ISOLATION, IDENTIFICATION AND BIOACTIVE PROPERTIES OF ENDOPHYTIC MICROORGANISMS FROM *Urtica dioica* AND *Cestrum nocturnum.*

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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CERTIFICATE

This is to certify that the Dissertation Report titled "**ISOLATION, IDENTIFICATION AND BIOACTIVE PROPERTIES OF ENDOPHYTIC MICROORGANISMS FROM *Urtica dioica* AND *Cestrum nocturnum***." That is being submitted by "BHAWANA TEWARI" 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, Bhawana Tewari hereby declare that the project that the project report entitled "**ISOLATION, IDENTIFICATION AND BIOACTIVE PROPERTIES OF ENDOPHYTIC MICROORGANISMS FROM *Urtica dioica* AND *Cestrum nocturnum***." 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 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

U.dioica	*Urtica dioica*
C.noctrunum	*Cestrum noctrunum*
D.W	Distilled Water
HPLC	High Pressure Liquid Chromatography
DNA	Deoxyribose Nucleic Acid
CNL	*Cestrum noctrunum* leaf

ABSTRACT

Endophytes may have bioactive products which can be useful in different areas. *U.dioica* and *C.noctrunum* are two plants whose endophyte study has not been carried out and they may have potential importance. Both plants were collected and grown in proper conditions and cultured in laboratory for isolating endophytes from it, followed by proper surface sterilization by the use of chemicals and plating in water agar. The endophytes were further inoculated for their subculturing. The identification of the subcultured endophyte was done with sequencing. And various enzyme assays were carried out. After running BLAST for similarity CNRDNA showed 72% similarity towards *candida, saccharomycetes and picha occidentalis.* Moreover, CNLDNA showed almost 80-90% similarity towards different species (*aspergillus flaschentraegeri, pencillum purpurogenum, talaromyces funiculosus, leptogium burnetiae*).

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Chapter-1
INTRODUCTION

Endophytes are the microorganisms (fungi or bacteria) which grow intercellular or intracellular within the tissue of a living plant, this relationship is called the symbiotic relationship where the endophytes colonize in the inner tissue of the plant without causing harm to host, known as endophyte-host interaction (1,2). Endophytes produce bioactive substance, they are an encouraging source of novel organic natural metabolites, both the partner's i.e. endophytes and the host get the benefit of this interaction (3, 4). Endophytes are a useful source of bioactive natural products with vast capability for the drug discovery, industrial application, medicinal and agricultural uses (5). In an ecosystem, the presence of many different kinds of endophytes plays an essential role in the plants to produce substances which are bioactive having low toxicity towards higher organism and thus endophytes can act as chemical synthesizer inside plants (6).

Endophytes provide potential utility in safety and concerns of human health by producing bioactive natural compounds, including chinones, alkaloids, benzopyranones, flavonoids, phenolic acids, quinones, steroids, terpenoids, tetralones, xanthones, and others. These are functional metabolites with a broad variety of bioactive secondary metabolites having wide range application in medical uses. By the use of endophytes, one can work on anticancer agents, antibiotics, antioxidants. Due to these listed applications of endophytes, there has been an interest in its origin, biodiversity and its economical role (5,6,7). In today's life, the threat of cancer is increasing day by day it enhances the need for new and beneficial compounds that have the ability to provide cure against diseases; microbial endophytes can be seen us a promising source of novel and natural products for human welfare.

There is a great need to study the bioactive properties of endophytic microorganism as there are many undiscovered endophytic species and the ranges of chemicals produced by endophytes are very diverse. They have the ability to synthesise secondary metabolites which have potential to treat many diseases and to obtain bioactive compounds by methods including microbial transformation called biotransformation, use of natural source, extraction

and collection of host plants, isolation and identification of bacterial strain endophytes, biological screening for determination of antimicrobial activity, chemical screening of host and bacterial extracts, genomic DNA extraction, amplification and sequencing, phylogenetic analysis, HPLC analysis, 2 dimensional paper chromatography analysis. Biotechnology techniques have been used for the production of volatile compounds which have sensory properties.

The popular methods used for antimicrobial activity are extraction, antibacterial assay, antifungal assay, micro-dilution method and phytochemical tests. It also has attractive properties like antimicrobial, antifungal, antioxidant, antiviral, blood pressure regulating, anti-inflammatory properties and others (8,9,10).

1.1.Nettle plant (*Urtica dioica*)

Nettle plant is the member of *Urticaceae* family. It grows up to 2m and is known for its medicinal use and also used as a food source. In ancient times it has been used for joint pains because of which is also known as a healing herb. However, the touching the herb causes inflammation at the contact area immediately giving a sharp needle pains followed by itching due to the presence of tiny hairs called stinging trichomes. They have a high amount of silicon and are rich in medicinal and nutritional value (11, 12, 13,14). It is being used for curing certain allergies and helps in treating sinus. Nettle plant consists of lectins and complex sugars (15). Mainly the location of the nettle plant is near river, they are known to adapt harsh condition, grow in hilly areas, it can bear cold temperature. |Leaves have pointed tips with sharp uneven edges.

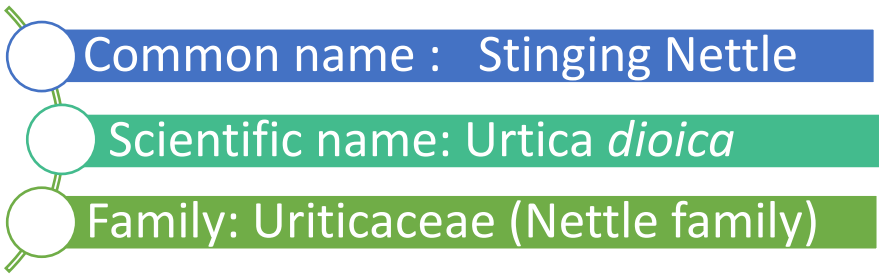

Fig.1- List of common name, Scientific name, Family

Flowers of nettle plant grow in rows, close to each other. Furthermore, leaves are much exposed, and stings are present all over in leaves as well as in stems.

Stings if touched release chemicals causing irritation and rashes. These pointed stings are painful to touch, but the pain does not last very long. In India, it is mostly found in the Himalayan region like Himachal Pradesh, Uttrakhand.

Image-1. Nettle plant, *Urtica dioica.*

Image-2.Nettle plant (*U. dioica*)

Photo: Wolfgang Stuppy

Nettle plant is full of many elements these all elements give it unique properties and highly increases its nutritional value. Nettle contains prostaglandins, high levels of protein, calcium, phosphorus, iron, magnesium, and beta-carotene. It is an excellent source of vitamin A, vitamin B, and vitamin D2 and thus having high nutritional value. It is known to treat muscle and joints pain, gout, hay fever, urinary tract infections, insects bites, arthritis, anemia, eczema and can also treat benign prostatic hyperplasia (16,17). Nettle plants favor hot, mild climate areas. Nettle plant is also known for its fiber content which is long, light and resistant. Nettle plant is full of many a beneficial property of which little is known. It has the potential to treat cancer and many diseases (18).

1.2.*Cestrum nocturnum*

Cestrum nocturnum may also have some endophytic property; less research has been done on *cestrum nocturum.* It is a well-known plant for its heavenly smell and said to be the world's strongest smelling plant. Its common name is night blooming jasmine, lady of the night, queen of the night, night cestrum etc as the name suggest it means it blooms and it’s smell overpowers at night, in hindi and manipurio it is called "raat ki rani" and moon flower, thus also known as for its nocturnal beauty. *Cestrum nocturum* belongs to the family Solanaceae.

Image-3. *Cestrum nocturnum* (raat ki rani/ night queen)

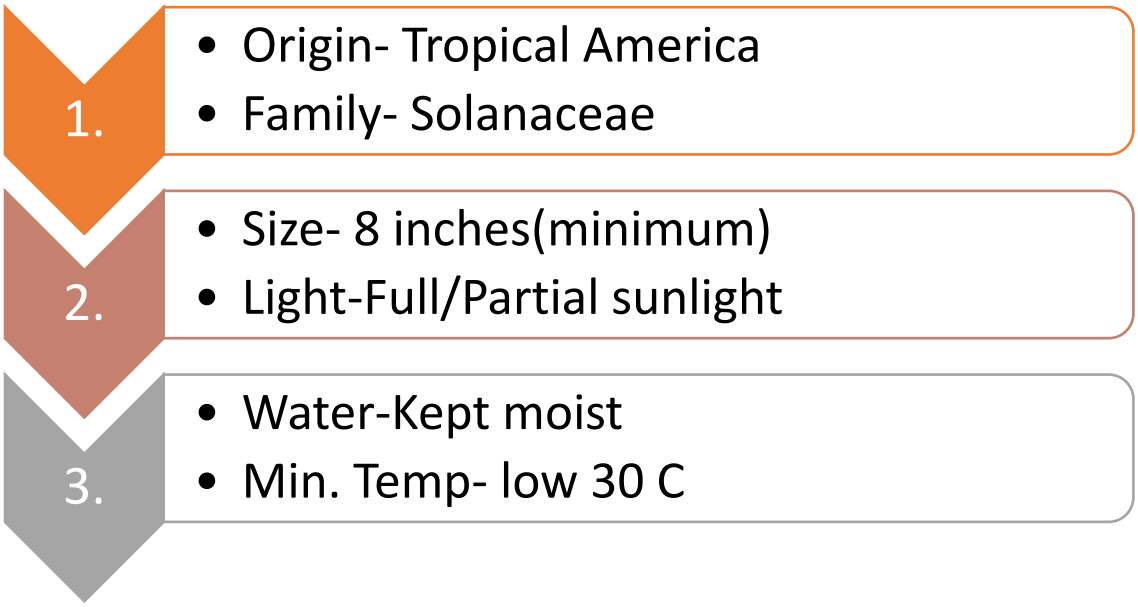

Fig.2. Origin, Size, Water and temperature required.

It is an evergreen and sprawling shrub, having leaves which are glossy, smooth and pale green. The flowers of this plant are known to bloom at night while it blooms it spread its overpowering fragrance everywhere and hence it is said that the smell of jasmine is heavenly, but some people are allergic to its smell as the smell diffuses very well with surroundings, one can smell it from 165 feet away. Chinisiwe folk used it as a treatment for burn and swelling, bactericidal activity was observed in the leafs of *C.nocturnum* plant. The leaves are simple and smoothly in touch. Leaf in its mature stage contains a carcinogenic glycoside which is known to have vitamin D toxicity and is responsible for raising the level of serum calcium.

Many parts of *C.nocturnum* are used like leaf, stem, and flowers for various purposes. Many compounds are being isolated from *C.nocturnum*. It is also known for its antifungal and antibacterial activity, due to these two main properties of *C.nocturnum* it became capable of treating disease, many medicinal uses are there of this plant. *C.nocturnum* is one of the best species, the elements present inside it fully makes this plant use in a human welfare. It can also fight with bacteria which are pathogenic to humans; many infectious diseases are being treated by *C.nocturnum*.

Chapter-2
REVIEW OF LITERATURE

A plant having endophytic property is beneficial to other organisms. This host interaction is a symbiotic relationship between them both, taking advantage of this interaction in their own way. Endophytes make colony with inner tissue of the host and give benefits to the host. Endophytes can be present in roots, leaves, and stems, a bioactive compound can be isolated from endophytes. The endophytic interaction is known as a mutualism as both the plant and organism provide benefit to each other. The bioactive compound has potential and can be used for medicinal purpose, industrial level etc (2). Endophytes are widely used to obtain bioactive compounds. Bioactive compounds are useful in the process of Biotransformation. Biotransformation is the change of chemicals in the body example- drugs, amino acid, nutrients, and toxins. Biotransformation of a drug, one of the most suitable example is liver, the liver does elimination of many drugs.

Nettle plant is known for its great medicinal value, traditionally being used for its nutritional value as well. It is an excellent source of vitamins. It is seen that nettle have a great capacity to treat digestion problem, anemia and the problem of aged people of joint pain (1). Many plants are having medicinal value, the chemical substance inside the plant judges the medicinal value some plant have little medicinal use some are known to have a magical touch. Many plants having medicinal value that means that antimicrobial or antioxidant elements are present in their tissues. There are some free radicals which show unwanted effect, antioxidants help by reversing the effect of free radicals. It is important to know the potential of various plant species and analyses of their properties for human welfare.

2.1.*Urtica dioica*

Nettle plant is an angiosperm which belongs to kingdom plantae, order Rosales, family urticaceae, genus *urtica* and species *urtica dioica.*

Nettle plant has a great capacity to full fill the needs, as nettle plant have many different constituents of chemicals. They are known to have sugar, starch, gum, choline, histamine, oleanol acid, steryl glycosides, flavanol glycosides, serotonin, homovinlyl alcohol neo-olivil

and secoisolariciresinol. Carotenoids are also being reported such as beta-carotene full of vitamin A, violaxanthin, zeaxanthin, xanthophylls, luteoxanthin, and lutein epoxide. Nettle stem, leaves, roots sting all having some significant chemical composition like from the leaf of nettle an anticoagulant was isolated and some new monoterpenoid, 18 phenolics were also detected. The leaves of nettle contain polyphenolic compounds and the amount is said to be less in female but more in male form of the plant. Nettle plant is also known to have enzymes which are useful for example, acetyl choline producing enzyme and choline acetyl-transferase.

Nettle plant is also useful in treating problems found in aging men like Benign prostatic hyperplasia (BPH). For treating this urinary infection nettle plant is researched, a source used was nettle plant roots. The alcoholic and aqueous extracts of *Urtica dioica* show antifungal activity against some fungi and anti-bacterial against some gram-positive and gram negative bacteria. Mainly disc assay gave anti-microbial activity against *P.vulgaris, L.monocytogenes* and *K. pneumoniae* cultures and inhibition zones were observed (21).

As nettle is known for many properties so it helps to treat many diseases, many tests have been conducted to explore the activity of nettle and use it for several reasons worldwide. Nettle is also known to treat asthma, it helps and is useful in some extent to treat prostate cancer the patients of prostate cancer have some activity of adenosine deaminase in their prostate tissue. *Urtica dioica* has been tested and its properties made it useful to overcome the problem of prostate cancer. *Urtica dioica*'s extracts have been used, the extract should be aqueous. This aqueous extract has been known to inhibit the activity of adenosine deaminase which is found in prostate tissue (22). The extract used in this test was leaves of *Urtica dioica*, aqueous leaves extract is taken having variation in their amount, along with the patients which have the prostate cancer are chosen for the study their adenosine deaminase activities were checked. ADA activities are checked with pre-incubation and some are checked without pre-incubation (22).

Urtica dioica has been used in Ayurveda it is generally used as a remedy for first-aid. Although *utica dioica* is rich in properties but the main two suggested one are the presence of histamine and formic acid. These two important agents give *Urtica dioica* one of its property called anti-infection, it can be used against any infection.

Traditionally people used *Urtica dioica* plant's part like leaves, root and stem one of the most used part is root. Roots were crushed and diluted then made into a cup, by having this remedy it is known to treat and have the ability to maintain prostate health, inflammation, allergies and hypertension. Huge no. of pharmacological work has been performed on *Urtica dioica*. Both in vitro and in vivo work are done on *Urtica dioica* one of the works which tell the consequence of *Urtica dioica* on cardiovascular tissue. Testai successfully did the work on this test.*Urtica dioica* has shown that blood vessels and heart diseases can be treated (23).

The parts of *Urtica dioica* are full with essential properties. With the help of the *Urtica dioica* leaf extracts, insulin secretions have been found in Langerhans i.e in its islets this effect was tested in both the normal one rat and the rats which are found to be diabetic (24). The derivatives of *Urtica dioica* are very crucial from the medicinal aspects. The metabolites and the roots of *Urtica* are used to treat diseases; lignans are used which are taken from the roots. The lignans and metabolites are known to have the ability to bind with human hormones eg. dilution bioassay of sex hormone binding globulin (SHBG) (25).

Dilution bioassay of agar is used for the anti-mycotic conditioning of ethanols. Extraction was done from nettle flower parts this test was conducted in vitro on various species of fungi. All the tested sample of fungi show the activity which is anti-fungal this test shows that nettle plant has antifungal properties. Other researches are also going on which shows the antifungal property of the nettle plant. Fungal diseases are widely spreading and increasing and cause much threat to the organism due to this anti-fungal property of nettle plant it can be used against various aspects of treating disease (27).

One of the threatening diseases name arterial thrombosis is generally a blood clot in an artery, it is very dangerous as it causes serious conditions. It blocks the flow of blood. The remarkable properties of nettle are widely used to treat various harmful diseases. In in vitro condition, different extracts of nettle plant are taken which are of different concentration and are incubated with rat platelets. This test support the use of nettle plant by ancient people for treatment of cardiovascular diseases (29).

A study on nettle plant in which *Urtica dioica* can be used as bioindicator by the help of uranium bio-indicator. Uranium bioindicator is an indicator of contamination. Nettle plant is said to have uranium (uranium is a radioisotope), this study shows the amount of uranium in

nettle plant, for this soil sample has been collected from the phosphor-gypsum stockpile (32). Uranium is also found in soil so to determine the concentration of uranium in nettle plant, soil concentration is checked, by knowing the concentration of the uranium in soil and root it can be used as a bioindicator which can indicate contamination. Nettle plant is highly economical, a medicinal plant which has the ability to treat many diseases, and it is a topic of concern for many scientists.

2.2.*Cestrum nocturnum*

It belongs to kingdom plantae, order solanales, family, solanaceae, genus *cestrum* and species *c. nocturnum*. *Cestrum nocturnum* is known for its heavenly smell and it is world's strongest smelling plant also called the queen of the night. Night blooming, night cestrum is said to have endophytic properties. Further *cestrum* is known for its liver protective activities, and ethanolic extract of the same is rich in hepatoprotective compounds. Those compounds are flavonoids and phenolics compounds (2). *Cestrum nocturnum* is known to have phytochemicals these phytochemicals are present in the form of secondary metabolites. *Cestrum nocturnum* are tested and concluded that it have cardiac glycosides, alkaloids, carbohydrates, tannis, phenolics (23).

The antimicrobial agents were effective against the bacterial and two fungal species *C.aurantiacum* was the most effective antimicrobial organism in bacteria and its activity in butanol extract was found in increasing amount against various species of *aspergillus*. The whole plant of *Cestrum nocturnum* is used and the extraction of crude methanol is done. Fractions are made and tested this testing was done against fungal and bacterial strains the result was positive, *cestrum nocturum* shows antimicrobial activity against bacteria like *bacillus subtils, Escherichia coli, staphylococcus aurous and Pseudomonas*, in this test, there was one exclusion against *which C.nocturnum* did not show antioxidant activity was *salmonella typhi* (26).

The pharmacological study is also been done on *C.nocturnum* by using the extract of its leaf for counteracting increasing amount of glucose in the blood (antihyperglycemic). When the level of glucose drops, sugar level also drops leading to a headache, a person is not able to talk and have confusion, this problem is known as Hypoglycemic. The study shows that *C. nocturnum* has the potential to treat this Hypoglycemia (30). Phytochemical analysis can also

be carried by *C.nocturnum* study, estimating the HIV -1 (human immunodeficiency virus) activity from the extracts of *C.nocturnum*. Syncytia formation assay and many extracts from the plant has been used and it looks that it have less anti-HIV-1 activity. When *C.nocturum* was investigated or analyzed it detected the existence of phytocomponents, sterols, saponins, coumarins, triterpenes, and flavonoids (31).

C.nocturnum is also known for larvicidal activity. Larvacide is an insecticide aimed against insect specifically at their larval stage. For this study to be conducted vector-parasite is used which is called Aedes aegypti, also called dengue parasite. The population of this parasite is increasing, new techniques should be used against them, for this extracts of *cestrum nocturnum* is used (33). The smell of it is so good that it attracts people towards it, in many cities of India its been used as gazra i.e a string is made from the flowers of *cestrum nocturnum* and girls of every age group wears it on their hair which beautifies their look. Many people use it for medicinal purposes, it is said to have much medicinal property thus researchers are attracted to this plant as it has medicinal value. Scientists are conducting research on this plant, as this plant is full of many benefits.

Cestrum nocturnum is full of goodness, but some people are allergic to it, that they start sneezing when coming in contact. They can grow simply by sprinkling seeds, so it's easy to grow *cestrum nocturnum*, the seed to propagate just need favourable conditions, it adds up more to *cestrum nocturnum* as seeds are tiny, easy to grow and convenient to transport. *Cestrum nocturnum* is known to have flavonoids, these flavonoids give it uniqueness and can be used as medicine to cure some diseases, it is known to treat burns especially its leaves are beneficial to treat a burn on the skin and also known to have bactericidal activity, it is also known to treat neurological disorders.

In many regions of America, the malaria is the serious cause, *cestrum nocturnum* can be used to treat malaria, its oil is extracted from the leaves which when applied to body will repel mosquitos hence can be used against mosquitos as *cestrum nocturnum* having antibacterial property in addition to antimicrobial property, it also been reported with antifungal, antimicrobial, anticancer and antioxidant. The flower and leaf are used to treat bad seat smell by bathing in hot water having a leaf of *cestrum nocturnum*, it also been recorded that *cestrum* can be used against a tumor. Not only as medicine but also has been used for decorations as the white color of *cestrum nocturnum* flowers look beautiful when used as

decorative piece and the additional point is not only it looks beautiful it also have good smell which defuses all in the room and gives fresh environment and helps in having positive mood because the smell is mesmerizing, some region people also eats its flower and in some region the flowers are used for worshiping god.

In some region the ingestion of *cestrum nocturum* plant parts like fruit, leaf is accepted but some people seems to be highly allergic specially asthma people as they are not able to tolerate its smell, when an asthma patient comes in contact with *cestrum nocturnum* they start having headache, nausea, difficulty in breathing and nose irritation. Some people who eat it's flowers get sick, their temperature get high, the sudden change in pulse is also reported (35). Many plants are known to have endophytic properties, it is simply a mutual understanding between plant and the organism both of them get benefited by this. Microorganism lies beneath the inter and intracellular tissue of the plant and thus help plant in their growth, health, also helps in their development and support the secondary metabolites produced by the plant, however, in return they take nutrition from plant to survive. This relationship is a treasure for a researcher as this symbiotic relationship can help scientists to study the plant and check for the associated microorganisms with that plant which can be used for further study for treating many diseases (36).

The endophytes are in huge diversity on our planet earth as there are numbers of plant and one plant can have many species, and many plants are found to have an endophytic property which means many organisms live mutually with plants hence there is so much diversity of endophytes called hyper-diversity. The proper study of endophytic biology can come up with many objectives; each plant can be a great treasure to find an endophytic property. Many plants can have novel endophytic microorganisms which can have novel property to treat many diseases. The objectives of endophytic biology are that it helps in various industries be it pharmaceutical industry, agriculture, drug industry, food industry , aroma industry, a study of secondary metabolites. The effect of microbial and plant association effect host and microbial secondary metabolites, the plant and microbe association for better productivity (36).

The huge diversity of endophytes leads to classifying endophytes based on physical structure, taxonomy, appearance, evolution, specific into fungal endophytes, microbial endophytes, nonfungal endophytes, actinomycetes endophyte and also virus endophytes. This

classification of endophytes is very necessary due to the complexity and wide diversity of endophytes, so classification is important for studying the endophytes for treating diseases.

2.3. Bacterial endophyte

Bacterial endophytes are found in wide diversity and in any part of the plant like a leaf, roots, internodes, stem, and barks of plant, seed, fruits and flower. Many plants are known to have an association with a bacterial property. Endophytic bacteria are known to be that endophyte which colonizes beneath inter and intracellular tissue of plant and benefit plant by helping in its growth and yield without showing any negative impact or symptoms on the plant (37).

Bacterial endophytes help in controlling harmful insect and many plant pathogens. Plants are known to help to balance atmospheric CO_2 by the process called photosynthesis, plant need support of many bacteria for its betterment in growth, one of the example is rhizobia, a plant needs this bacteria to grow, it shows an association of plant with bacteria. bacteria take nutrition from the plant and give support to plant by helping it grow and give an example of a symbiotic relationship (38).

Isolation of bacterial endophyte can be done from any part of the plant like seed, stem, leaf, roots, flower and seed. Endophytic property can be found in a herbaceous plant as well as in species of woody trees and bacterial endophytic property can be obtained from both mono and dicotyledon plants (37).

2.4. Fungal endophytes

Fungal endophytes have application in the drug industry, agricultural industry, seed industry and much more. Fungal endophytes are known to produce large number of enzymes which are very effective in treating many diseases thus enzyme produced by fungal endophytes are extracellular enzymes like lipases, cellulases, pectinase, amylase, proteinase and laccases this comes under fungal enzymes. Many fungi contain gene which produces these enzymes and serves many applications like hydrolysis process and biodegradation in which enzyme which is basically a protein degrade the complex sugar into simple and this property is useful for many industries. Fungal endophyte also provides support, growth, yield to the plant and help in reducing stress it’s an example of plant and fungal association. Fungi come under the group of eukaryotes and can be used to treat diseases. Fungi have been known for its

bioactive property many bioactive properties are being isolated from fungi, for example, antimicrobial and antifungal agents (39).

2.4.1. Isolation of fungal endophytes

Isolation of fungal endophyte is essential as it produce many enzymes which can be used in various industries. Fungal endophyte can be found in any part of the plant like stem, leaf, fruit, internodes, root and flower etc. Firstly the source plant need to be sterilized so for this surface sterilization is done where the plant is washed under tap water and then in tween 20 or another chemical like 70 % ethanol can also be used followed by washing with distilled water and with filtered mercury chloride plants are then cut into small parts under laminar to maintain aseptic conditions and placed in petri dish containing sterilized media and the plates are incubated so that fungal grows and further can be studied.

After isolation of fungal endophyte, we need to identify it. The plant has fungal endophyte or microbial endophyte, sometimes fungal endophyte can be misunderstood by contamination which is grown on the plant so to check these many tests are followed up which confirm m that the coming growth is of fungal endophyte or any other. The first basis of identification is based on appearance, many funagal endophytes are being isolated from a plant so the basic appearance of them is known so just looking at the morphology one can tell is it fungal endophyte, taxonomy is also play role in identification many fungal endophytes are being characterized according to their taxonomy. The identification is not just based on looking via naked eyes, microscopes are also being used, and microscopic examination gives a broader look to the endophyte.

Fungal endophyte is known to have many properties and activities like antimicrobial activities, proteolytic activities, cellulase activity, amylolytic activity, tyrosinase activity, xylanolytic activity and amylolytic activity. Amylase is used in many starch producing industries, it breaks starch by breaking the bond present in starch i.e 1,4 linkage. Starches gets degraded and thereby convert it into simple sugar. It is being used in many industries like alcohol industry, sugar industry, brewing industry, bread industry, paper industry, baking industry, detergent industry, drug industry, and food industry also help in medical chemistry.

Fungi is one of the major sources of amylolytic enzyme, bacteria, yeast is also known to yield amylase enzyme but the enzyme production from enzyme is more stable than that of bacteria and yeast. To check the amylolytic activity the fungi is plated on agar with 1% soluble starch

then plates are subjected to 1% iodine after incubation if amylase is present clear zone of circles around the fungi will be seen and are measured. Starch is the source of energy and to check amylolytic activity starch is very .

2.5. Importance of isolating endophyte

Almost all plants are known to have endophytic property, and this property gave them potential to be used for human welfare, these tiny microrganims which live beneath the tissue of the plant help the plant to grow and also take nutrition from them and thus live as a symbiotic relationship these microorganisms not only help plant with the association with a plant they also help human in many ways like in many industries. And thus it's become important to study this wonderful relationship and isolate them study them for further use. Once the endophytes are isolated they are characterized, as well as purified and checked their potential to be used to having a novel drug, in alcohol industries, in agriculture, novel antibiotics, an anticancer compound, novel immunosuppressant and antimycotics.

Nowadays humans are facing many diseases some diseases are easy to cure and some are not. Many diseases are there whose drug is being formed but initially, it work and in long run, the disease is not able to be cured, scientists are concerned with this type of diseases and also the diseases which have no drug as such it’s a big issue. Many diseases like arthritis, cancer, aids which are known to be major disease and which are affecting human since past, till now we don’t have any specific and effective drug for that, long ago when there were no drugs no hospital, people used to treat every disease with plant , many philosophers said that many plants have potential to treat many diseases no matter how much dangerous, now scientists also believe that almost all plants have endophytic property which gives it the power to treat disease many scientists said that it can treat disease like cancer, aids.

Endophytes are useful for us as they produce bioactive compounds; these bioactive products can be used to find a novel drug, from this bioactive compound one, can have a drug with minimum side effect or no side effect and low toxicity.

Bioactive products also help in many agriculture sectors, as nowadays the quality of food is decreasing day by day, food are becoming toxic due to high use of pesticide, in many areas govt have taken a serious step, eliminating use of pesticide in agriculture now the importance of bioactive compounds is increasing, so now plants need to be protected from pest by the use of bioactive compounds these bioactive compounds can be very useful as they can control the

pest without being toxic to human, it will not decrease the value of food and thus by isolation endophyte one will have a natural product which does not spread toxicity (40).

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Endophytes are also known to treat heavy metal pollution which is again a serious cause in the environment the ratio of heavy metal is increasing heavy metal is basically increase of metal which is normally should not be present in soil and when concentration of these heavy metals like Pd, Cu, Cd, As increase they cause pollution and have adverse effects in agriculture field. Due to the presence of these heavy metals in soil, they lead to major effects in plants like as follows- they reduce the growth of a plant, the plant also needs these metals but when present in large quantity it hinders their growth for example cu help lipid membrane but due to excess of it cause instability. The heavy metal directly effects the metabolism of the plant. Due to which plants are not able to grow to its full length and lack the essential nutrition it requires.

2.5.1. Anticancer agent

Cancer is one of the major problems many people are suffering from cancer and proper cure is not there, a plant having endophytic property have potential to treat cancer. Scientist just needs to check the best possible use of this association of plant and microorganism. Isolating endophyte reveal that it have potential to produce anticancer agents including taxol, camptothecin, vinblastine, podophyllotoxin, vincristineand camptothecin (41, 42)

2.5.2. Antidiabetic agents

Endophytes also promise to produce antidiabetic agents which are known to treat diabetes which is a major disease increasing day by day. Maybe due to the bad diet of people the level of glucose in human gets affected and the high glucose level in person cause diabetes. Theses antidiabetic agents produced by the plant and microbes association have potential to treat diabetes and side by side are inexpensive, less toxic, less or no side effect and environmentally (43). These antidiabetic agents can decrease the blood glucose levels and by the help of endophyte scientists can have an effective way to treat and have a new alternative to treat diabetes (40).

2.6.Enzymes

Enzymes catalyze chemical reactions converting substrates to products. Enzymes are selective to their substrates. An enzyme's activity can be affected by conditions like pressure, temperature etc (44). Amylase is used in food processing, it helps in producing sugars from starch for fructose corn syrup. It is also used for brewing beer. Protease is useful in biscuit manufacturing as it lowers the protein content in flour and is also used in detergents. Cellulase breaks down cellulose to monosaccharides, it is widely used in textile, laundry detergents, pulp, and paper industry. Lipases are used in dairy industry for ripening of blue mold cheese (45).

Chapter-3

SCOPE OF STUDY

Endophytes can have anti-microbial or anti-fungal properties which can be useful. The studies of endophytes from *C.noctrunum* and *U.dioica* have important characteristics which can have the potential to be useful. The main study includes isolating the endophytes by culturing the explants and their identification. Characterizing the endophyte can give its potential use and any important bioactive compound produced from it. Any novel compound from it having the therauptic use like anticancer compounds can be useful commercially.

Chapter-4

RESEARH OBJECTIVE

4.1. Collection of samples from *U.dioica* and *C.noctrunum.*

4.2. Isolation of endophytic fungi from roots leaves and stems of plants *U.dioica* and *C.noctrunum.*

4.3. Purifying the fungal strains by further subculturing.

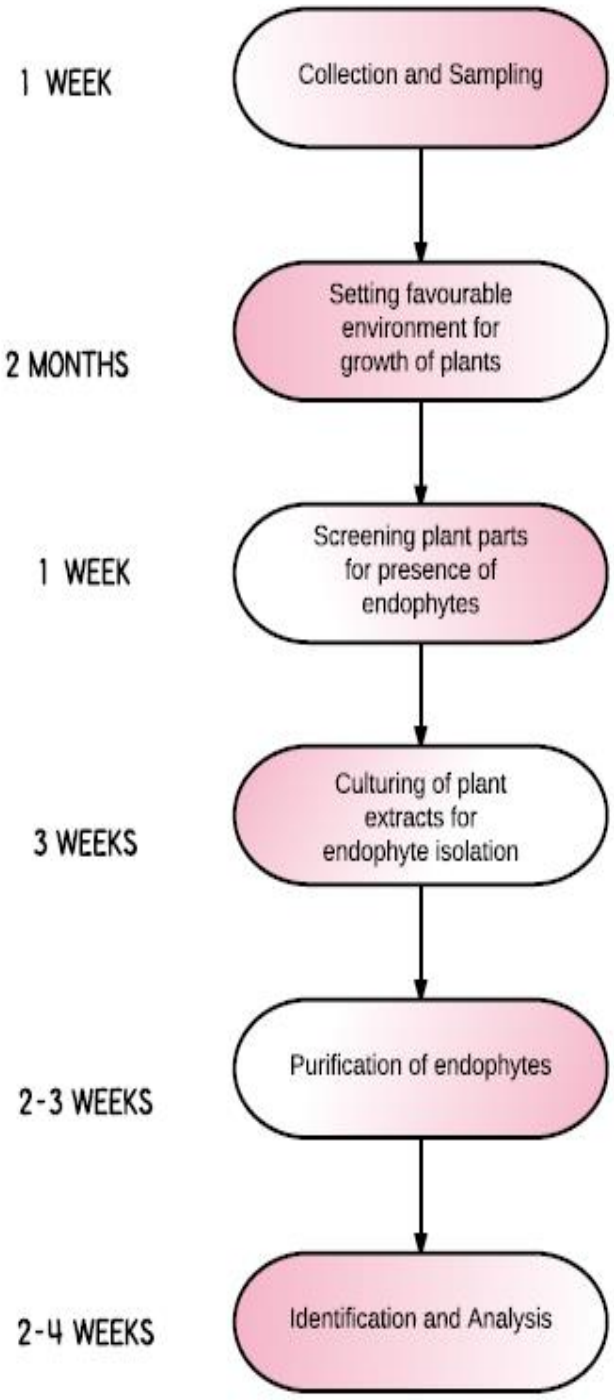
4.4. Morphological features of isolated endophytic fungi.

4.5. Screening of the isolates for the presence of bioactive molecules

4.6. Identification of the selected fungal endophytes based on amplification and sequencing of the ITS region of the fungal rRNA using PCR with universal primers (ITS1 and ITS2).

Chapter-5

WORK PLAN



Chapter-6

MATERIAL AND METHODOLOGY

6.1. Material collection and preparation

Cestrum noctrum and *U.dioica* sampling was bought from Phagwara nursery and grown in a pot. Proper environmental conditions were provided for their growth. Plants were grown sufficiently before isolating endophytes from them.

Proliferative stems, roots and leaves maintained and established on culture media in standardized laboratory.

6.2. Glassware preparation

"Borosil" glasswares were used for experiments. Before use they were washed thoroughly with detergent followed by their rinsing with water. The flasks were sealed with muslin cloth wrapped cotton plugs and one-fifth of the flask was filled with distilled water and then subjected to steam sterilization in autoclave. The objective of sterilization was to make the glassware free of unwanted impurities like microorganisms and spores such as bacteria, fungi etc.

6.3. Sterilization of media

Plant tissue culture media was sterilized by steam sterilization in an autoclave at 121^0 C under 1.05 kg cm^{-1} (15-20 psi) for 15 minutes.

6.4. Laminar air flow set up

All aseptic manipulations were done inside laminar air flow hood. The working surface was cleaned with 70% ethanol followed by irradiation with UV for 20 mins. For carrying out the experiment Bunsen burner kept close for flame sterilization. Surgical instruments like forceps, spatula, scissors, scalpel and surgical needle were dipped in 70 % ethanol and flamed prior to use.

6.5. Surface sterlization and inoculation

Various explants (leaves, roots and stems) of *C.noctrunum* and *U.dioica* were thoroughly washed with tap water .The roots of shoot tip explants were excised. The explants were washed with 1% Tween-20 detergent (v/v) solution and then kept under running tap water for

30 minutes. Then they were surface sterilized by treatment with 0.01% mercuric chloride ($HgCl_2$) (w/v) solution for 1 minute in laminar chamber. 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. Then they are individually transferred on to Petriplates containing water agar medium. Each plate was carefully marked with code and date of inoculation and each plate was sealed with parafilm (to prevent contamination), prior to incubation.

6.6. Incubation conditions

-Petriplates were inoculated with particular endophytes and were kept at 28^o c for 1week until the entophyte have grown all over the plate.

-After the completion of incubation period, further sub culturing and purification of the fungi was done by transferring piece of fungi to a freshly prepared water agar plate under the laminar air flow.

-These new plates were further incubated at 28^oc in an incubator.

-Each plate was carefully marked with code and date of inoculation and each plate was properly sealed with parafilm, prior to incubation.

-Further subculturing was done in case of a contamination in the plate. Thus, cultures were purified by subculturing and plates were kept at incubation at 28^0 C.

6.7. 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

6.8. Enzyme assays

Cellulose, amylase, lipase, and protease tests were performed on the plates having endophytes.

- **For cellulase**, 1% CMC was dissolved in PDA and with sharp needle the inoculum was taken and placed onto the cellulose containing plate. Further, after few days of incubation the plates were stained with 0.25% Congo red for 15-20 mins followed by destaining with 1MNaCl. Wherever cellulase enzyme was present clear zones were observed.
- **For amylase**, 1.5% Starch was dissolved in PDA and the sub cultured sample was inoculated followed by incubation. After the growth was observed, the plated were flooded with iodine solution. Dark spots were observed meaning those spots have amylase.
- **For protease**, Skimmed milk was autoclaved separately along with PDA later inside LAF 0.5% skimmed milk solution was made. Plates were made and incubated. After a day, growth was observed with clear zones showing presence of proteases.
-**For lipase,** 0.5% Tributyrin was dissolved in PDA, further plates were made and inoculated with cultures. After incubation clear zones were measured for lipase detection.

6.9. Anti-microbial acitivity

To check anti-microbial activity of the isolated endophytes, the samples were cultured in Potato dextrose broth and centrifuged separating supernatant from pellet. To check if there was extra cellular and intra cellular enzymes, supernatant was collected in a separate and the pellet was 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.

Four different bacterial cultures were taken- *Halobacterium, Bacillus subtilis, Bacillus megaterium and E.coli*. These were spread (200 microlitres) onto the luria agar with a spreader and holes were created in which 50µL and 200µL of supernantant and cell free extract each was loaded along with the controls.

6.10. DNA isolation

-Firstly the overnight grown culture of endophyte was taken and centrifuged at 6000rpm for 10 mins, supernatant was discarded and centrifugation was done multiple times to obtain 50 mg of pellet

-The pellet was then dissolved in 500µLof TES buffer, 5µL of proteinase K and RNase was added and the tubes were incubated at 60^{o}C for 1hr.

-After incubation for 1 hour, 140µL of 5M NaCl was added with 64µL of CTAB followed by incubation again for 15 mins at 65 ^{o}C.

-Equal volume of phenol:choloform:isoamyl alcohol was added in (25:24:1) and centrifuged at 1000 for 10 mins.

-Separate layers were observed out of which aqueous layer was taken in another eppendorf to which 0.6 volumes of cold isopropanol and 0.1 volume of 7.5 M ammonium acetate was added. This preparation was then stored in -20 ^{o}C for 30 mins.

-After removing the tubes from deep freezer, centrifugation was done at 1000 rpm for 10 mins and the supernatant was discarded, dissolving pellet in 70% of ethanol for washing.

- After centrifugation ethanol was removed and eppendorfs were air dried followed by adding 50µL of TE.

-To confirm that pure and good yield of DNA was isolated, gel electrophoresis was carried out.

6.11. DNA amplification

After confirmation of DNA bands its amplification was carried out.

-Autoclaved PCR tubes were taken and amplification was done with ITS primers

Solution	Quantity (µL)
Nuclease water	7.5
10x buffer	2.5
$MgCl_2$(5mM)	2
ITS1 primer(10pmole)	0.5
ITS4 primer(10pmole)	0.5
dNTP(10mM)	1.5
Template	4
Taq polymerase(1U/µL)	1.5

The reaction was then immediately set with reaction window of 2 mins at the start at 94 oC, 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 DISSCUSION

7.1. Sample Collection

Cestrum nocturum and *U.dioica* sapling were collected from phagwara nursery and grown in a pot with controlled watering and sunlight.

Image-4. Grown *Cestrum nocturum* and *U.dioica.*

Water agar plate were made for culturing both the plants. Explants from stem, root, leaves were obtained and plated on the prepared plates. Endophytic growth was observed on leaves and roots of *C.noctrunum*, it was then isolated for further culturing.No endophytic growth was visible with leaves, stem and roots of *U. dioica* therefore not processed further.

Image-5. Root of *C.noctrunum* plated on Water Agar.

Image-6. Leave of *C.noctrunum* inoculated on Water Agar showing initial growth of endophytic fungi

Subculturing of endophytes from excised plant was done on PDA for their growth and proliferation for doing further studies on them.

Image-7. Subcultured endophytes from leaf of *C.noctrunum* on PDA showing initial growth.

Image-8. Subcultured endophytes from root of *C.noctrunum* on PDA showing initial growth.

7.2. Enzyme Tests

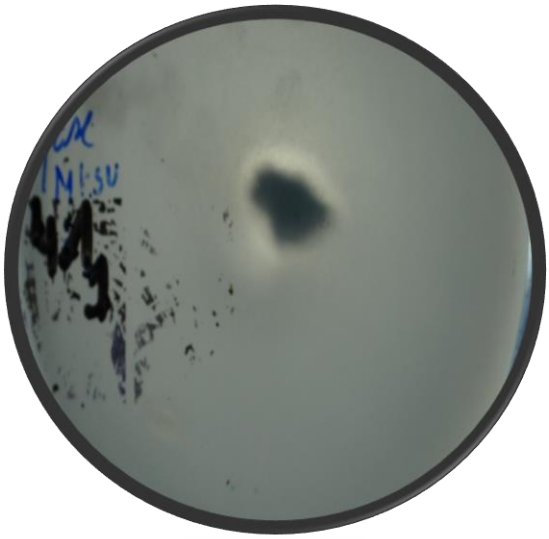


Image-9. Endophyte from root of *C. noctrnum* plated on skimmed milk shows presence of protease with clear zone

Image-10. Endophyte from leaf of *C. noctrnum* plated on starch shows presence of amylase with clear zone

7.3. Endophytic isolate of *C.noctrunum* for Antimicrobial Activity

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

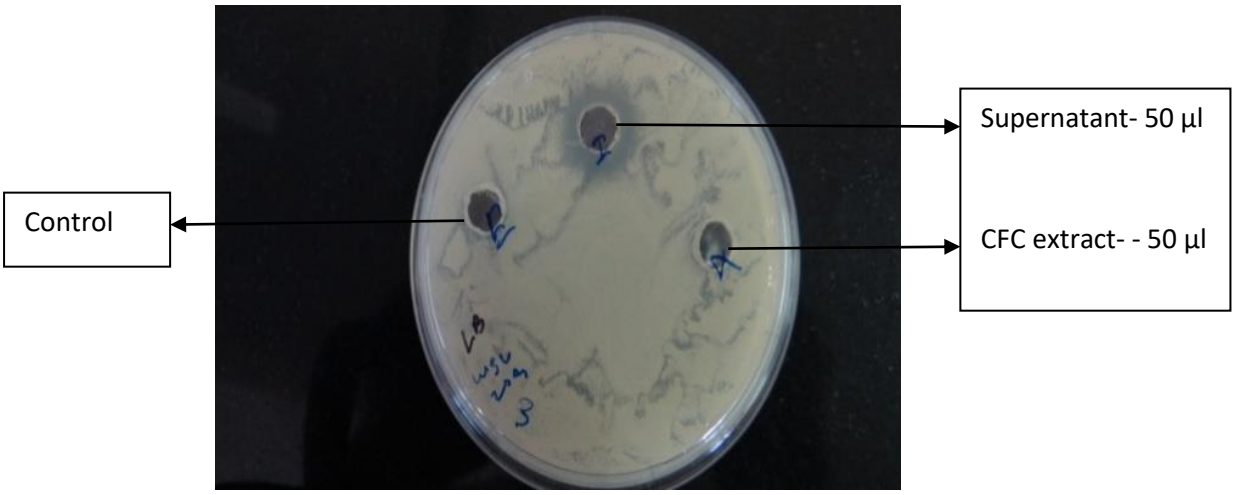


Image-11. Plate showing primary screening of isolate from *C.noctrunum*(leaf) against *E.coli*

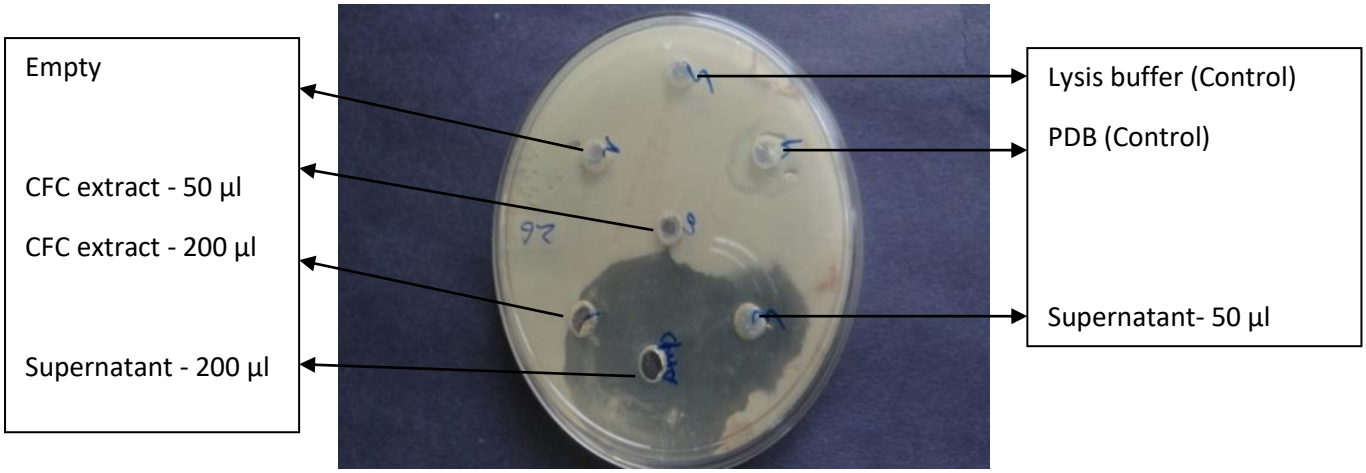


Image-12. Plate showing primary screening of isolate from *C.noctrunum* (leaf) against *B.megaterium*

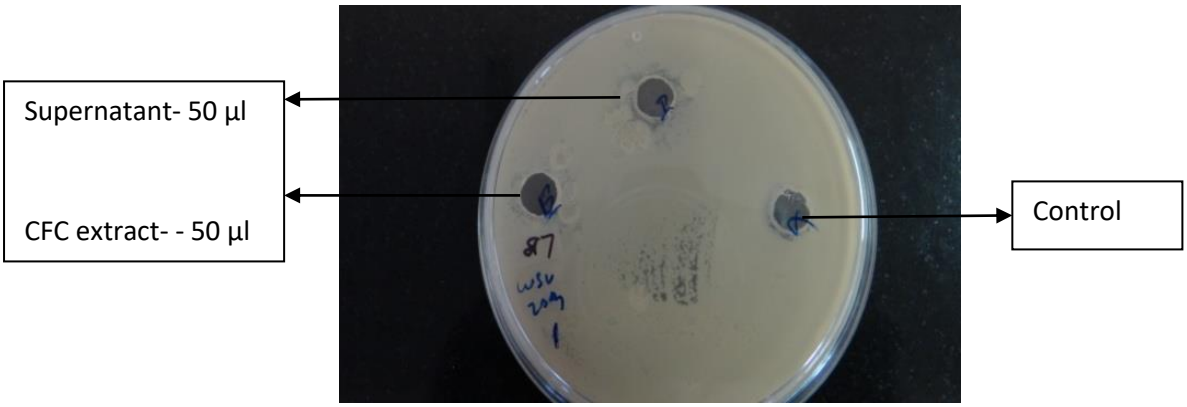


Image-13. Plate showing primary screening of isolate from *C.nocturum*(root) against *E.coli*

7.4. DNA Isolation

Image-14. 0.8% Agarose gel electrophoresis showing isolated endophytic DNA(CML DNA)

Well1: From leaf

Well2: From root

7.5. Amplified DNA

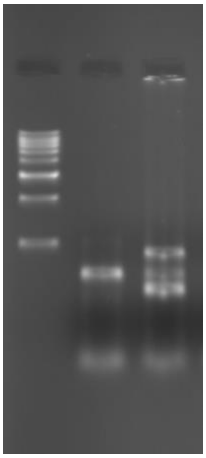


Image-15. 1% Agarose gel showing amplification of isolated endophytic DNA from leaf & root of *C.noctrunum* (CNL & CNRDNA).

7.6. Lactophenol Test

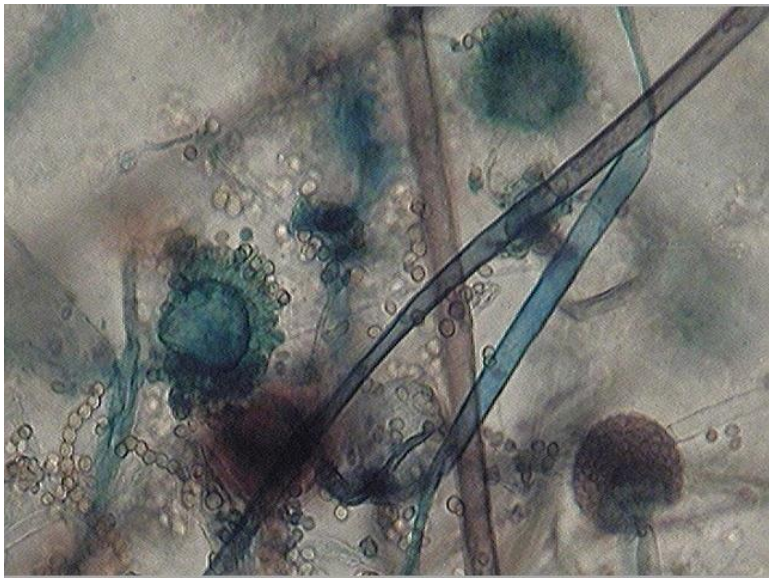


Image-16. Lactophenol test of root of *C.noctrunum*

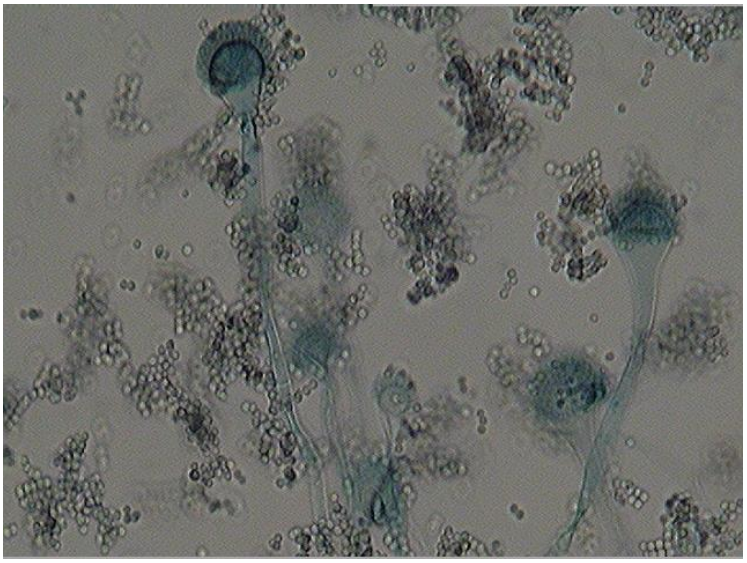


Image-17. Lactophenol test of leaf of *C.noctrunum*

7.7. Sequencencing Results

>15115-CNRDNA-ITS1

GGGCCGGGGGACTTGGGTATTGTGCCTTCTCGTCCGTATGCAGTATTGCATGCACTGTTA
CCCAAGTGTCAGTTGCACCACTTGAAAAACCCCCAACCACTGAGCTATCCGTTCTAGGAT
CGATGAAGAGCGCAACCAAGTGCGATACCTAGTGTGAATTTTACCCATCGCGAATCATCC
GGTTCTTGCTCGACCTTGGGCCCTCTAGAATTACGGTATGCAGCATCGCTCGCTCGATCCT
TTTGGCTTTTAGACAAAGGTGGGGCCCTGGCATTGGGGAGATTCTTAAAAGAAACTTTTT
TTGTTAAGTTTGTTTTGAGTAGGAGAAAGGCCACCGAAATGAATGCAAAACCCCGACCTC
AATGGCCGCCGCAAACCCGCTAAATTTCCCTTCATCAATGCCGGAGGAAAGAAAGCATC
CGGCCTGGCTCTCCTGTTTGCCTCCAGTGGGCCGAACCCACCATCGCAGCGATTCGCGTC
CGGGGCATATTTTCAAGCAAACATTAAACCTGGCGCCTTAAGGTAATCGCTGAATGAAA
GCTTGGGAAGCTTCCTAGGGGTTGTAGGCCTTTCCCACTGAACCATGTTCACCGGTTTAA
GGGCCTTGCCCCCCACGAACTACTTTGAAGGGTATCCTCTAGGCCCAAGATGGGCCCCAA
ACCATTCTAAAGGGCTAATATTTTGTGGTCCCCCACCCCCCCCGAGCCGACCCCCCTTCC
AGGGGTGAAAATCGACTACGATGTAGCATCGACCCCGCTTCCTTCTGGGCCTACCCCAGT
GTATCCCCCCGGGAGAAA

>15115-CNRDNA-ITS2

TTAAAGGGGCAATTTCGTGTTTGGAGTTACGGAGAATAACTGGATAGCGGTTTTTTTGGG
GCAATGGACTAAGAGGGGGGGTTTCAATAAAGGAGGTTATGGTTACGGGGGGCTGGCGA
GTTACGTAGGGGGCTCCTGAGGGAATTCTAACTGGAGGACCACCGGGTTCTCGAACGGA
GGAGTGGGCGTTAAAAATTGGTTATTCAAGAATGTCTGCGCTTCGTTTCCGTCTGGCTTTT
CGCGCGTTTTGGGGGCCCTGGTAATGAGGCCGTTCTTAATAGAAACTTTTCTTGCGAGGT
TGATTATGATTAGGACGCTGGGCCGCCTAATTGGAGGGTAAGCTCGACCTCAAATGCAA
GCGGAATAACGCTTAAATTAATATTGTAAAGGGCGACAGAAAGGGAAGGTTCATTGCCT
GGTTGTCTTCTTGCCCCCGGGGGGACCAACCCACCTAAACAAAGGTTATGATTCCTGGGC

CTCGTGCTCGGAAAACCTTACGCTAAGGTTATATGGAAACTCTGAATAAAACCGTGAGC
GGGATCCCTAGGGGATTGTGGTCCGTTTACCCCGGGCTATGGTCACCTGTTTTCGGCGCT
CTGCGCCCCAAAGGACCCCCTTTGAAAAGTTTTTCCCCTTGGCCCCCTGTAGGCCATCAA
AGCACCCCTTAGGGGTAAAATATGTGTGAAAGCCCCCTGCCCCCCGAACTCACCCATCTT
GCGGGGGCCTAAACCCCAAAAAAAATGGAGAAACCGCCCCCCCTTGGGTTCGGGGCCCT
CCCCCAGTGTCCCCTCTCGGAGGATG

>15115-CNLDNA-ITS1

CGGGGTTTTTTTCGGTCGTAAGCGATGCAATGGCGCCTAGTTCTTCGGGTTTTAGGTGCTT
GGGCGGGCCGAAAGAAAATGGGGAGGGCTCTCAGCCCCGGGCAGGCGGCGTTCGACAA
GAATTGGTCGCTTCCCCGAGGGGGTGGATTCACCAAACCCCAGACAGGAAGAAGGGTAT
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GTTTTTTTTAAAAATGGGTGAATTTTCTCCGTGGGATGTTCTGAAACTCAGTTTCTCCATG
AGCTTGCCTGTGTGCGGGTCATCCCCCACTCCTGAGGGGACAGAAACAATTTGGGGTTCC
CCCCCGGGGCGGAGCCCGGGTCTTAGGGGCCCGTAAGCCTTTTTGGCGGCCCCGGAAAT
AACTAAGGTAATTAAATCGGGGGCGGGTGGACTCGAACAGTAGGGGATACCCGCTAATT
TAAGCATTTAATACGGAAAAGCAGAAACCAACCGGGATTGCCTCGTAACGGCAA

>15115-CNLDNA-ITS2

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GGCGAAAAAGAAGGGGAAAAGAGAAAGAGAGGGGGAAGGGCGGGCGGGGGGCCAAAA
CTTTGGGGAGTAACCGACGGAACAGGACCAATACCCCACACAACATCCTTTTTTTAAATG
GGGATATTGGGGCCGGAATGGGTTTGCCCCCGGAAAAGAAAGGGGGGATCATGTTTCAT
TTAAAAAATTTCTTTACTAAAGGAATTCCTTATTCCCACCTTTTTACCCCATTTTCTTCCGG
GGGGAATGCCTCCCCGACCCAAAATTTCCTTGCTTTAAAATTTTTTAATTGTTTTTTGGGT
CATCCACCCCTCTCCCGGGGGGAAAGGACCAGATTGTTGCTGTCCCCCCGGGGCCCAGCC
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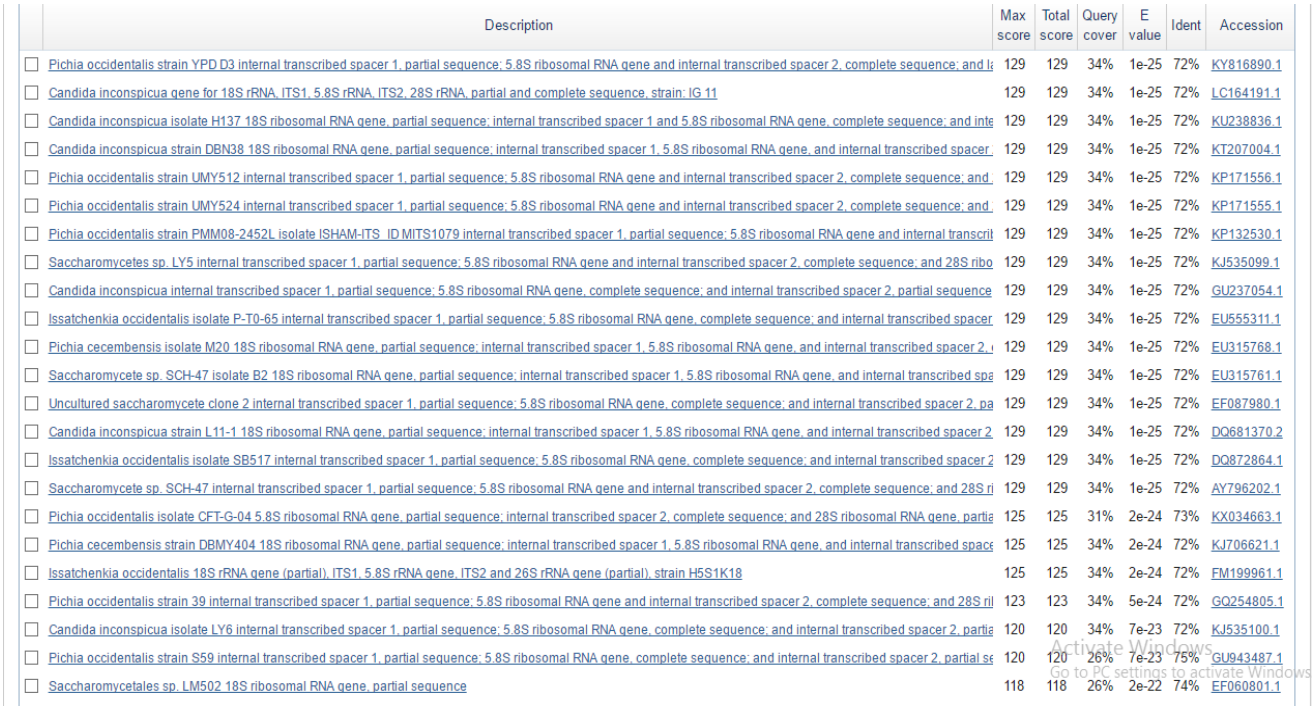

Description	Max score	Total score	Query cover	E value	Ident	Accession
Pichia occidentalis strain YPD D3 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and la	129	129	34%	1e-25	72%	KY816890.1
Candida inconspicua gene for 18S rRNA, ITS1, 5.8S rRNA, ITS2, 28S rRNA, partial and complete sequence, strain: IG 11	129	129	34%	1e-25	72%	LC164191.1
Candida inconspicua isolate H137 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1 and 5.8S ribosomal RNA gene, complete sequence; and inte	129	129	34%	1e-25	72%	KU238836.1
Candida inconspicua strain DBN38 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer.	129	129	34%	1e-25	72%	KT207004.1
Pichia occidentalis strain UMY512 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and.	129	129	34%	1e-25	72%	KP171556.1
Pichia occidentalis strain UMY524 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and.	129	129	34%	1e-25	72%	KP171555.1
Pichia occidentalis strain PMM08-2452L isolate ISHAM-ITS_ID MITS1079 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcrit	129	129	34%	1e-25	72%	KP132530.1
Saccharomycetes sp. LY5 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and 28S ribo	129	129	34%	1e-25	72%	KJ535099.1
Candida inconspicua internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene, complete sequence; and internal transcribed spacer 2, partial sequence	129	129	34%	1e-25	72%	GU237054.1
Issatchenkia occidentalis isolate P-T0-65 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene, complete sequence; and internal transcribed spacer	129	129	34%	1e-25	72%	EU555311.1
Pichia cecembensis isolate M20 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, (	129	129	34%	1e-25	72%	EU315768.1
Saccharomycete sp. SCH-47 isolate B2 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spa	129	129	34%	1e-25	72%	EU315761.1
Uncultured saccharomycete clone 2 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene, complete sequence; and internal transcribed spacer 2, pa	129	129	34%	1e-25	72%	EF087980.1
Candida inconspicua strain L11-1 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2	129	129	34%	1e-25	72%	DQ681370.2
Issatchenkia occidentalis isolate SB517 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene, complete sequence; and internal transcribed spacer 2	129	129	34%	1e-25	72%	DQ872864.1
Saccharomycete sp. SCH-47 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and 28S ri	129	129	34%	1e-25	72%	AY796202.1
Pichia occidentalis isolate CFT-G-04 5.8S ribosomal RNA gene, partial sequence; internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partia	125	125	31%	2e-24	73%	KX034663.1
Pichia cecembensis strain DBMY404 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed space	125	125	34%	2e-24	72%	KJ706621.1
Issatchenkia occidentalis 18S rRNA gene (partial), ITS1, 5.8S rRNA gene, ITS2 and 26S rRNA gene (partial), strain H5S1K18	125	125	34%	2e-24	72%	FM199961.1
Pichia occidentalis strain 39 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and 28S ril	123	123	34%	5e-24	72%	GQ254805.1
Candida inconspicua isolate LY6 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene, complete sequence; and internal transcribed spacer 2, partia	120	120	34%	7e-23	72%	KJ535100.1
Pichia occidentalis strain S59 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene, complete sequence; and internal transcribed spacer 2, partial se	120	120	26%	7e-23	75%	GU943487.1
Saccharomycetales sp. LM502 18S ribosomal RNA gene, partial sequence	118	118	26%	2e-22	74%	EF060801.1

Image-18. CNRDNA-ITS1 showing 72% identity with *candida, saccharomycetes and picha occidentalis.*

Description	Max score	Total score	Query cover	E value	Ident	Accession
Pichia occidentalis isolate ZJB-09162 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed space	86.0	86.0	21%	1e-12	72%	JN872840.1
Pichia occidentalis strain YPD D3 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and la	80.6	80.6	21%	6e-11	73%	KY816890.1
Candida inconspicua gene for 18S rRNA, ITS1, 5.8S rRNA, ITS2, 28S rRNA, partial and complete sequence, strain: IG 11	80.6	80.6	21%	6e-11	73%	LC164191.1
Pichia occidentalis isolate CFT-G-04 5.8S ribosomal RNA gene, partial sequence; internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial	80.6	80.6	21%	6e-11	73%	KX034663.1
Pichia cecembensis isolate CFT-G-01 5.8S ribosomal RNA gene, partial sequence; internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partia	80.6	80.6	21%	6e-11	73%	KX034661.1
Candida inconspicua strain DBN38 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2	80.6	80.6	21%	6e-11	73%	KT207004.1
Uncultured fungus clone SACCH1 5.8S ribosomal RNA gene, partial sequence; internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial se	80.6	80.6	21%	6e-11	73%	KP167628.1
Pichia occidentalis strain UMY512 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and 2	80.6	80.6	21%	6e-11	73%	KP171556.1
Pichia occidentalis strain UMY524 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and 2	80.6	80.6	21%	6e-11	73%	KP171555.1
Pichia occidentalis strain PMM08-2452L isolate ISHAM-ITS_ID MITS1079 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcrib	80.6	80.6	21%	6e-11	73%	KP132530.1
Saccharomycetes sp. LY5 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and 28S ribos	80.6	80.6	21%	6e-11	73%	KJ535099.1
Issatchenkia occidentalis isolate P-T0-65 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene, complete sequence; and internal transcribed spacer 2	80.6	80.6	21%	6e-11	73%	EU555311.1
Pichia cecembensis isolate M20 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, c	80.6	80.6	21%	6e-11	73%	EU315768.1
Saccharomycete sp. SCH-47 isolate B2 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spa	80.6	80.6	21%	6e-11	73%	EU315761.1
Uncultured saccharomycete clone 2 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene, complete sequence; and internal transcribed spacer 2, par	80.6	80.6	21%	6e-11	73%	EF087980.1
Candida inconspicua strain L11-1 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2,	80.6	80.6	21%	6e-11	73%	DQ681370.2
Issatchenkia occidentalis isolate SB517 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene, complete sequence; and internal transcribed spacer 2,	80.6	80.6	21%	6e-11	73%	DQ872864.1
Candida inconspicua isolate H137 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1 and 5.8S ribosomal RNA gene, complete sequence; and inter	78.8	78.8	17%	2e-10	73%	KU238836.1
Pichia cecembensis strain DBMY404 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer	78.8	78.8	17%	2e-10	73%	KJ706621.1
Candida inconspicua internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene, complete sequence; and internal transcribed spacer 2, partial sequence	78.8	78.8	17%	2e-10	73%	GU237054.1
Saccharomycete sp. SCH-47 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and 28S rit	78.8	78.8	17%	2e-10	73%	AY796202.1
Pichia cecembensis strain DBMY245 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; an	73.4	73.4	17%	9e-09	72%	KJ706462.1
Issatchenkia occidentalis 18S rRNA gene (partial), ITS1, 5.8S rRNA gene, ITS2 and 26S rRNA gene (partial), strain H5S1K18	73.4	73.4	17%	9e-09	72%	FM199961.1

Image-19. CNRDNA-ITS2 showing 72% identity with *candida, saccharomycetes and picha occidentalis.*

Description	Max score	Total score	Query cover	E value	Ident	Accession
Physma byrsaeum voucher NK-273 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer	77.0	77.0	14%	5e-10	84%	KM887874.1
Penicillium purpurogenum strain NRRL 58011 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete seq	77.0	77.0	16%	5e-10	83%	HM045289.1
Rhinocladiella aquaspersa CBS 313.73 ITS region; from TYPE material	75.2	75.2	16%	2e-09	82%	NR_119760.1
Ochroconis minima CBS 510.71 ITS region; from TYPE material	73.4	73.4	15%	6e-09	82%	NR_145366.1
Aspergillus flaschentraegeri NRRL 5042 ITS region; from TYPE material	73.4	73.4	12%	6e-09	86%	NR_135345.1
Aspergillus bombycis strain LCF29 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2	73.4	73.4	16%	6e-09	82%	FJ867931.1
Penicillium cecidicola strain NP118S internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; an	73.4	73.4	15%	6e-09	82%	JX524135.1
Ochroconis minima 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete seq	73.4	73.4	15%	6e-09	82%	HQ667522.1
Venturiaceae sp. BN-Lon33-3 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, cor	73.4	73.4	15%	6e-09	82%	HQ634610.1
Aspergillus flaschentraegeri isolate NRRL 5042 internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S	73.4	73.4	12%	6e-09	86%	EF652150.1
Ochroconis sp. CBS 140316 28S ribosomal RNA gene, partial sequence	71.6	71.6	14%	2e-08	83%	KX668259.1
Gibbosporina sphaerospora voucher NK-175 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribe	71.6	71.6	15%	2e-08	83%	KM887877.1
Uncultured fungus clone SW015_H04 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spac	71.6	71.6	14%	2e-08	83%	KP889709.1
Pezizomycotina sp. KO-groupE 2014 gene for ITS1, 5.8S rRNA, ITS2 and LSU, partial sequence, strain: GF1017	71.6	71.6	14%	2e-08	83%	AB986461.1
Pezizomycotina sp. KO-groupD 2014 gene for ITS1, 5.8S rRNA, ITS2 and LSU, partial sequence, strain: LWg2002	71.6	71.6	14%	2e-08	83%	AB986460.1
Uncultured fungus clone 123_NA11_P31_C23 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcrit	71.6	71.6	16%	2e-08	81%	KC966152.1
Uncultured fungus clone 103_NA10_P32_I9 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribe	71.6	71.6	15%	2e-08	83%	KC965878.1
Uncultured fungus clone 59_NA11_P33_D7 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed	71.6	71.6	15%	2e-08	83%	KC965260.1
Uncultured fungus clone 106_NA7_P32_F6 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed	71.6	71.6	16%	2e-08	81%	KF297161.1
Uncultured fungus clone 67_NA7_P32_J12 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed	71.6	71.6	15%	2e-08	83%	KF296918.1
Uncultured fungus clone 3237M6 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2,	71.6	71.6	12%	2e-08	85%	KF618014.1
Sclerophora farinacea voucher WEDIN 6414 (UPS) internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete	71.6	71.6	14%	2e-08	83%	JX000113.1
Chaenotheca furfuracea UPS Wedin 6366 ITS region; from verified material	71.6	71.6	14%	2e-08	83%	NR_120128.1

Image-20. CNLDNA-ITS1 showing 86% similarity with *aspergillus flaschentraegeri*, 82% with *pencillum purpurogenum*.

Description	Max score	Total score	Query cover	E value	Ident	Accession
Leptogium pedicellatum isolate Lep040388 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete seque	95.3	95.3	15%	2e-15	85%	KJ409611.1
Leptogium cf. pedicellatum Lep121412 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence;	95.3	95.3	15%	2e-15	85%	KJ409608.1
Leptogium burnetiae var. hirsutum isolate Lep070725 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, compl	95.3	95.3	15%	2e-15	85%	KJ409607.1
Leptogium cf. burnetiae Lep100602 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; an	95.3	95.3	15%	2e-15	85%	KJ409602.1
Leptogium burnetiae var. hirsutum isolate Lep091112 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, compl	95.3	95.3	15%	2e-15	85%	KJ409601.1
Leptogium cf. burnetiae Lep091095 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; an	95.3	95.3	15%	2e-15	85%	KJ409600.1
Leptogium cf. burnetiae Lep090509 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; an	95.3	95.3	15%	2e-15	85%	KJ409599.1
Talaromyces marneffei strain CBS 141765 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed s	93.5	93.5	14%	6e-15	87%	KY115196.1
Talaromyces diversiformis strain CBS 141931 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcrib	93.5	93.5	14%	6e-15	87%	KX961215.1
Talaromyces funiculosus isolate 14R-2-F01 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequer	93.5	93.5	14%	6e-15	87%	KX958062.1
Talaromyces funiculosus isolate 14R-2-F06 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequer	93.5	93.5	14%	6e-15	87%	KX958026.1
Penicillium sp. strain F0236 internal transcribed spacer 2 and 28S ribosomal RNA gene, partial sequence	93.5	93.5	14%	6e-15	87%	KU747578.1
Penicillium sp. strain F0197 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and 28S rit	93.5	93.5	14%	6e-15	87%	KU747577.1
Talaromyces udagawae CBS 579.72 ITS region; from TYPE material	93.5	93.5	14%	6e-15	87%	NR_145156.1
Talaromyces primulinus CBS 321.48 ITS region; from TYPE material	93.5	93.5	14%	6e-15	87%	NR_145151.1
Eurotiomycetes sp. genotype 631 JMUR-2016 voucher ARIZ:AK1694 small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal	93.5	93.5	14%	6e-15	87%	KX909058.1
Eurotiomycetes sp. genotype 699 JMUR-2016 voucher ARIZ:FL1886 small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal F	93.5	93.5	14%	6e-15	87%	KX908849.1
Eurotiomycetes sp. genotype 699 JMUR-2016 voucher ARIZ:FL1574 small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal F	93.5	93.5	14%	6e-15	87%	KX908680.1
Talaromyces siamensis CBS 475.88 ITS region; from TYPE material	93.5	93.5	14%	6e-15	87%	NR_103683.2
Talaromyces rubicundus CBS 342.59 ITS region; from TYPE material	93.5	93.5	14%	6e-15	87%	NR_103682.2
Talaromyces aurantiacus CBS 314.59 ITS region; from TYPE material	93.5	93.5	14%	6e-15	87%	NR_103681.2
Talaromyces aculeatus CBS 289.48 ITS region; from TYPE material	93.5	93.5	14%	6e-15	87%	NR_103679.2
Talaromyces funiculosus CBS 272.86 ITS region; from TYPE material	93.5	93.5	14%	6e-15	87%	NR_103678.2

Image-21. CNLDNA-ITS2 showing 87% identity with *talaromyces funiculosus*, 85% with *leptogium burnetiae*.

Chapter-8

CONCLUSION AND FUTURE SCOPE

The endophytes from *C.noctrunum* had been isolated and subcultured, further studies were done which included their identification by isolating their DNA, rDNA amplification and sequencing.

Both the plants were further studied by carrying out their endophyte isolation and identification which may reveal presence of bioactive compounds.

Both the plants showed important characteristics for endophyte study and their study may give novel results, e.g. endophytes having characteristics for treating a disease or used in other areas.

Chapter-9

OUTCOMES

Endophytes have a great importance in plants as they have in defence mechanism, pathyway regulation , diseases resistance etc. enzymes from endophytes can be extracted and used in detergent , textile industry , pulp industry etc. Endophytes have the capability to break larger complexes example starch , cellulose , lignin , chitin etc . due their major role researchers are working to isolate and genetically engineer endophytes which can help to solve the problem of gasoline leakage ,waste treatment and for industrial purposes many novel drugs has been isolated from endophytes famous example include taxol from the bark of Taxus plant present in wild forest it has the anticancer properties . many other drugs for antimicrobial , antifungal, treatments have also been produced. Removing pollutants are the major issue endophytes can be utilised by changing their metabolism so that they can break the harmful products into simpler products this technique is called Bioremediation(34).

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APPENDIX

11.1. Water Agar (for 200ml)- Himedia

2g Agar in 200ml of D.W

11.2. 1% Tween 20 (for 10ml)- Atlas Chemie

0.1ml tween 20 in 10ml of D.W

11.3. 0.1% Mercuric Chloride (for 10ml)- Qualikams

0.01g mercuric chloride in 10ml of D.W

11.4. Potato Dextrose Agar (for 100ml)– Himedia

3.9 g in 100ml of D.W

11.5. Potato Dextrose Broth(for 100ml)– Himedia

2g in 100ml of D.W

11.6. 1% Carboxy Methyl Cellulose(for 100ml)– Himedia

1g in 100mil

11.7.1.5% Starch (for 100ml)– Qualikams

1.5g in 100ml

11.8. 10%Skimmed milk (for 100ml)– Nestle

1g in 10ml

EQUIPMENTS USED

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