for testing and identification by sequencing. The sequence showed 99% similarity to *meyerozyma caribbica* after carrying out BLAST.

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CHAPTER-1

INTRODUCTION

Plants serve as a receptacle of vast number of organisms which are called endophytes. Endophyte show a mutual relationship between microorganisms like bacteria and fungi and the plants also called endophytism. In nature, endophytes are omnipresent. Microbes gain from plants with the accessibility of nutrients, and plants earn by help in growth enrichment or stress reduction (Pablo et al., 2008). Endophytes play diverse roles in plants, example for defense they can modulate host's secondary metabolic pathway gene expression. "Endophytic technology can be employed for the efficient production of agricultural and economically important plants and plant products" (Wani et.al., 2015). Moreover, bioactive endophytic compounds have been utilized in novel drug discoveries for their wide variety of biological activity as anticancer, antibiotic, anti-inflammatory and antioxidant agents (Mehanni, 2010).

Endophytic bioactive compounds research elevated with the discovery of taxol from *Taxomyces andrenae* endophyte. The anti-cancer agent formerly was extracted from the Pacific yew tree which leads to the mass destruction of the yew trees but with the discovery of *Taxomyces andrenae* a taxol-producing endophyte it has been hypothesized that endophytes from anticancer plant have possibility to synthesize anticancer compounds. This has promoted the further studies on study of various compounds from various endophytes(Chow et.al., 2015).

Other bioactive compounds produced from endophytes includes- a unique tetramic acid produced by *C.quercina,* Cryptocin show activity against plant and human pathogenic fungi i.e it act as an anti-fungal agent (Li et.al., 2000). Pseudomycins is produced by plant-associated pseudomonad and has anti-fungal characterstics (Harrison et.al; 1991). Colletoric acid produced in *Colletotrichum gloeosporioides* of an endophytic fungus *Artemisia mongolica* show both anti-microbial and anti-fungal activity on *Helminthsporium sativum* (Zou et. al.,2000). A metabolite derived from endophyte *Phomopsis Sp.*, phomopsichalasin display antibacterial activity in disk diffusion assays against *Salmonella enteric* (11-mm inhibition zone), *Bacillus subtilis* (12-mm inhibition zone) and *Staphylococcus aureus* (8-mm inhibition zone) (Horn et.al., 1995).

In order to isolate an endophyte first the plant having some potential trait of having an endophyte is selected. Mainly plants growing in tropical or in semitropical regions have the tendency to inhabit diverse endophytes. Also, plants growing in harsh environment and able to resist infections give an idea of endophyte-plant relationship, as an endophyte may help in protection of the plant. After selecting the plant for study and isolating endophyte it is important to identify the endophyte. For identification, different biological, biochemical and morphological aspects are studied (Strobel et.al; 2003). Also taxonomical identification is done with the help of DNA amplification and sequencing. For achieving this, endophyte is sampled from agar plate, lyophilized and DNA isolated by lyses, followed by centrifugation to remove cell debris, and precipitate DNA. Purified DNA is then amplified by PCR then sequencing can be done and compared with publicly available databases (Coombs et.al., 2003).

Distinct endophytic and epiphytic microorganisms associate with plants in nature. Regardless of long history of studies on symbiosis the aspects of the synergy are still unclear. Many studies have been performed in various plant species for their endophytic study but no such study has been reported on *Antirrhinum majus* and *Polianthes tuberose* so, these plants are the appropriate choice for carrying out their endophytic analysis which may give some potential insight.

Antirrhinum majus (snapdragon) belongs to the family plantaginaceae. It is the native plant of Mediterranean region which flowers annually (Fawzy, 2013). *Antirrhinum majus* is an ornamental flower used for decorating gardens. Different flower colors, shape and size in *Antirrhinum* inflorescences are found (Abdul et.al., 2011). It has been used as a model plant to study the effects of inheritance by both Darwin and Mendel. One of the key reason for using it as a model plant is its growth cycle as it can be easily cultivated and can be grown in the span of three months. The information about the genetic control of floral asymmetry, floral organ identity and petal cell-type specification in *Antirrhinum* has given the peek into basic molecular mechanisms (Davies et. al., 2006). The confirmation of genetic linkage, correlation of recombination with CHIASMA formation, environmental effects on recombination and mutation rate and the identification of cytoplasmic inheritance was first observed in *Antirrhinum* by Erwin Baur and his colleagues during the first decade of twentieth century. Where the model plant *Arabidopsis thaliana* does not show much genetic variability *Antirrhinum* gave insight to the role

of transposable elements. *Antirrhinum* transposons have been proved to be an effective and a versatile tool for studying the aspects of plant genetics. To attract bees many flowers emit volatile scent compounds, *Antirrhinum* flowers emit methyl benzoate to attract pollinators. The emission takes place usually during the day to ensure successful pollination and once ovary is fused then no further demand is there for attracting pollinators and scent production diminishes (Davies et.al; 2006).

With the use of molecular techniques major *Antirrhinum* mutants have been studied. Also, the vast mutant study can be accessed in *Antirrhinum* database. Where transposon tagging fails in *Antirrhinum*, a map-based cloning approach can be considered. DNA samples can be screened by polymerase chain reaction (PCR), for integrating any gene in its genome (Schwarz-Sommer, 2003).

Polianthes tuberose belongs to family Agavaceae. It is a perennial plant whose common name is 'Rajnigandha' in India. Its extracts are used in perfumes and cosmetics. It is a night blooming native plant of Mexico. It has properties of antisepsis, act as sedative, anti-inflammatory, anti-spasmodic, anti-nausea and it's flowers are the source of essential oils which are used in aromatherapy as anti-depressant and for removing lethargy (Rawani et.al., 2012; Sood et.al,, 2005). *P. tuberose* is known to show antimicrobial properties against *Proteus mirabilisc* and *Esherichia coli* by the study carried out in 2011 (Anu et.al., 2011).

CHAPTER-2

REVIEW OF LITERATURE

Diverse fungal and bacterial endophytes can be found in the nook of plant tissues. Some endophyte species have the ability to limit the growth of fungi, bacteria or can shield host against damage by herbivores. Endophytes are being industrialized as bio-control agents or pharmaceutical tools. They are believed to be an unexploited source of natural products and show an industrial promise. Endophytes are the least researched field which has a wider scope. Endophyte has the ability to produce bioactive compounds; these bioactive compounds can be isolated and can further be used in discovering a drug, industrial production. Bioactive compounds have an economical importance. The mutual association between host and organism can be intracellular and intercellular. Endophyte can be present in any part of the plant i.e. it may be present at roots, stem, leaves. Mainly endophytes are present in roots. They form a symbiotic relationship in this interaction and both are benefited. The microorganism present in this association is very useful as they can be important in the production of bioactive compounds like alkaloids, flavonoids, and phenolic compounds. These compounds have some medicinal properties which can be used in various research. Many studies are done on the isolation and identification of the endophytic organism.

Many species have the potential of endophytic properties, these properties are needed to be studied and identified. Because they have the ability and can be used for human welfare.

2.1. *Antirrhinum majus*

KINGDOM	Plantae
ORDER	Lamiales
FAMILY	Plantaginaeae/Veronicaceae
GENUS	*Antirrhinum*
SPECIES	*A.majus*

It is an ornamental plant belonging to angiosperm on which various studies has been done it is used as a model plant for studying transposable gene elements. Flavonoid study has been done on *A.majus*; one such flavonoid aurone is 6-glucosidase which imparts a yellow color to *Antirrhinum majus*. Flavonoids have significant importance in plants for reproduction system as they provide alluring colors to flowers to fascinate insects for carrying out pollination also flavonoids play an important role in resistant to disease when attacked by pathogens. (Harborne, 1979). Research on oil yield showed that *A.majus* seeds provide a considerable amount of oil yield and are suitable source of lipid-soluble bioactive and essential fatty acids. The oil is nutritionally beneficial due to its high level of oleic acid and linoleic acid content (Fawzy et.al., 2013). Genes for the floral arrangement of *A.majus* has been identified which are GLO, DEF and PLE mutation in these genes can cause a change in the pattern of floral arrangement (Schwarz-Sommer et al., 1990). *A.majus* has many transposable elements because of which vast genetic diversity is observed. The transposition is controlled by environmental and genetic factors which also contribute to the natural variation and adaptation. The callus of *A.majus* hypocotyls can be stimulated significantly by adding antibiotics like penicillin G and carbenicillin to the medium, it acts as growth regulators when incorporated in media other plant parts like shoots can also be significantly stimulated but not roots. This happens due to the presence of beta-lactam side chain which gets cleaved and releases act as auxin (Davies, 2006; Holford, 1992). Mulching of *A.majus* plant show high growth levels and high blooming period also radioactivity effect is low when cultivation in biodegradable mulch is done (Schettini et.al; 2007). A gene, olive has the capability of reducing chlorophyll levels by producing a structural change in it. The gene's expression is limited to photosynthetic cells only. It is a recessive nuclear mutation and is not commonly observed.

The chemotactic activity of pollen tubes of *A.majus* towards calcium has been observed also the presence of calcium increases the growth rate of pollen tubes (Joseph et.al., 1962). The scented plant's emission of volatile compounds occurs from the petals. Like other flowers, *A.majus* also possess permeable cuticles which allow methyl benzoate emissions (Oyama, 2004).

Image-1. *Antirrhinum majus*

(Photo by. Fotolia)

2.2. ***Polianthes tuberose***

KINGDOM	Plantae
ORDER	Asparagales
FAMILY	Asparagaceae
SUB-FAMILY	Agavoideae
GENUS	*Polianthes*
SPECIES	*P.tuberosa*

It is a herb known for its perfume extracts and used in cosmetics. It requires warm weather for four to five months and high moisture for its growth. Not much study has been done on *Polianthes tuberose* so far. But few studies have been done like a study carried out in

supercritical carbon dioxide *Polianthes tuberose* extracts at 40°C and 60°C was screened against various microorganisms and significant activity was observed against *C.albicans, P.aeruginosa, and E.coli*. The solvent extraction of extracts was used to check their antimicrobial activity (Probir et.al; 2014). Cytotoxicity measurement by using methanol extracts implied the presence of compounds like spirostanol pentaglycosides in the extract. It is known to show activity against human carcinoma cells (Rumi et.al; 2014). Abscisic acid (ABA) has been detected in the dormancy period in relevantly high levels but with ending of dormancy the ABA levels decreases which suggest that ABA could be involved in maintaining and inducing of dormancy in the bulbs of *Polianthes tuberose* (P.K. Nagar, 1995). Research carried out in 1999 suggested that prolificity of eggs decreases by hecogenin, tigogenin, and sapogenin present in *Polianthes tuberose* (K. Singh et.al., 1999). The heteropolysaccharide which can constitute mannose, xylose, galactose, arabinose and glucuronic acid as its structural units from this plant can be used in various applications. Polysaccharides from the callus culture can be used in detergents as they improving cleaning and also provide moisturizing effects and a rich and smooth feeling upon use (Kazuya et.al; 1994).

Image-2. *Polianthes tuberosa* (Photo by Spalding Bulb)

Image-3. *Polianthes tuberosa* flowers

(Photo by Asha Susan)

Image-4. *Polianthes tuberosa* leaves

(Lim, 2013)

Many plants have potential to treat deadly diseases. In ancient times also, plants had been used in many countries like India, China for treating diseases. They used herbs, shrubs to treat various kinds of diseases (Wani et.al., 2015). Endophytic technology is being used in many fields, this association of plant and microorganism with each other play important role in agriculture, pharmaceutical, and in various industries. The use of toxic pesticides in agriculture which directly affects the quality of food, and cause new diseases. The use of excess pesticides has been banned due to its toxic nature but is still being used despite the downsides. Bioactive compounds from endophytic fungi and endophytic bacteria promise low toxicity and also it doesn't affect the quality of food. In pharmaceutical companies due to increasing deadly diseases not only new drugs are needed also a new method to treat them, so the need for new antibiotics is important which will be more effective and less toxic and endophytes can play a role in it (Strobel et. al., 2004).

2.3. Importance of endophytic microorganism

The endophytic microorganisms can be fungi or bacteria which live beneath the tissue of plants like intracellular tissue or intercellular tissue there they take nutrition from a plant and provide growth and support to plant from abiotic stress. Thus endophytic microorganisms are very much important to plant.

The endophytic microorganisms like bacteria or fungi is a source of new bioactive products which can be used to treat diseases and can be used in many sectors like agriculture, industrial and pharmaceutical, they also have the ability to produce secondary metabolites like alkaloids, flavonoids, steroids, peptides etc. These all secondary metabolites play important role in pharmaceutical industry and have potential to produce a novel drug which can be used for treating deadly diseases.

Cancer is one of the major problem which is increasing day by day, many bacteria which are becoming drug resistance is also a huge problem, the presence of deadly viruses and fungal infections, these all serious problems are affecting human, to fight with these problems new compounds are needed. A compound which has the ability to fight, the endophytic microorganism can be a solution; the phytochemicals they secrete can be used as a novel compound to treat diseases. There are a large number of plants and almost all plant have endophytic property so it is important to isolate this property from these plant (Gutierrez et.al., 2012).

An antimicrobial and anticancer agent from endophytic fungi or bacteria, the association of plant with microorganism like fungi which give support and help in the growth of plant take nutrition from plant produce phytochemicals and this association can be used as the antimicrobial and anticancer agent. Plants from which endophytes are needed to be isolated are taken and surfaces sterilized then are grown onto the nutrient agar plate.

Endophytic fungi produce secondary metabolites and phytochemicals when provided suitable temperature and proper medium composition. In comparison to endophytic fungi from endophytic bacteria, the isolation of endophytic fungi is in huge numbers only a few species of bacteria have been isolated from a plant, but like endophytic fungi, endophytic bacteria also produce secondary metabolites and can be used as novel bioactive compounds. Both endophytic

fungi and endophytic bacteria have potential to produce phytotoxic compound and antibiotics plant (Gutierrez et.al., 2012).

2.3.1. Antiviral compounds

The antiviral compounds are also one of the properties of endophytic microorganisms. The product of endophytic fungi can be used for virus i.e. inhibition of viruses. The product isolated from the plant having endophytic property and this isolated product is used as an antiviral compound.

2.3.2. Antioxidant compound

From endophytic microorganism antioxidant compound can also be isolated, the endophytic fungi produces secondary metabolites, from this product, can be obtained which is antioxidant in nature and can be used for human welfare (Strobel et. al., 2004).

2.4. Collection and Isolation of endophytic activity

Endophytic activity can be isolated from a plant part, like a root, stem, leaf, seed, flower, internodes, and buds. To isolate an endophyte the plant parts first need to be surface sterilized. For surface sterilization many steps are needed to follow, first the part of a plant from where endophyte can be obtained is to be isolated and washed with tap water and then a sterilizing chemical agent is used followed by plating in an appropriate medium for endophyte growth at 28 degree celsius.

2.5. Importance of enzymes

Enzymes are biocatalyst. They can escalate the rate of a reaction. Present and produced by living organisms they take part in chemical reactions which are important for living be it DNA replication, metabolism, protein synthesis and signal transduction etc. Due to their capacity of chemical transformations, they are highly useful in various fields, including animal nutrition, food industry, medication etc. (Li et al. 2012). Proteases are largely used because of its use in

detergent and dairy industries. Carbohydrases, mainly cellulases and amylases are the second largest group to be used in industry. They are used in detergent, starch, baking and textile industries (Kirk, Borchert, and Fuglsang 2002). Lipases play important role in breakdown and mobilization of lipids in the cells and also in transferring lipids from an organism to other. A Large part of earth's biomass is constituted by lipids (Hasan, Shah, and Hameed 2006).

CHAPTER-3

SCOPE OF STUDY

Polianthes tuberose and *Antirrhinum majus* show characteristics of producing endophytes. The endophyte can be studied for their useful traits which may have some commercial use. The bioactive compounds from the endophytes can be used to study their anti-microbial and anti-fungal nature.

Many studies have been done on both the plants their endophytic study can show their diverse nature. As endophyte show importance in various fields be it textile, therapeutic etc. any useful endophyte obtained is a great potential.

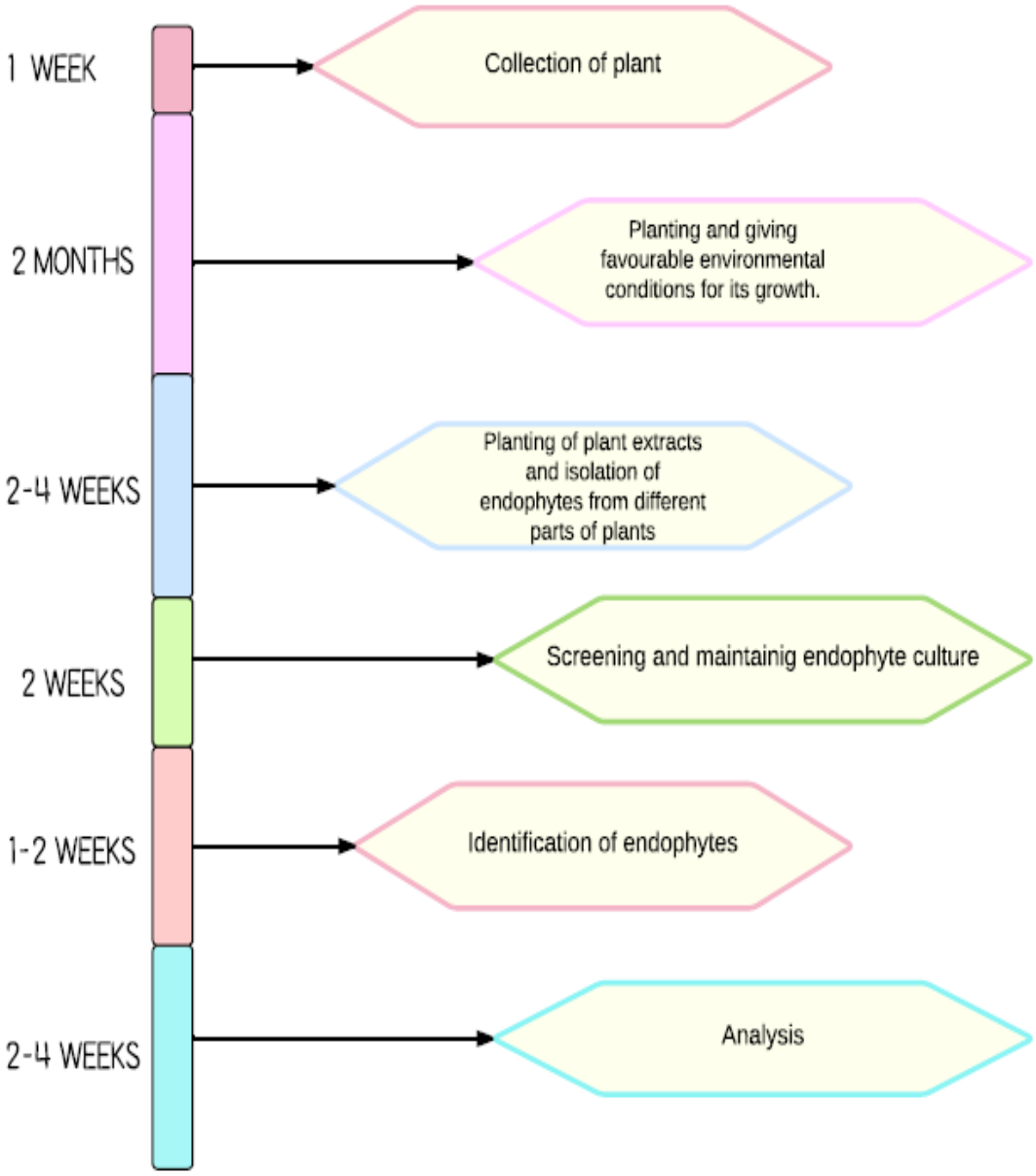
CHAPTER-4

RESEARCH OBJECTIVE

a. Collection of samples from *A.majus and P.tuberose.*
b. Isolation of endophytic fungi from leaves, roots and stem of plants *A.majus and P.tuberose.*
c. Purification of the fungal strains by further subculturing.
d. Morphological features of isolated endophytic fungi.
e. Screening of the isolates for the presence of bioactive molecules
f. Identification of the selected fungal endophytes based on amplification and sequencing of the ITS region of the fungal rRNA using PCR with ITS primers (ITS1 and ITS2).

CHAPTER-5

WORKPLAN



CHAPTER-6

MATERIAL AND METHODOLOGY

6.1. Collection and planting

The plant *Polianthes tuberosa* and *Antirrhinum majus* were collected from a nursery in Jalandhar. They were then planted in a pot. Their regular maintenance was done by providing water regularly. Proper amount of sunlight exposure i.e about 5-6 hours a day and shade with fertile soil. Plants were made to grow for few weeks before isolating endophytes from them.

6.2. Sterlization of glasswares and media

Glasswares made of Borosil were used. Sterlization of glasswares was done by autoclaving at 121^0C and 15psi.After sterilizing the flasks the 150ml of water agar prepared and autoclaved.

6.3. Plating for isolating endophytes from different parts of the plant

About seven petriplates, one control and two plates each for root, stem and leaves were autoclaved with 250ml of Nutrient Agar for making the plates for both the plants *A.majus and P.tuberose*.

6.3.1 From roots

- The roots were cut from the plant following proper washing with water to remove any soil.
- After washing with tap water the root samples were washed with 1% Tween 20 (Atlas Chemie) for about 10 mins
- Froth by tween 20 removed by washing with water 3-5 times, to obtain clean root samples.
- Then the root sample was brought in laminar air flow hood where it is surface sterilized with membrane filtered 0.05% (w/v) mercuric chloride (Qualikams) for 1 min.
- After surface sterilization, the explants were blotted between sterile filter paper discs and excised to 0.5-1 cm length so that their internal tissues get exposed and their endophytes are able to grow

- The dried root with the help of flame sterilized forceps placed in agar prepared plates.
- Then the plates were sealed with parafilm followed by incubation at 28^{o}C.

6.3.2 From leaves and stems

- The leaves and stems were sliced from the plant and washed.
- After water washing, 1% Tween 20 (Atlas Chemie) was used for washing for 10 mins.
- Followed by washing with water 3-5 times to remove any froth left by Tween 20.
- The washed explants were then surface sterilized with membrane filtered 0.05% (w/v) mercuric chloride (Qualikams) for 1 min inside laminar air flow hood.
- After surface sterilization, the explants were blotted between sterile filter paper discs and excised to 0.5-1 cm length so that their internal tissues get exposed and their endophytes are able to grow.
- The excised explants were plated and sealed with parafilm followed by incubation at 28^{o}C.

6.4. Incubation conditions

Prepared plates were incubated at 28^{o}C for one week until endophyte growth was observed. Then further subculturing of the endophyte was done by inoculating the endophyte and placing into a freshly prepared potato dextrose agar (PDA) plates. These plates were then incubated at 28^{o}C. All the prepared plates were labeled with date, plant name and its explants used for proper identification of the endophyte obtained.

6.5. Morphological study of fungi:

Morphological study of isolated fungi was done by using Lactophenol cotton blue staining

Requirements :A young culture (5 to 7 days old), Lactophenol cotton blue in a dropper bottle and Mounted needle

PROCEDURE

1. Placed a drop of lactophenol blue on a clean slide.

2. Transferred small piece of fungus, preferably with spores and spore bearing structures, into the drop, using a flamed, cooled needle.

3. Gently teased the materials using two mounted needles.

4. Mixed gently the stain with the mold structures.

5. Placed a cover- glass over the preparation taking care to avoid trapping air bubbles in the strain.

6. Observed the slide under the microscope carefully.

6.6 Primary screening:-

In the present study, endophytic fungal species from the leaves of plants were selected for the qualitative analysis of four different extracellular assays such as cellulase, amylase, protease, and lipase (Maria et. al., 2005). Based on the digestion of the dissolved or suspended appropriate substrate supplied in agar medium by the extracellular enzyme produced by endophytic fungi which was incubated for 3-5 days at room temperature resulted in clear zone formed around the fungal colony.

Four enzyme tests were performed on the subcultured endophytes.

- 6.6.1. <u>For amylase</u>

  1.5% starch was dissolve in PDA and autoclaved. The plates were then prepared by inoculating the selected isolates. After 3-5 days incubation time, the fully formed cultures were flooded with 1% iodine in 2% potassium iodide. The clear halos should be visualized around the colony.

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- 6.6.2. For cellulose

  1% carboxy methyl cellulose (CMC) was dissolved in PDA and autoclaved. After preparing the plates and inoculating the plates were incubated at 28°C. After 3-5 days incubation time, the fully formed cultures were flooded with with 0.1% congo red dye for 30 mins. Then destained with 1M Nacl for 30 mins. The clear halos should be visualized around the colony.

- 6.6.3 For protease

  10% stock solution of skimmed milk was prepared and autoclaved with PDA in separate flasks. After autoclaving, 0.5% of skimmed milk stock solution was added in PDA inside the LAF. The plates were prepared and incubated at 28°C. After 3-4 days of incubation zone of clearance was seen which shows the protease enzyme activity on the skimmed milk PDA plates.

- 6.6.4 For lipase

  0.5% of tributyrin solution was prepared and added to autoclaved PDA and the plates were incubated at 28^0C. After 6 days of incubation zone of clearance should be seen. This would indicate the presence of lipase.

6.7 Antimicrobial Activity

6.7.1 Preparation and Storage of Supernatant and Cell Free Extract:

For primary screening , pure fungal strain was inoculated in a test tube containing 5mL of potato dextrose broth which was used for primary screening Cultivation was done at 150 rpm at 28^0C in an incubator shaker .After 6 to 7 days, strains that had grown properly in the test tube, were processed for fractionating the supernatant and cell free extract. For separation, samples from test tubes were transferred to 50 ml centrifuge tubes and labelled. Now the samples were centrifuged at 10,000rpm for 30 minutes at 28^0C. After centrifugation, supernatant was collected in another centrifuge tube and the tubes were properly sealed with parafilm .Pellets were further processed by adding 1mL of lysis buffer(Tris 50mM, pH7.5, TritonX and glass beads) in the

eppendorf. The eppendorfs were vortexed for 45 minutes followed by centrifugation at 10,000rpm for 30 mins at 28^0C. The cell free extract and supernatant were stored at -20^0C.

6.7.2 Agar Well Diffusion Method.

Antimicrobial activity was checked against four bacterial strains- *Halobacterium, Bacillus subtilis, Bacillus megaterium and E.coli.*

For carrying this out first, the endophyte samples were cultured in PDB at 28^oC. Luria agar plated were made onto which the four bacterial cultures (200 µL) were spread in different plates. Holes were made in the plates with the help of 1mL of microtip. 200µL of each supernatant and cell free extract were loaded in wells. Plates were incubated O/N at 28^0C.Zone of clearance was observed and results were recorded.

6.8 Isolation of DNA from Fungal Mycelia

- The culture was inoculated in 5mL of PDB and incubated for 3-4 days at 28^0C in a shaking incubator for proper growth.
- The culture was centrifuged at 6000rpm for 10 mins.
- Supernatant was discarded and to the pellet added 500 µl of TES buffer, 5 µl proteinase K and 5 µl of RNase followed by incubation at 60 ^{o}C for 1hr.
- After incubation, 140 µl of NaCl and 64 µl of CTAB were added in the eppendorf and were incubated for 15 mins at 65 ^{o}C.
- Phenol, chloroform and isoamyl alcohol was added in equal volume (in ratio 25:24:1) and centrifugation was done at 1000rpm for 10 mins.
- Aqueous layer was removed and transferred to a fresh eppendorf.
- In the aqueous layer 0.6 volumes of cold isopropanol and 0.1 volumes of 7.5M ammonium acetate was added.
- After incubation at -20 ^{o} C for 30 mins the eppendorfs were centrifuged at 10,000rpm for 10 mins.
- The pellet obtained was submerged in 70% ethanol and centrifuged at 10,000rpm for 10 minutes.
- Ethanol was discarded and the eppendorfs were air dried followed by addition of 50µL of TE buffer for storing the DNA.

6.9 Agarose Gel Electrophoresis

After isolating DNA, gel electrophoresis was carried out, for that 0.4g of agarose was dissolved in 1XTAE by heating. After cooling it down a bit of EtBr was added and the gel was poured in the chamber by placing the comb for casting.

After the solidification of gel, it was placed in electrophoresis chamber and it was filled with 1XTAE. The samples were loaded in the well with a DNA ladder. The migration of isolated DNA showed band under Gel documentation confirming the isolation of DNA from endophytes.

6.10 PCR Amplification

With the confirmation of DNA, next step was amplification. For that autoclaved PCR tubes were taken for preparing the solution.

In the tube a total of 20µl reaction was set containing 6.5 µl water, 2.5 µl 10x buffer, 2 µl $MgCl_2$(5mM), 0.5 µl(10pmole) ITS forward and reverse primers (ITS1 & ITS4),1.5 µl dNTPs(10mM), 5 µl template and 1.5 µl(1U/µl) Taq polymerase. The reaction was the immediately set with reaction window of 2 mins at the start at 94 ^{o}C, then at 94 ^{o} C for 30secs, 58 ^{o} C for 30 secs, 72 ^{o} C for 1min 30secs, and 72 ^{o} C for 5 mins. The reaction was carried for total 35 cycles.

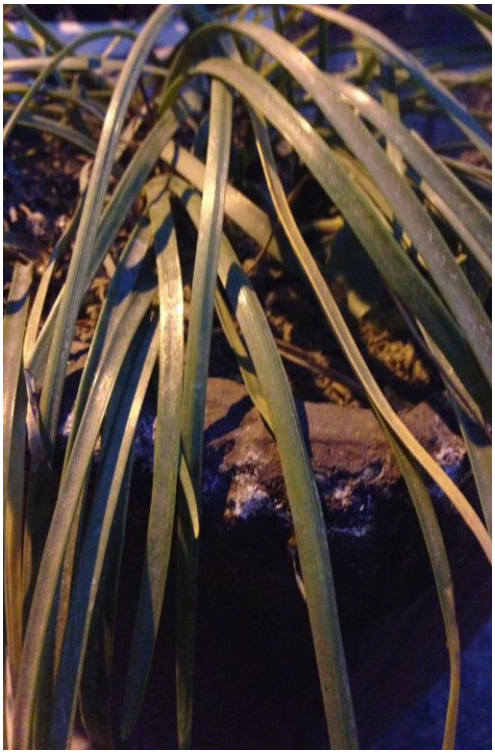
The amplified product was sent for sequencing to Bioserve, Hyderabad.

CHAPTER-7

RESULT AND DISCUSSION

7.1. Sample collection

Plant *Polianthes tuberose* and *Antirrhinum majus* were collected from Jalandhar nursery and grown under controlled conditions for their proper growth.



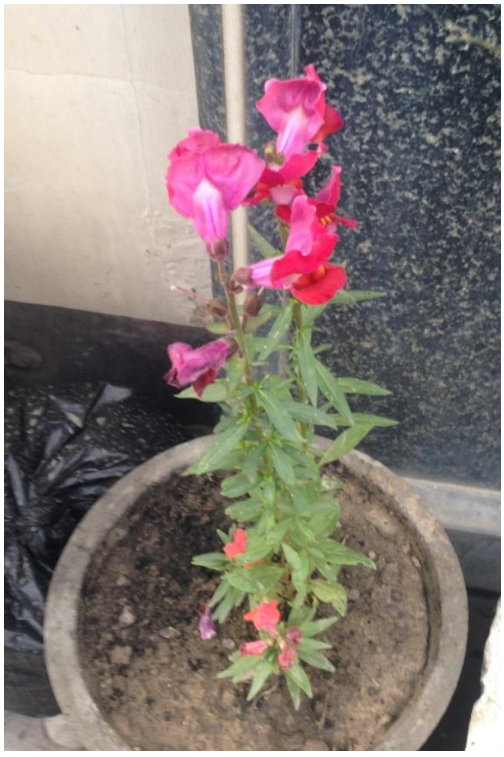


Image-5,6. Grown *Polianthes tuberose* and *Antirrhinum majus.*

Polianthes tuberose and *Antirrhinum majus* were grown on water agar. Different explants from both plants i.e- stem, roots and leaves were grown in different water agar petri plates for isolating endophytes from them. Endophyte growth was observed in leaves of *Antirrhinum majus* but not in *Polianthes tuberose*, hence not processed further.

7.2. Plating

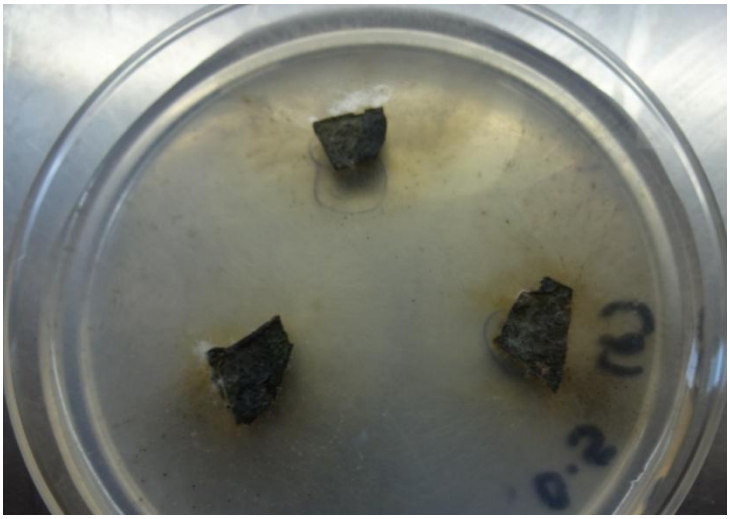


Image-7. Leaf of *Antirrhinum majus* inoculated on Water Agar showing initial growth of endophytes.

7.3. Subculturing

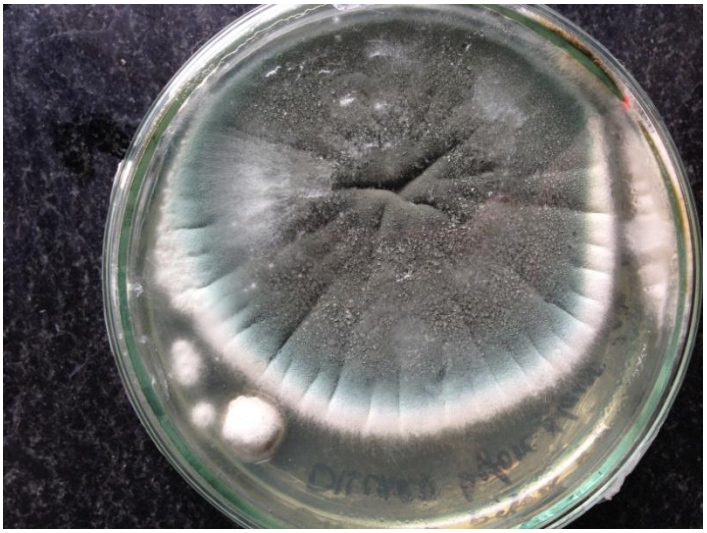


Image-8. Isolated endophytic strains from leaves of *Antirrhinum majus* on PDA.

Endophtyes from leaves were inoculated and sub cultured into freshly prepared PDA petri-plates for their growth.

7.4. Results of microscopic identification.

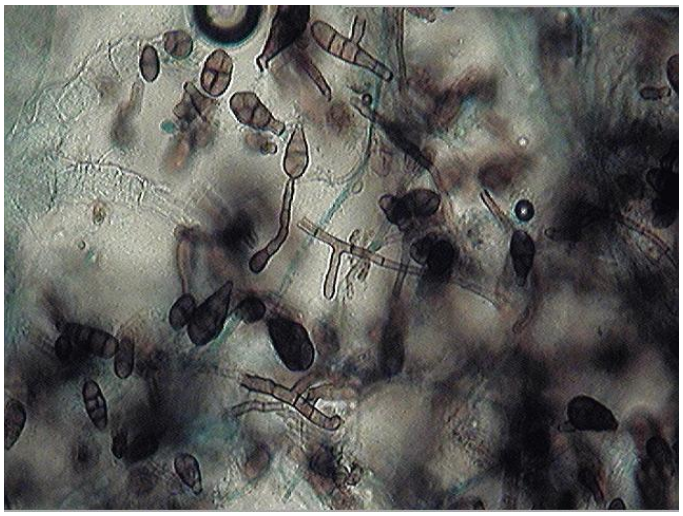


Image-9. The endophyte isolated from AML were of *meyerozyma caribbica* species.

7.5. Primary Screening

7.5.1. Cellulase Test

Image-10,11. Plates showing cellulase activity of endophyte isolated from *Antirrhinum majus* (AML)

7.5.2. Amylase Test

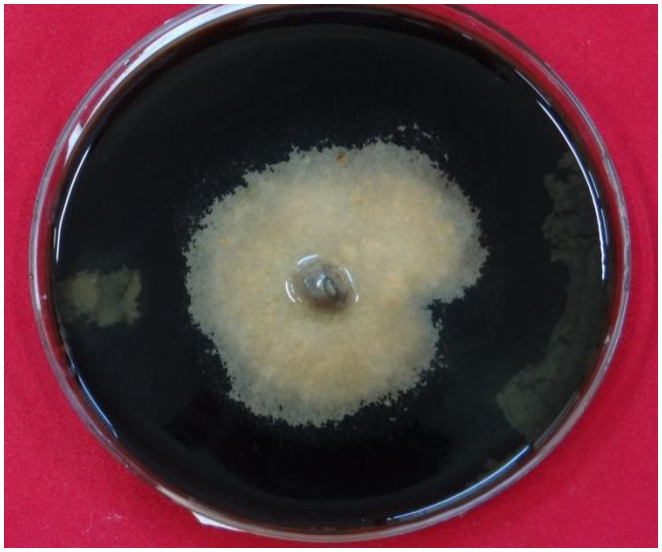


Image-12. Plate showing amylase activity of endophyte isolated from *Antirrhinum majus* (AML)

7.5.3. Protease Test

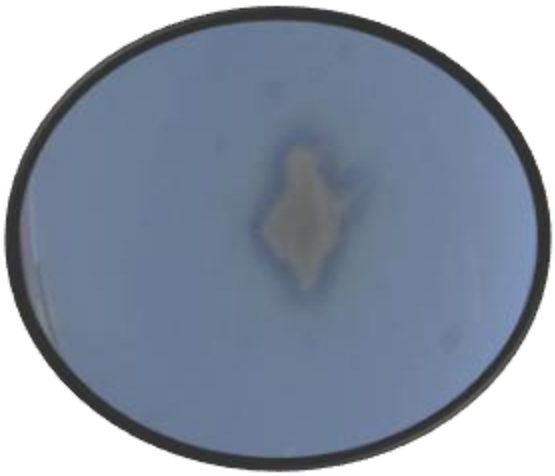


Image-13. Plate showing protease activity of endophyte isolated from *Antirrhinum majus*(AML)

7.6. Endophytic isolate of *Antirrhinum majus* (AML)for Antimicrobial Activity

Plant part used	*E.coli*	*Halobacterium*	*Bacillus subtilis*	*Bacillus megaterium*
Leaf	+++	-	+++	-

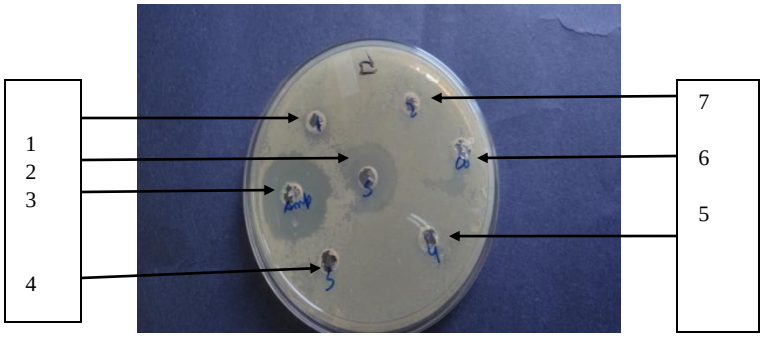


Image-14. Plate showing primary screening of isolate from *Antirrhinum majus* against *E.coli.*

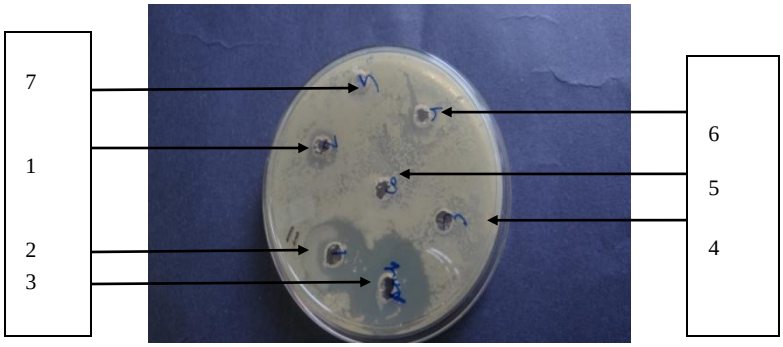


Image-15. Plate showing primary screening of isolate from *Antirrhinum majus* against *Bacillus subtilis*

1- CFC extract (200 µl)
2- Supernatant (50 µl)
3- Supernatant (200 µl)
4- Lysis buffer (CFC control)
5- PDB (Supernatant control)
6- Empty
7- CFC extract (50 µl)

7.7. DNA Isolation

Image-16. 0.8% Agarose gel electrophoresis showing isolated endophytic DNA(AML DNA)

7.8. PCR Amplification

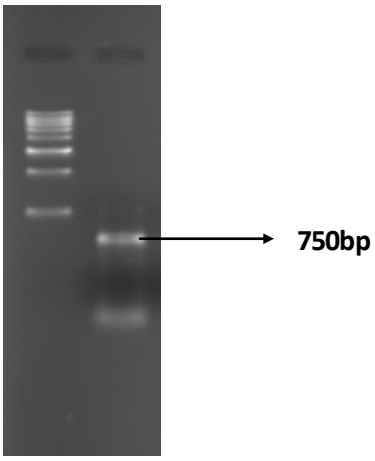


Image-17. 1% agarose gel showing amplification of isolated endophytic DNA (AMLDNA).

7.9. Sequencing Results

>15115-AMLDNA-ITS1

GGGGTTTTCTCCTTTCGGCGTCTGCCTGCGCGGCGAAAACCTTACACACAGTGTCTTT
TTGATACAGAACTCTCGCTTTGGTTTGGCCTAGAGATAGGTTGGGCCAGAGGTTTAA
CAAAACACAATTTAATTATTTTTATTGATAGTCAAATTTTGAATTAATCTTCAAAACT
TTCAACAACGGATCTCTTGGTTCTCGCATCGATGAAGAACGCAGCGAAATGCGATAA
GTAATATGAATTGCAGATTTTCGTGAATCAACGGGGGTCTGGGGGCCCATGGGGGC
CCCTGGGGCCCCGAAGGGGGCGGCTTTTTGGGAGGGATGTTATTTGGGGACACCCCC
GGGGGGGGGTGTGGGGGAGGCTCTTTGTTAAATGCTTTAAGGTTGAAAGCA

Description	Max score	Total score	Query cover	E value	Ident	Accession
Meyerozyma sp. 1 CS-2017 genomic DNA sequence contains ITS1, isolate JY117	429	429	59%	3e-116	99%	LT795053.1
Meyerozyma caribbica strain GKVK internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene, complete sequence; and internal transcribed spacer 2, pa	429	429	59%	3e-116	99%	KY810496.1
Meyerozyma caribbica strain m67b internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; anc	429	429	59%	3e-116	99%	KP675261.1
Meyerozyma caribbica strain h63a internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and	429	429	59%	3e-116	99%	KP674752.1
Meyerozyma caribbica isolate LY11 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; an	429	429	59%	3e-116	99%	KJ535105.1
Meyerozyma caribbica isolate B-NC-12-OZ21 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequ	429	429	59%	3e-116	99%	KF728801.1
Meyerozyma caribbica isolate B-NC-12-OZ13 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequ	429	429	59%	3e-116	99%	KF728794.1
Meyerozyma caribbica isolate B-NC-12-OZ01 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequ	429	429	59%	3e-116	99%	KF728784.1
Meyerozyma caribbica isolate B-NC-12-OM24 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete seq	429	429	65%	3e-116	97%	KF728783.1
Meyerozyma caribbica isolate B-NC-12-OM16 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete seq	429	429	59%	3e-116	99%	KF728778.1
Meyerozyma caribbica isolate B-NC-12-OM08 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete seq	429	429	59%	3e-116	99%	KF728771.1
Meyerozyma guilliermondii isolate SZ-28 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequenc	429	429	59%	3e-116	99%	KF472173.1
Meyerozyma caribbica isolate B-WHX-12-26 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequ	429	429	59%	3e-116	99%	KC544483.1
Meyerozyma caribbica strain Hb36b internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene, complete sequence; and internal transcribed spacer 2, pa	429	429	59%	3e-116	99%	KC422423.1
Meyerozyma guilliermondii internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and 28S rib	429	429	59%	3e-116	99%	JN974905.1
Meyerozyma guilliermondii strain PYSS1 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequenc	429	429	59%	3e-116	99%	JF920159.1
Meyerozyma caribbica strain F49572-08 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequenc	429	429	59%	3e-116	99%	HQ693777.1
Pichia guilliermondii internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and 28S ribosoma	429	429	59%	3e-116	99%	DQ088676.1
Meyerozyma guilliermondii voucher UFLA YRCS21 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene, complete sequence; and internal transcrib	427	427	59%	9e-116	99%	KU892645.1
Meyerozyma caribbica isolate 22F small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1 and 5.8S ribosomal RNA gene, complete sequenc	424	424	59%	1e-114	99%	KY848364.1
Meyerozyma caribbica isolate 20F small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1 and 5.8S ribosomal RNA gene, complete sequenc	424	424	59%	1e-114	99%	KY848363.1
Meyerozyma caribbica isolate 19F small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1 and 5.8S ribosomal RNA gene, complete sequenc	424	424	59%	1e-114	99%	KY848362.1
Meyerozyma caribbica isolate 17F small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1 and 5.8S ribosomal RNA gene, complete sequenc	424	424	59%	1e-114	99%	KY848361.1

Image-18. Showing the blast results for AML

The endophyte isolated from AML showed 99% similarity and 59% query coverage for *meyerozyma caribbica*.

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CHAPTER-8

CONCLUSION AND FUTURE SCOPES

The isolated endophyte was grown and subcultured which showed presence of important bioactive compounds.*Antirrhinum majus* endophyte had been isolated and subcultured, study on its identification by isolating the DNA of the endophyte and sequencing was also done. Although, *Polianthes tuberosa* was also cultured on PDA but no endophyte was seen on the plate, hence not processed further for any experiments.

As no endophyte study on them had been carried out their endophyte isolation and identification may provide importance. Further studies from the endophyte was carried out by screening, for different anti-microbial activities. Also any important bioactive compounds if present can be isolated.

CHAPTER-9

OUTCOMES

Endophytic organism shares a mutual relationship with plants and contributes to its health. There have been many endophyte isolations and their analysis has provided strong evidence that they can play a role against biotic stress, secondary metabolite pathways, resistance to environmental stresses, promoting plant growth etc. The endophytes survive onto the nutrients supplied by plants so anything affecting the plant nutrition can have an impact on the endophytes also. The physical factors like- temperature, UV radiation, rainfall etc can affect the endophytic communities indirectly (Tadych, & White, 2009). The endophytic microorganisms can produce natural bioactive products which can be useful in industry, medicine and agriculture. Bioactive compounds like terpenoids, steroids, alkaloids and flavonoids from endophytes are few such examples. The compounds can act as anti-cancer agents, antibiotics, immune-suppressants, biological control agents etc. Endophytes have a large potential in various fields.

Endophytes are useful in phytostimulation (help plants in nutrient uptake); pigment production which can be used as natural colorants in food industry; enzyme production example- *Acremonium zeae* from maize which extracellularly produces hemi-celluase, this hydrolytic enzyme has application in conversion of lignocelluloses to fermentable sugars; antimicrobial activity; source of novel compounds like Diaportheone A and B, two benzo-pyanones are secondary metabolites from *Diaporthe* species. An endophytic fungus present in *Pandanus maryllifolius* leaves, it can inhibit the growth of *Mycobacterium tuberculosis* (Bungihan et.al., 2011). Taxol used for various cancer treatments is a product of *Metarhizium anisopliae* organism obtained from the bark of *Taxus chinensis* (K.Liu et.al,2009); bio-control agents which can be used as alternative to chemical controls and can be also genetic engineered for increasing efficiency; bioremediation as endophyte can break complex compounds (this can be employed in treating waste); production of volatile compounds (Wani and Ashraf, 2015).

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APPENDIX

11.1. Water Agar (Himedia) for 150ml

1.5g in 150ml of D.W

11.2. Tween 20 (Atlas Chemie) for 10 ml

1% Tween 20- 0.1ml in 10ml of D.W.

11.3. Mercuric Chloride (Qualikams) for 10ml

0.1% mercuric chloride- 0.01g in 10ml of D.W.

11.4. Potato Dextrose Agar (Himedia) for 100ml

3.9 g in 100ml of D.W

11.5. Potato Dextrose Broth (Himedia) for 100ml

2g in 100ml of D.W

11.6. Carboxy Methyl Cellulose (Himedia) for 100ml

1% CMC- 1g in 100ml of PDA

11.7. Starch (Qualikams) for 100ml

1.5% Strach- 1.5g in 100ml of PDA

11.8. Skimmed milk (Nestle) for 10ml

10% Skimmed milk- 1g in 10ml of D.W

EQUIPMENTS USED

- Laminar Air Flow (Rescholar)
- Weighing Balance (Adventurer)
- PCR (eppendorf)