Antioxidant and antimicrobial activity of extracellular polymeric substances isolated from endophytic fungus (*Debaromyces hansenii*)

A Dissertation

Submitted in partial fulfilment of the requirements for the award of degree of

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IN

BIOTECHNOLOGY

Submitted by

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DECLARATION

I, the undersigned, hereby declare that the research work presented in this thesis entitled **"Antioxidant and antimicrobial activity of extracellular polymeric substances isolated from endophytic fungus (*Debaromyces hansenii*)"** is an authentic record of the work carried out by me under the supervision of Dr. M.S Reddy, Professor and Dr. Manoj Baranwal, Assistant Professor Department of Biotechnology, Thapar university, Patiala.

Further, I declare that no part of this dissertation has been submitted for a degree or any other qualification to any other university or examining body in India/elsewhere.

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Date: 15 July, 2016

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CERTIFICATE

This is to certify that dissertation entitled "**Antioxidant and antimicrobial activity of extracellular polymeric substances isolated from endophytic fungus (*Debaromyces hansenii*)**" submitted by **Ms. Kanika Mehta** in partial fulfilment of the requirements for the requirements for the award of Masters of Science degree in Biotechnology at Thapar University, Patiala is an authentic work carried out by her under our supervision and guidance.

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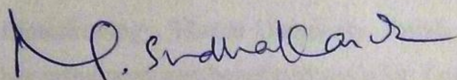

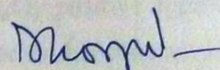

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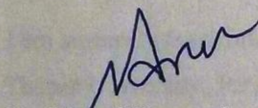

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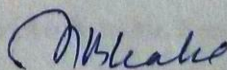

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(KANIKA MEHTA)

ABSTRACT

Fungal endophytes are microorganisms dwelling in coalition with their host plant without causing any harm. Infact endophytes protect their host plant from diseases and make the plant tolerant to undesired environmental conditions. Till date many vascular plants have been reported as the reservoir of endophytes. Endophytes produce many bioactive compounds having wide applications in pharmaceutical, agricultural and industrial areas. In the present study, endophytic fungus (IN2) isolated from *Camellia sinensis* leaves, procured from Palampur, is identified and characterized for the production of extracellular polymeric substances exhibiting antioxidant activity. The PDB broth inoculated with endophytic fungus for 14 days was concentrated using lyophilization and purified by membrane dialysis. The extract so obtained was used for the estimation of exopolysaccharide (EPS). The dry weight of mycelium is 42 mg. The 258 µg/ml glucose concentration was obtained from 300 µg/ml extract. The protein concentration was estimated to be 20 µg/ml. The free radical scavenging activity of the EPS extract was calculated by DPPH antioxidant assay. The maximum DPPH scavenged was 53.2% at 500 µg/ml concentration. This clearly depicts the antioxidant activity of extracellular polysaccharides produced by IN2 endophytic fungus. Also the antimicrobial activity of the extract was evaluated using 96 well plate antimicrobial assay and agar well diffusion method. The extract showed little activity (8%) against *Bacillus megaterium* at 500 µg/ml concentration but no antimicrobial activity was observed against *Pseudomonas aeruginosa* and *Staphylococcus aureus* even at 2000 µg/ml. This shows that the IN2 endophytic fungus produced very little antimicrobial components. Further the IN2 endophytic fungus was identified by both morphological analysis and by molecular biology techniques. The morphological studies identified IN2 as *Debaryomyces*. The genomic DNA was isolated and amplified by ITS1 and ITS4 primers. The amplified product was sequenced and its homologous sequences were retrieved by BLASTn analysis. The phylogenetic analysis revealed that the isolated endophytic fungus clustered with *Debaryomyces hansenii.* Present study results suggest that *Debaryomyces hansenii* produce high level of extracellular polysaccharides having antioxidant activity.

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LIST OF ABBREVATIONS

M	Molar
ROS	Reactive oxygen species
EDTA	Ethylenediaminetetraacetic acid
PCR	Polymease chain reaction
DPPH	2,2-diphenyl-1-picrylhydrazyl
PDB	Potato Dextrose broth
PDA	Potato dextrose agar
TBE	Tris-borate-EDTA
RNase	Ribonuclease
OD	Optical desnsity
Dna	Deoxy ribonucleic acid
DNTP	Deoxynucleotides
Kb	Kilo base

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1. INTRODUCTION

Plants are found to be a treasure of endophytes. Endophytic fungus dwell in coalition with their host plant mainly leaves, stems, roots (Bacon and White, 2000). Endophytes cause no harm to their host plant, infect they prevent them from diseases and make the plants capable to tolerate the undesired environmental conditions. The numbers of vascular plants that are explored till today are found to be a reservoir of endophytes (Zhang *et al*., 2006).

The growth of host plant is not only influenced by climate and area in which it reside, it also show their impact on endophytes . The main roles of endophytes in host plant are increase in the resistance of their host plant against insects, worm, herbivores, pests and also improve their tolerance against heat, drought, heavy metal presence.

Endophytes produce number of bioactive compounds that are found to be important and have lots of necessary applications in pharmaceutical, agricultural and industrial areas (Strobel and Daisy 2003; Qudri *et al*., 2013; Specian *et al*., 2013; Tan *et al*., 2001). Endophytic fungi are type of microorganism that has capability of producting maximum bioactice compounds when comparing to other endophytic microorganisms (Zhang *et al*., 2006). The different properties of endophytic fungus are, they produce antioxidant compounds, antiviral compounds, antimicrobial compounds, anticancer compounds (Strobel 2003; Gutierrez *et al*., 2012).

Studies have shown that endophytes possess bioactive extracellular polymeric substances. Hence in the present study, extracellular polymeric substances (EPSs) were isolated from endophytic fungus isolated from *Camellia sinensis* plant. Isolated EPS were tested for carbohydrate and protein contents by phenol sulphuric method and lowry method, respectively. DPPH was carried out to assess the antioxidant activity. Further, antimicrobial activity of EPS was evaluated based on MTT assay.

1.1 Endophyte:

To understand the word endophyte, it is split into two parts ‘endo’ and ‘phyte’, the meaning of ‘endo’ is within and phyte is plant (Fig 1). Endophytes are isolated from different plants. These plants pertain from different families; different classes and lives in different geographical locations. The relationship between the endophytes and their host plant are considered as mutualism mean endophytes protect their host plants from biotic and abiotic stresses and it return they gain nutrients and shelter from their host plants.

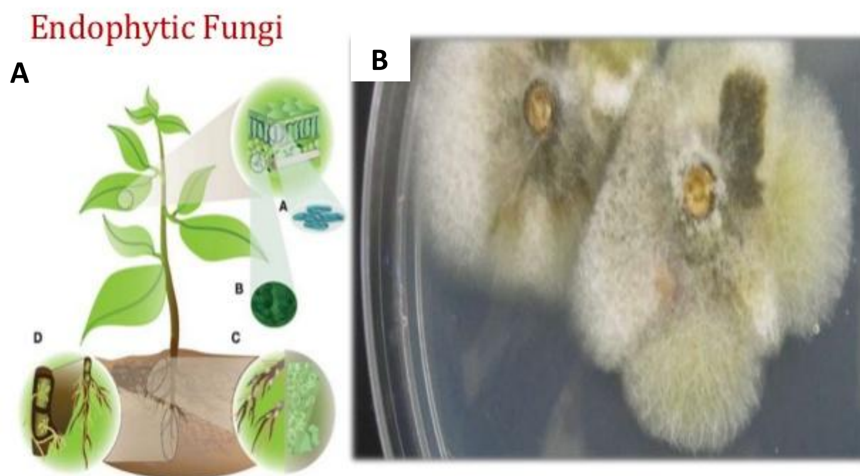

Fig.1 Endophytic fungi: A- Endophytic fungi in different locations of plants. B- Endophytic fungi on media plates (Firdaus *et al*., 2013).

1.2 Types of Endophytes

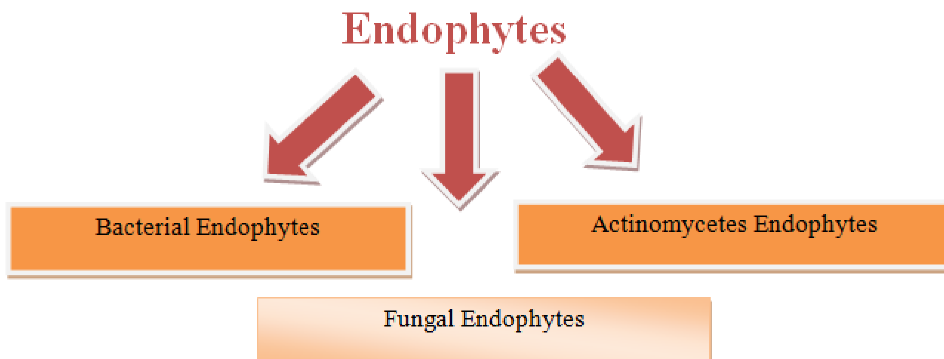

Fig.2 Different types of endophytes

Bacterial Endophyte

Bacteria that dwell in plants and have mutualism association with plants are called as bacterial endophyte (Fig 2)

Table 1. Some bacterial endophytes and their host plants

Sr.No.	Endophytes	Host plant	Reference
1.	*Azorhizobium caulinodans*	Rice	Engelhard *et al*., 2000
2.	*Enterobacter sakazakii*	Maize	Kuklinsky-Sobral *et al*., 2004

Endophyte *Azorhizobium caulinodans* plays important role in their host plant. Many studies proof that this endophyte promotes the growth of host plant and also help in increasing seed production in rice. These functions of endophyte *Azorhizobium caulinodans* enables less use of chemical fertilizers in case of *Enterobacter sakazakii*, it also known for supporting the growth of maize (Table 1).

Fungal Endophytes

Fungi that dwell in plants and have mutualism association with plants are called as bacterial endophyte (Fig 2).

Table2. Some fungal endophytes and their host plants

Sr. No.	Endophyte	Host plant	Reference
1.	*Pestalotiopsis versicolor* *Pestalotiopsis neglecta*	*Taxus cuspidate*	Kumaran *et al*., 2009
2.	*Alternaria tenuissima*	*Ocimum Sanctum*	Bhagat *et al*., 2012

Pestalotiopsis versicolor and *Pestalotiopsis neglecta* are taxol producing fungal endophytes. These fungal endophytes are resided in Taxus cuspidate plant (Table 2).

Actinomycetes Endophytes

Actionmycetes that dwell in plants and have mutualism association with plants are called asactinomycetes endophyte. For example- Streptomyces actinomycetes are found in Wheat (Fig 2)

1.3 Endophyte-Host Interactions:

Endophytes prevent their host plants from pests, worm, insects, hervobores. They are all considered harmful organisms for plants. Plants are prevented by endophytes by producing toxins that are harmful for these all grazing organisms that attack the plants for their feed example endophytic fungi produce alkaloids. These toxins control and suppress the growth of harmful organisms (Bacon and White, 1994). Endophytes also increase the expression of plant genes who are responsible for defense against pathogens. Increase in expression of these defense associated genes make the plant capable to resist against infectious agents.

Plants get more nutrients when endophyte present in it. Increase uptake of nutrients mean good growth of plant following better resistance to diseses and pathogensand adaptation to harsh environmental conditions (Kageyama *et al*., 2008). More numbers of fungal endophytes also accountable for loss of water in leaves of host plant (Fig. 3).

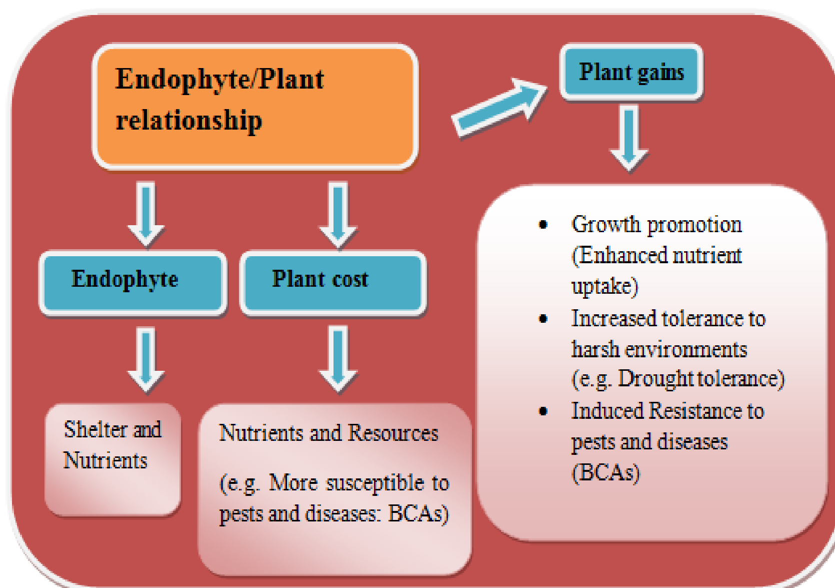

Fig.3 Endophyte/Plant relationship

A number of endophytes are known for nitrogen fixation, Phosphate solubilization, Siderophore Production. These properties have beneficial effect on growth of plants (Malinowski & Belesky 2000). Plants for their certain benefits including growth, nutrient uptake, tolerance to biotic or abiotic stresses, tolerence to heavy metals make connection with particular endophyte (Bae *et al*., 2005).

1.4 Classification of fungal endophytes:

Fungal endophytes are divided into two main groups:

Clavicipitaceous endophytes:

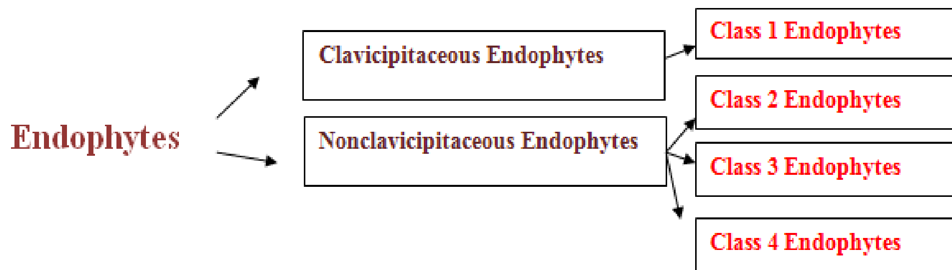

Fig.4 Groups of fungal endophytes

Class 1 endophytes

Clavicipitaceous endophytes are related with grasses and are class 1 endophytes dwell their entire life expectancy within the aerial section of host grass. They cause no harm to host grass, in spite of it protect plants from pathogenic organisms and make them tolerable to biotic and abiotic stresses. Class 1 endophytes and plant have non pathogenic associations. Tall fescue growth increased when it infected with endophytes (Fig. 4).

Nonclavicipitaceous Endophytes:

Class 2 Endophytes

Class 2 Endophytes the different locations of their host plants like stems, roots, leaves. They can transfer either vertically or horizontally through seeds, seeds coats. Class 2 endophytes infect mainly those plants that are grown in high stress environmental areas.

Class 3 and 4 endophytes

The difference between class 3 endophytes and class 4 endophytes is that class 3 endophytes reside in the shoot of plants while Class 4 endophytes reside in roots of plants and the difference is that both of them are transfer horizontally (Fig. 5).

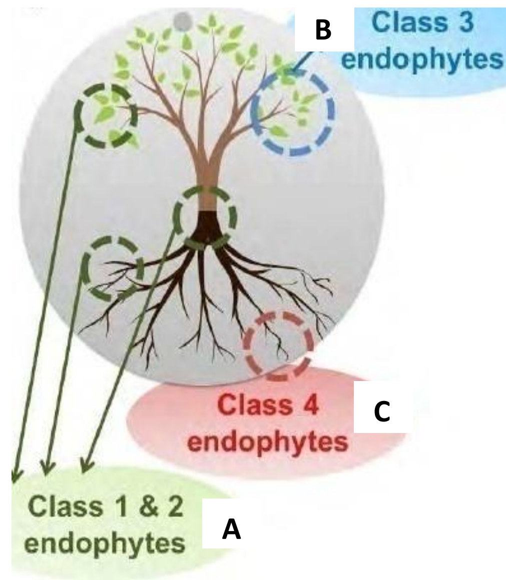

Fig.5 Classes of fungal endophytes A. Class 1 and Class 2 endophytes reside in stems, leaves, roots. B. Class 3 endophytes reside in shoots. C. Class 4 endophytes reside in roots of their host plants (Bae *et al*., 2005).

1.5 Gain of entry into host plants:

The central site through which endophytes access entry into plants is root portion, where the maximum numbers of endophytic population reside as compared to other plant tissues. The use of microscopy is applied for the detection of, how endophytes gain entry in plants through this portion, so it is found that they gain entry through open sites on roots or through wounds. There are also many zones in the plant from whish endophytes can gain access such as the aerial portions of plants or by infection of seed (Fig. 6). The endophytes get access into fruits through flowers.

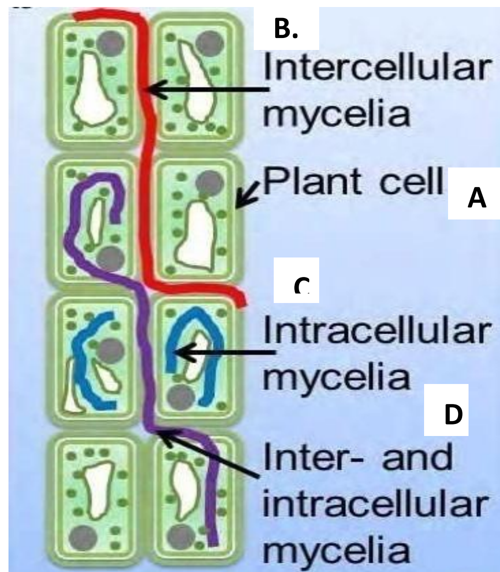

Fig. 6 Entry of endophytes into plants A. Plant cell B. Intercellular mycelia C.Intracellular mycelia D. Inter- and Intracellular mycelia (Bae *et al*., 2005).

In this study we have focused on the identification of endophytic fungi isolated from *Camellia sinensis* plant. Further isolation and characterization of exopolysaccarides (EPS) from endophytic fungus (IN2) was carried out and free radical scavenging activity of EPS was evaluated.

2. REVIEW OF LITERATURE

De bary introduced the term endophyte in 1866. He initially applied this term to any microorganism present within plant. Afterwards when the meaning of endophyte was cleared, it is declared that endophytes include any organism which lives inside the plant, mainly leaves, roots, branches and stems, without causing any definite damage to its host plant (Fig. 7).

Fig.7 Growth of endophytic fungus (IN2) on PDA media

2.1 Classes of fungal endophytes

Clavicipitaceous (Class 1 endophytes) reside in only some species of grasses. Non-Clavicipitaceous (Class 2 endophytes) includes large number of fungal endophytes which are defense mutualists. They increase the strength of plants against botic or abiotic stresses and make them capable to easily tolerate the harsh environmental conditions.

Clavicipitaceous grass endophytes are well known to decrease hervibory by producing toxic alkaloids against insects, herbivores. One of the example of class I fungal endophytes is *Neotyphodium* endophytes. The tolerance of cadmium is increased by *Neotyphodium* endophytes, when it infected with plant *F. pratensis* when compared to non infected plant.

Neotyphodium sp. Endophytes produce loline alkaloids for the prevention of fescue grasses from herbivores. Endophytic fungus *Neotyphodium lolii* promote the growth of perennial ryegrass plant which indirectly make it tolerable to abiotic stresses.

There is a variety of endophytic population in the different parts of the leaves like midrib, lamina and petiole, and these alterations are even seen in cell type. Different factors are responsible that affect the distribution and species diversity of fungal endophytes such as age of leaves and plant, position and dampness of the site and weather.

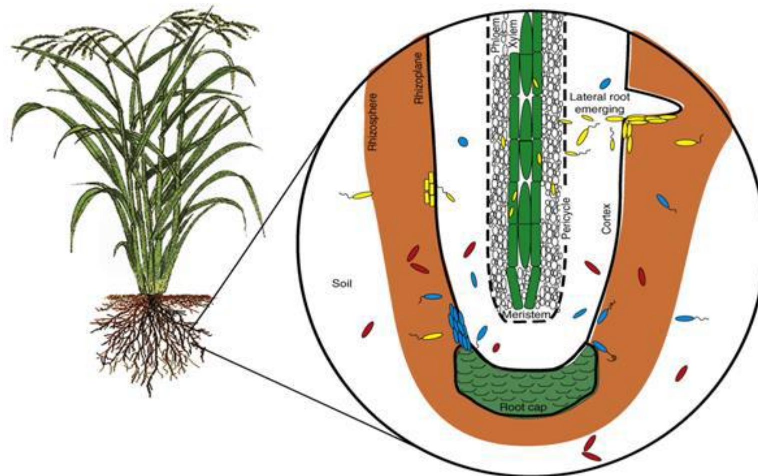

Fig.8 Gain of entrance of endophytes in most of the tissues of roots like cortex, phloem, rhizoplane (Hardoim *et al*., 2008)

2.2 Protection from Insects, Worm, Pests and Herbivores

A lot of research has been focused for increasing the resistance of plants against herbivores through the roles of endophytes.

First reported method relies on the total upgradation or reform the functions of plants by endophytes, following decrease herbivory (Gehring & Whitham 2002) or survive to any kind of damage. This method does not affect the productivity of plant.

Second reported method is change in plant nutrients and resources by endophytes. When endophytes enters the plant system, so for their survivor, they get food from plants following

change in chemical composition of plants. When the chemical composition of plants changes, its direct impacts shown in herbivores.

Third repored method is production of toxins by endophytes. The foliar endophytes that are found to be present in grasses produce toxic alkaloids for decresing herbivory (Clay 1990, Clay & Holah 1999; Brakhage *et al*., 2003). Mandyam & Jumpponen (2005) noticed that from the total numbers of endophytes present in plants, the maximum endophytes plays role in preventing the root potion from grazing animals. Endophytes produce melanin for this purpose as melanin does not allow microbial grazing.

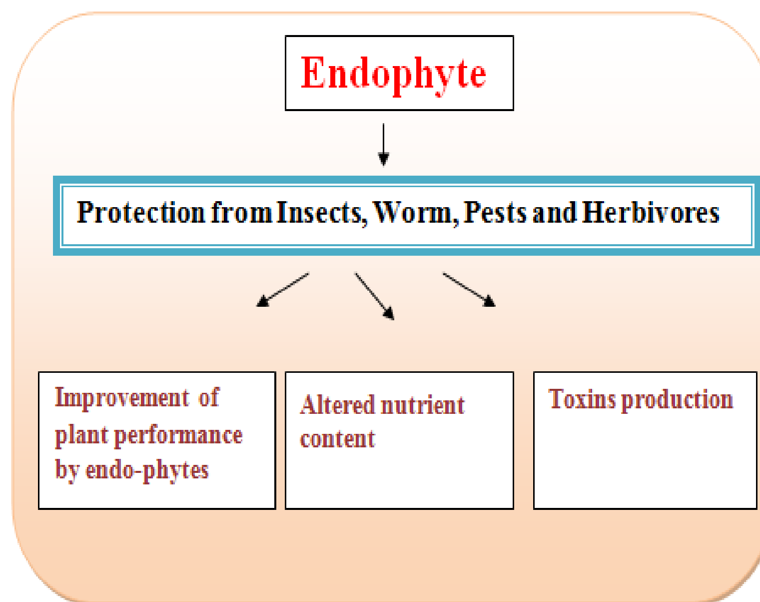

Fig.9 Three reported methods for prevention of plants from Insects, Worm, Pests and Herbivores (Mandyam & Jumpponen 2005)

2.3 Protection from Pathogens

There are three mechanisms through which endophytes can improve resistance of their host plant against pathogens (Mandyam & Jumpponen 2005).

The first mechanism is competition between endophyte and pathogen for the same nutrients and resources (Lockwood 1992). This is proved in the given example, *F. oxysporum* Fo47 endophyte inhibit pathogen *F. oxysporum* f. sp. *radicis-lycopersici,* and improve resistance of their host

plant against this pathogen. This causes the decrease in root rot symptoms of tomato (Bolwerk *et al.*, 2005). Fo47 spores compete with the pathogen for the same C source, and then reducing nutrient availability to the pathogen.

In the second mechanism endophytes can improve resistance of their host plant against pathogens by two ways. One of them is ability of endophytes to spurring the host to produce phytoalexins and biocidal compounds and other is the ability of the endophyte itself to produce fumigants and antimicrobial agents. *Spilanthes calva* is a medicinal plant. When this plant inoculated with endophyte *Piriformospora indica*, it produces a number of antifungal compounds (Rai *et al.,* 2002).

The third mechanism is inducing host defense responses to improve resistance of host plants against pathogens by localized endophytes.

2.4 Role of endophytes in Hosts Tolerance to Stress

The role of endophytes and the mechanisms through which endophytes make plants more fit for tolerating biotic or abiotic stresses are well known. They are considered defensive mutualists that stimulate host plants to withstand high temperatures, salts, drought (Malinowski & Belesky 2000). A herbal plant, *Dichanthelium lanuginosum* ,dwell in the areas where temperatures of the soil can reach 57 °C, the presence of the endophytes may improve the plant potency to tolerate high temperatures. Plants inoculated with endophytic fungus *Curvularia* sp., can survive high soil temperature and water stress better than endophyte-free plants (Redman *et al.,* 2002).

2.5 Biotechnological applications of endophytes

Endophytes are also known for the production of enzymes. In vitro it is proved that endophytes for their survival and for their growth utilize many plant nutrients and cell components. Endophytes grown on xylan and pectin agar medium produce chitinase, glucanase (Sieber *et al.,* 1991. Fungal endophytes produce xylanase and extracellular cellulase enzymes hemicellulases, pectinases, amylases.In biodegradation as well as hydrolysis processes, these fungal enzymes have important role against pathogenic infection and to get food from host plants.

2.6 Properties of endophytic fungus

2.6.1 Anti Cancer Agents from Endophytes

Cancer is a disease whisch is the result of uncontrolled divisions of abnormal cells. There are numbers of bioactive compounds are found from endophytes that have anticancer property (Firáková *et al*., 2007, Guo *et al*., 2008, Debbab *et al,*. 2011; Duarte *et al.,* 2012).

Chandra (2012) noticed certain endophytic fungi as a treasure of anticancer compounds.

More and more research has been done for the discovery of anticancer agents after the discovery of taxol producing endophyte. Cytotoxic quinone dimer, torreyanic acid are examples of the anticancer agents that are isolated from the endophytic fungi *Pestalotiopsis microspora* which have mutulism relationship and associated with tree *Torreya taxifolia.* These anticancer agents are found to be express robust cytotoxicity in cell lines which are critical to protein kinase C, following programmed cell death (Lee *et al*., 1996).

Recently an enzyme was found which is responsible for starting the first step of taxol synthesis. This enzyme is encoded by the *Taxus* taxadiene synthase (ts) gene which is isolated from endophytic fungi *Stemphylium sedicola* SBU-16. This is txol producing endophytic fungi isolated from *Taxus baccata.* (Mirjalili *et al*., 2012).

Wagenaar *et al.,* (2000) noticed cytochalasins alkaloid; having antitumor activity these antitumor compounds are isolated from the endophytic genus *Rhinocladiella*.

There are many studies has been done for the discovery of new anticancer alkaloids. camptothecin and its derivatives has antineoplastic activity for the treatment of cancers. In china for prevention of skin diseases camptothecin is used as a drug (Guo *et al*., 2008).

Endophytic fungus *Fusarium solani* which is also produce compounds that have anticancer activity. These bioactive anticancer agents are camptothecin and two analogues (9-methoxycamptothecin and 10-hydroxycampto-thecin). This endophytic fungus are isolated from *Camptotheca acuminate* (Kusari *et al*., 2009b. Pandey *et al*., 2014).

In many studies other camptothecin are also discovered with antineoplastic activity, isolated from endophytes (Amna *et al*., 2006).

Cytoskyrins express antibacterial activity, and also proved to be a potent anticancer compound. This cytoskyrins agent was produced by endophytic fungus *Curvularia lunata* isolated from *Niphates olemda* (Brady *et al*., 2000).

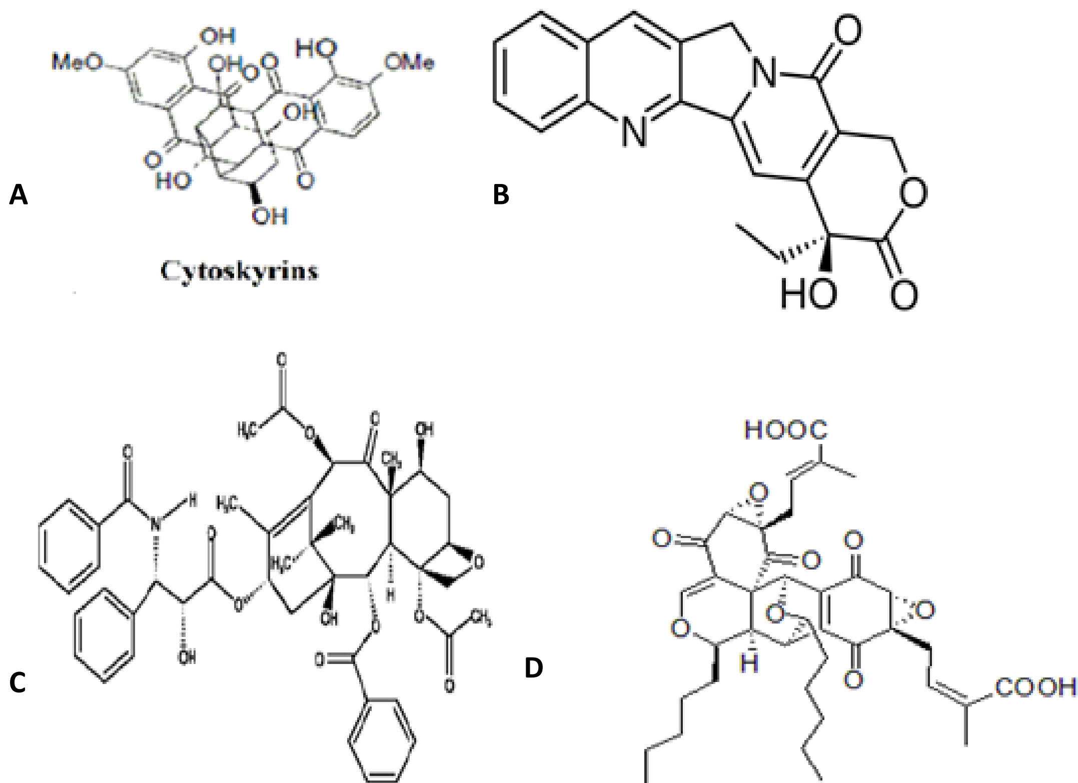

Fig.10 Structure of some anticancer agents isolated from endophytic fungi A. cytoskyrins B. Torreyanic acid C. Taxol D. camptothecin

2.6.2 Antimicrobial Agents from Endophytes

There are numbers of endophytes isolated from plants that express antimicrobial property. These antimicrobial agents are low molecular weight organic compounds. They are helpful for inhibiting the growth of microbial pathogens or prevent plants from their attack (Kumar *et al.,* 2014). From prevention to harmful microorganisms they are used in very minute concentrations (Demain 1981, Strobel & Daisy 2003, Guo *et al.,* 2008; Yu *et al*., 2010).

There are different uses of antimicrobial compounds by human beings. They can be used as drugs. They have main role as food preservative in the control of food spoilage and also in control of food borne diseases, which is the serious matter in the world food chain.

Schulz *et al.,* (2002) reported a compound having antifungal activity. This compound is known as fusidikactones isolated from endophytic *Fusidium* species.

In vitro and *in vivo* griseofulvin isolated from endophyte is reported with antifungal activity against plant pathogenic fungi. It is effective for delay the development of various food crops diseases (Park *et al.,* 2005).

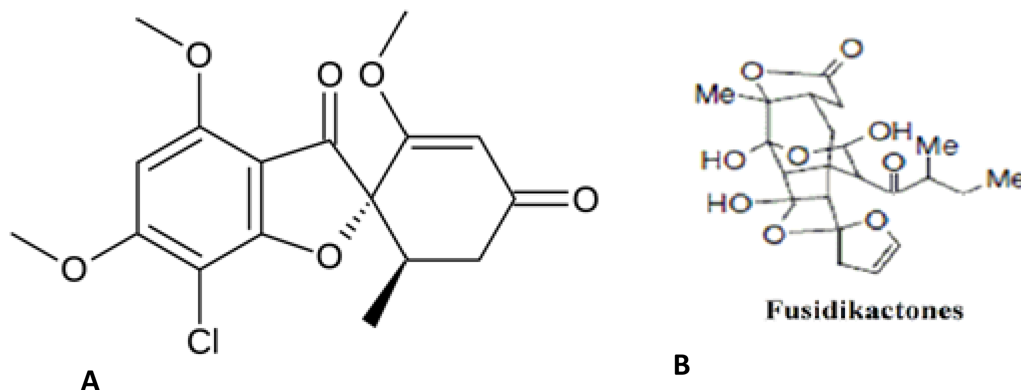

Fig.11 Structure of Some Antimicrobial Metabolites from Endophytes A. Griseofulvin B. fusidikacetones

Table 3 Endophytic microorganisms isolated from medicinal plant *Tylophora indica* have antimicrobial activity against *Sclerotinia sclerotiorum* and *Fusarium oxysporum*

Endophytic microorganisms with Antimicrobial activity	Host plant	Pathogen
Dothideomycetes sp	*Tylophora indica*	*Sclerotinia sclerotiorum* *Fusarium oxysporum*
Alternaria tenuissima	*Tylophora indica*	*Sclerotinia sclerotiorum* *Fusarium oxysporum*
Thielavia subthermophila	*Tylophora indica*	*Sclerotinia sclerotiorum* *Fusarium oxysporum*
Alternaria sp.	*Tylophora indica*	*Sclerotinia sclerotiorum* *Fusarium oxysporum*
Nigrospora oryzae	*Tylophora indica*	*Sclerotinia sclerotiorum* *Fusarium oxysporum*
Colletotrichum truncatum	*Tylophora indica*	*Sclerotinia sclerotiorum* *Fusarium oxysporum*

2.6.3 Antioxidant Agents from Endophytes

The value of those compounds having antioxidant activity relies in the fact that, they are totally useful against spoilage caused by reactive oxygen species (ROSs) and oxygen-derived free radicals. like DNA damages, carcinogenesis (Huang *et al*., 2007; Valko *et al*., 2007).

These antioxidant activity compounds are used for the prevention and treatment of reactive oxygen species related diseases. These diseases include cancer, hypertension, diabetes (Valko *et al.,* 2007).

Table 4 Endophytic fungus produces bioactive compound graphislactone A with antioxidant activity

Host plants	Endophyic fungi	Bioactive compound	Activity	Reference
Trachelospermum jasminoides	*Cephalosporium sp*. IFB-E001	graphislactone A	Antioxidant scavenging activity	(Hormazabal *et al.*, 2005, Song *et al.*, 2005).
Pilgerodendron uviferum	*Microsphaeropsis olivacea*	graphislactone A	Antioxidant scavenging activity	(Hormazabal *et al.*, 2005, Song *et al.*, 2005).

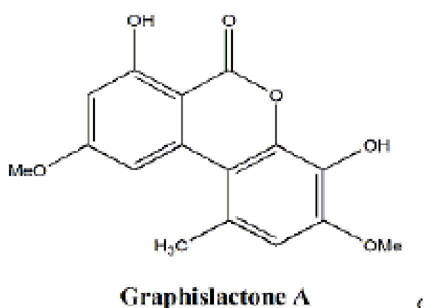

Fig. 12 Structure of graphislactone A with antioxidant activity

2.6.4 Antiviral Activities of Endophytic Fungi

Viruses are infectious agents that cause diseases in plants and animals. Endophytes increase the resistance of plants against viral infections. Antiviral compounds are also produced by the endophytic fungi. Human cytomegalovirus is a pathogen. It has enveloped virus and has double stranded DNA. From the solid state fermentation of endophytic fungus *Cytonaema* sp., human cytomegalovirus (hCMV) protease inhibitors cytonic acids A and B have been isolated (Guo *et al.,* 2000).

Human immunodeficiency virus type 1 (HIV-1) protease is inhibited by the bioactive compound hinnuliquinone. This compound is produced by the endophyte which is isolated from the leaves of *Quercus coccifera* (Singh *et al*., 2004).

2.6.5 Other Biological Activities of Endophytic Fungi

Endophytic fungi are also involved in the production of different other bioactive agents like anti-inflammatory, anti-diabetic, anti-malarial and immunesup-pressant agents, as well as insecticidal and anti-nematodes agents (Pimentel *et al*., 2011. Endophyte *Fusarium subglutinans* can produce compounds Subglutinol A and B. It is responsible for influencing the immune system of animals. Some compounds are produced by the endophyte *Pestalotiopsis leucothes.* This endophyte is isolated from *Trypterygium wilfordii.*these compounds are used for the treatment of human immunerelated diseases (Kumar *et al*., 2005). Some antitumor agents are found which are involved intuor cell apoptosis these antitumor agents include Gliocladicillins A and B (Chen *et al*., 2009)

3. OBJECTIVES OF STUDY

- Isolation and characterization of extracellular polymeric substances (EPSs) from endophytic fungi isolated from *Camellia sinensis* plant
- Estimation of antioxidant and antimicrobial activity of EPS
- Identification of endophytic fungus producing bioactive EPS

4. MATERIALS AND METHODS

4.1 Materials

4.1.1 Biological Materials

Endophytic fungus was (IN2) isolated from *Camellia sinensis* leaves procured from palampur. The IN2 culture was maintained on potato dextrose agar (PDA) (Appendix) plates at 25°C in dark conditions.

4.2 Methods

4.2.1 Isolation of Endophytic Fungus

Camellia sinensis leaves procured from palampur were used to isolate endophytic fungus. Leaves were cut into pieces of 5x5mm and were surface sterilized using 70% v/v ethanol and then dipped in 0.5% v/v sodium hypochlorite solution for 1 minute the leaves were washed with sterilized water and blotted with sterilized filter paper. Pieces of leaves were placed on PDA plates and incubated at 28^0C for 10 to 15 days. The hyphae emerging out from the leaves were excised and transferred to the fresh PDA plate. Ten fungal isolates (IN1 to IN10) were obtained from the *Camellia sinensis* leaves, amongst which IN2 was selected for the present study based on preliminary study.

4.2.2 Extraction of Exopolysaccharides (Mahapatra *et al*., 2013)

500 ml of PDB was inoculated with six to eight 7mm IN2 discs and incubated at 25^0C for 14 days. After 14 days, the broth was filtered by using sterilized muslin cloth and centrifuged at 10,000×g for separation of remained fungal biomass. The biomass was then dried and its dry weight was recorded. The broth was further concentrated by lyophilizing at -55^0C. In the concentrated broth, absolute ethanol was added and mixed for precipitation (5:1, v/v). The extract was kept at 4^0C for 24 hr. The precipitates formed were recovered by centrifuged at 10,000 × g for 10 min. Further purification was done using dialysis membrane against distilled water for 24 hours.

4.2.3 Purification by dialysis

Dialysis membrane was tied from only one side with thread, and the extract was transferred carefully in dialysis tubing from the other side of membrane. The membrane was tied from other side. The dialysis membrane was then placed in a beaker with double distilled water and stirred constantly using magnetic stirrer. After every two hours the double distilled water was changed till twenty four hours (Fig. 13).

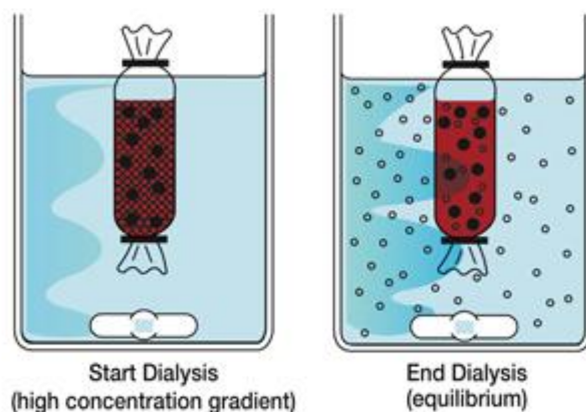

Fig.13 Schematic diagram showing dialysis of EPS sample.

4.2.4 EPS estimation by Phenol / sulfuric acid method

Phenol sulfuric method is mainly used for the estimation of carbohydrate concentration. 2 ml of extract was taken in a test tube; subsequently 1 ml of 5% aqueous solution of crystal phenol was added and then mixed properly. In the next step 5 ml of concentrated sulfuric acid was added and incubated at room temperature for 10 min, the tubes were then vortexed for 30 seconds. Tubes were then placed in water bath at room temperature for 20 min, for the development of color. Concentration of EPS was recorded at 490 nm on a spectrophotometer.

4.2.5 Protein estimation by Bradford method

Bradford method of protein estimation was used for measuring the protein concentration. 5 serial dilutions of BSA were prepared for standard curve. 3 ml of Bradford reagent was added to 8 test tubes followed by addition of 100 µL of each BSA dilution and the extracted sample. Two tubes

were kept as blank. The test tubes were vortexed properly and incubated at room temperature for 15 minutes for color development. Protein concentration was measured at 595 nm.

4.2.6 Estimation of antimicrobial activity

4.2.6.1 Antibacterial assay (NCCLS, 2004)

The antibacterial activity of sample IN2 was tested on the following bacterial strains by Agar well diffusion method (ref).

Material Required: Muller Hinton Agar plate, test antibiotic solutions, culture, autoclave, incubator, pipettes, micropipettes, laminar air flow.

Test antibiotic: Streptomycin and Ampicillin

Test culture: *Escherichia coli*, *Bacillus megaterium*, *Staphylococcus aureus* and *Pseudomonas aeruginosa*

1. **Preparation of culture:** Fresh culture of test organism was prepared in MHB (Muller Hinton Broth) and optical density was set according to the Mc Farland Standard.
2. **Preparation of Medium:** Muller Hinton Agar Medium was prepared in distilled water. The dissolved medium was autoclaved at 15 lbs pressure at 121°C for 15 minutes. The autoclaved medium was mixed well and poured into 100mm petriplates (25-30ml/plate). After solidification 100µL of the test organism was spread on to the plates. It was dried.
3. **Preparation of sample:** Stock solution (5mg/mL) of test antibiotic and sample (IN2) was made. From the stock solutions, dilution of test antibiotic was made 50µg/mL, sample was made 1000 µg/mL and 2000 µg/mL.
4. Took the above prepared solidified agar plates. Wells were created by removing circular plugs of agar. With the help of pipette 0.1ml of test antibiotic as well as samples were added into the wells. It was left for 1 hr at room temperature.
5. Petriplates were incubated at 37°C for 24 hrs. After the incubation period zone of inhibition was recorded.

4.2.6.2 96 well plate Antimicrobial Assay (NCCLS, 2004)

Test antibiotic: Ampicillin

Test culture: *Bacillus megaterium*, *Staphylococcus aureus* and *Pseudomonas aeruginosa*

Preparation of bacterial culture

Using aseptic techniques a single colony was transferred into a 100mL bottle of broth capped and placed in incubator overnight at 37 °C. After 12–18 h of incubation, optical density of the bacterial culture was recorded at 600 nm, and serial dilutions were carried out with appropriate aseptic techniques until the optical density was set according to the Mc Farland standard.

Preparation of antibiotic *solution*

The antibiotic solution was prepared by dissolving a 1mg powder in 1mL of sterile distilled water. A vortex mixer was used to ensure that it was a well-dissolved and homogenous solution.

Preparation of the plates

A sterile 96 well plate was labeled. To the wells MHB was added as media. To the wells media, bacterial cells and sample (IN2) was added. As a positive control, media was added to the wells along with bacterial cells and test antibiotic. As a cell control only media and cells were added to the wells. Initial reading was taken at 600nm on microtiter plate reader. The plates were placed in an incubator set at 37 °C for 24 h. After 24h MTT assay was performed. MTT was added in each well. It was incubated at 37 °C for 4 h. After 4 h DMSO was added and absorbance was recorded at 540nm.

4.2.7 Estimation of antioxidant activity by DPPH method

DPPH assay was done to know the antioxidant activity of EPS extract. DPPH is a stable free radical. The reaction of antioxidant with DPPH results in its reduction. As a result the purple colour of DPPH reduced to yellow colour and this is called discoloration (Fig.14).

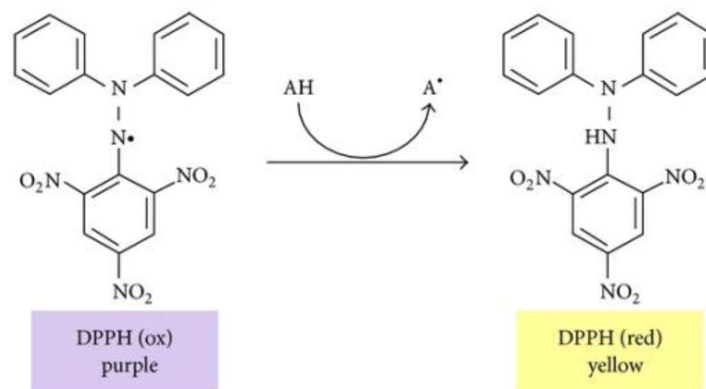

Fig. 14 Reduction of DPPH in the presence of antioxidants.

$$\textbf{DPPH Scavenged (\%)} = \frac{A_{control} - A_{test}}{A_{control}} \times 100$$

50 µL of each serial dilutions of endophytic fungus extract was mixed with150 µL of DPPH (100 µM) and added in 96 well micro-titre plate. Ascorbic acid (100 µg/ml) was added as positive control and water as negative control. The plate was wrapped in aluminium foil and kept for 45 minutes in dark. The change in color (from deep violet to light yellow) was measured at 517 nm using microplate reader.

4.2.8 Identification of Endophytic fungus

The endophytic fungus (IN2) isolated from *Camellia sinensis* leafs was identified based on morphologiocal studies and molecular studies.

4.2.8.1 Morphological based identification

The microscopic examination of fungus (IN2) was done using lactophenol cotton blue solution. Initially, microscope slides were cleaned using 70% alcohol. Two to three drops of lactophenol cotton blue stain were added on slide using dropper. Mycelial fragements were pricked using inoculating loop/ needle. Inoculating loop was heated and cooled before pricked the spores. A sterile glass cover was placed on the slide. The slide was examined under microscope.

4.2.8.2 Molecular based identification of endophytic fungus

IN2 fungus was grown on PDA plates overlaid with cellophane sheets (treated with EDTA) for 5 days. After 5 days, the mycelium was scrapped from the cellophane sheets and crushed with liquid nitrogen. The crushed samples were stored at -80°C for further isolation of genomic DNA.

4.2.9 Genomic DNA Isolation

100 mg of liquid nitrogen crushed mycelium was taken in 1.5 ml centrifuge tube. 0.5 ml extraction buffer (Appendix) was added and mixed well. The tube was then kept at 65°C for 15 to 20 minutes. Then 0.5 ml of equilibrated phenol was added and mixed well. The tubes were then incubated at room temperature for 15 minutes. 0.5 ml chloroform:isoamyl alcohol (24:1) was added and mixed by inversion. It is then incubated at room temperature for 15 minutes and centrifuged at 12000 g for 20 minutes. The upper aqueous layer was collected in new tube. To the aqueous layer 400 µl of chloroform:isoamyl alcohol (24 : 1) was added and mixed by inverting gently. It is then centrifuged at 12000 g for 10 minutes. Supernatant was collected in new tube. 0.54 volumes of isopropanol was added and incubated at room temperature for 15 minutes. The samples were then centrifuged at 12000g for 10 minutes. The pellet was washed with 100 µl of 70% cold ethanol. Pellets was resuspended in 300 µl of 0.2 M ammonium acetate and incubated overnight at 4°C. The DNA was further precipitated by adding 600 µl ethanol and centrifuged at 10000g for 15 minutes at 4°C. Supernatant was discarded and pellet was washed with 300 µl of 70% ethanol. Finally, the pellet was dissolved in 50 µl autoclaved MQ water.

4.2.10 Agarose gel electrophoresis for DNA

For 1% agarose gel 0.4 gm agarose was mixed in 40 ml 0.5xTBE buffer (Appendix). Agarose is insoluble at room temperature so the agarose solution was heated in microwave oven until it dissolved completely and become clear. The solution was cooled down and 2 µL of ethidium bromide (Appendix) was added and mixed properly. The comb was placed above the casting tray in a gel caster and agarose gel was poured. The gel was allowed to solidify for about 30 to 40 minutes at room temperature. The comb was carefully removed so that the wells are not damaged, and the intact wells are formed for the prevention of any type of leakage. The gel was then placed carefully on to the electrophoresis chamber. The electrophoresis buffer was added to cover the gel. Ensure that each well is filled with electrophorsis buffer. The dna sample was

mixed with 6x loading dye (Appendix) and loaded into the wells. The gel was run at 70V for 1 hour 40 minutes. The bands were observed in a UV-Transilluminator.

4.2.11 DNA Amplification through PCR

The genomic DNA was further amplified for 'Internal transcribed spacer' by using ITS1 and ITS4 primers:
ITS1: TCCGTAGGTGAACCTGCGG
ITS4: TCCTCCGCTTATTGATATGC
The genomic DNA was diluted in 1:20. 1 µl of diluted sample was used as a template in PCR reaction consisting of following reaction mixture (Table 5).

Table 5. Reaction mixture for PCR

Components (µL)	Amount (µL)
DNA	1Ml
Distilled water	13.2Ml
PCR buffer	2Ml
DNTPs	1.5 µL
Forward primer	1µL
reverse primer	1µL
Taq polymerase	0.3µL
Total (µL)	20µL

The PCR was run in thermocycler with following PCR program:
Initialization: at 94° C for 5 min, Denaturation: at 94° C for 1 min, Annealing: at 54° C for 1 min, Extension: at 72° C for 1 min, Final extension: at 72° C for 10 min. The PCR was run for 35 cycles. The amplified product was run on 1% agarose gel electrophoresis and visualized in gel documentation system.

4.2.12 Purification of gene amplified product:

The amplified gene was purified using Thermo Scientific GeneJET PCR purification kit. 100 µL of binding buffer was added to 100 µL of amplified product. It was mixed thoroughly. 200 µL of the solution was added to GeneJET purification column. It was centrifuged for 30-60 s and flow-through was discarded. 700 µL of wash buffer was added to the GeneJET purification column. Centrifuged for 30-60 s, flow-through was discarded and the purification column was placed back into the collection tube. The empty GeneJET purification column was centrifuged for an additional 1 min to completely remove any residual wash buffer. The GeneJET purification column was transferred to a clean 1.5 ml microcentrifuge tube and 50 µl of MQ water was added to the center of the GeneJET purification column membrane and centrifuged for 1 min. The purified DNA was quantified using nanodrop and was sent for sequencing.

4.2.13 Bioinformatic analysis for identification of isolated gene

The DNA sequence obtained after sequencing was used to identify the IN2 strain. The sequence was submitted as query in BLASTn (http://blast.ncbi.nlm.nih.gov/) analysis. The homologous sequences so obtained were aligned using multiple alignment tool MultAlin (http://multalin.toulouse.inra.fr/multalin/). The evolutionary relationship of the predicted sequence was studied using phylogenetic tree construction by MEGA.7 software.

5. RESULTS AND DISCUSSION

5.1 EPS estimation

Phenol sulfuric acid method is the mostly applied colorimetric method for analysis of carbohydrate concentration. Standard curve was generated by taking glucose as standard in Table 6 (Fig 15). 300µg/ml sample of EPS were used and the carbohydrate contents was calculated using standard curve. The carbohydrate content in EPSs is given in Table 7.

Table 6 O.D of extract and different concentrations of glucose as standard

Standard Concentration (µg/ml)	O.D.at 490nm
100	0.344
200	0.640
300	1.044
400	1.356
450	1.532

Table 7 Glucose concentration of extract

Sample concentration (µg/ml)	O.Dat490nm	Glucose concentration (µg/ml)	Glucose con per mg (µg/ml)
300	0.7	258	860

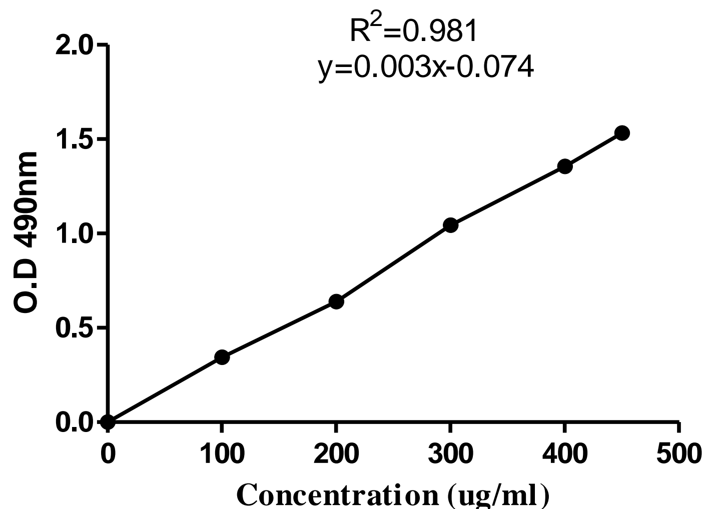

Fig15. Standard curve of glucose

5.2 Protein estimation

The Bradford protein assay is used to measure the concentration of protein in a solution. Marion M. Bradford developed the Bradford protein assay. This method is subjective and relies on the amino acid composition of the measured protein. The principle of this assay is that under acidic conditions when protein molecules bind to the Bradford reagent, the change in colour occurs, the colour change from brown to blue.

Standard curve was generated to calculate the protein content by taking BSA as standard Table 8 (Fig 15). 5 mg/ml sample of EPS were used and the proteins contents was calculated using standard curve. The proteins content in EPSs is given in Table 9.

Table 8 O. D of different concentrations of BSA as standard

Standard Concentrations (µg/ml)	O.D.at 595nm
125	0.166
250	0.332
500	0.685
1000	1.285
2000	2.45

Table 9 Protein Content of extract

Concentrations	O.D.at 595nm	Protein content
5mg/ml	0.049	20µg/ml

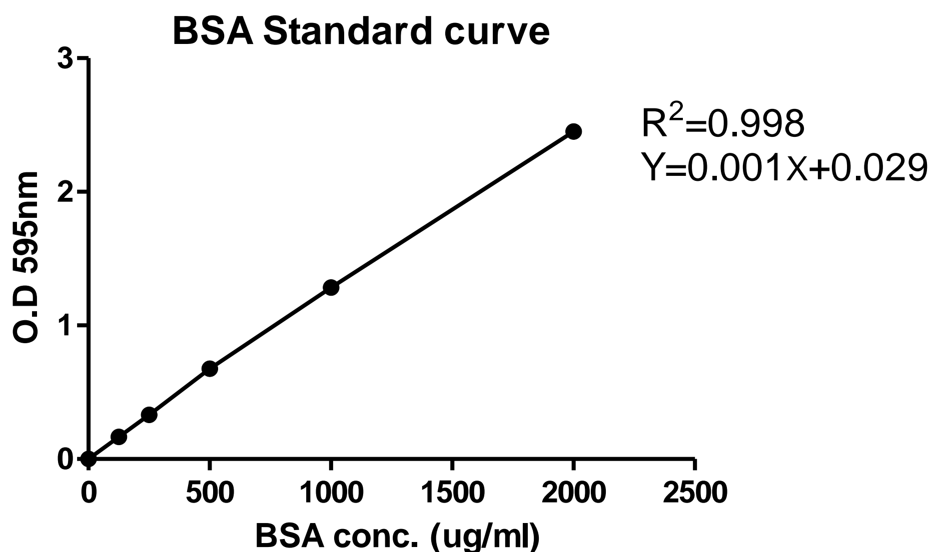

Fig 16 Standard curve of BSA

5.3 Antioxidant and free radical scavenging activity by DPPH assay

DPPH (2,2-diphenyl-1-picrylhydrazyl) assay was performed to know the antioxidant activity of EPS isolated from endophytic fungus. EPSs (50 µL) with different concentrations (1000µg/ml, 500µg/ml, 250µg/ml, 125µg/ml) was mixed with150 µL of DPPH (100 µM) added in wells of a 96 micro-titre plate. Ascorbic acid (100 µg/ml) was added as positive control. The change in color (from deep violet to light yellow) was measured at 517 nm using microplate reader. Free radical scavenging activity was found to be increased with concentration.

$$\textbf{DPPH Scavenged (\%)} = \frac{A_{control} - A_{test}}{A_{control}} \times 100$$

Table 10 Antioxidant activity by DPPH method

Concentrations (µg/ml)	Experiment 1	Experiment 2	Mean
125	11.500	1.340	6.4
250	17.000	26.800	21.9
500	43.300	63.000	53.2
1000	56.400	47.000	51.7
100 µM Ascorbic acid (Positive control)	56.200	78.520	67.4

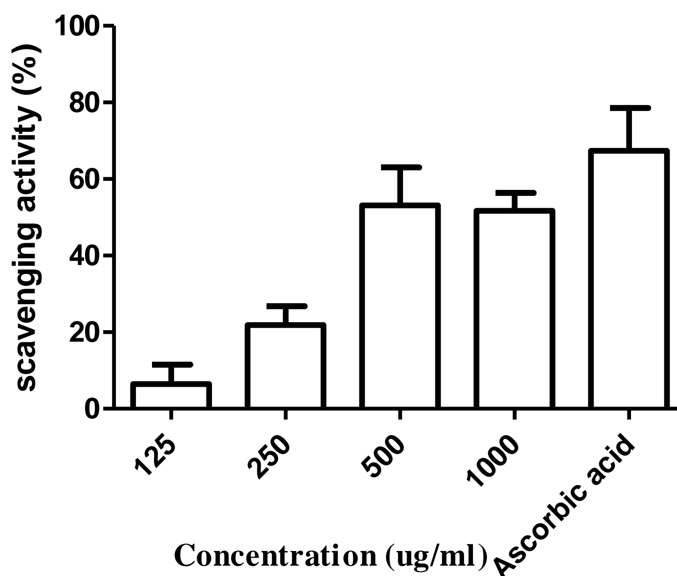

Fig17 Free radical scavenging activity of exopolymeric substance

5.4 Antimicrobial Assay

The Antimicrobial activity was determined using MH broth (HiMedia) and using a color indicator, (MTT), 3-(4, 5- dimethylthiazol-2-yl)-2, 5 diphenyltetrazolium bromide which was reduced to formazan (purple color) by living cells. Antimicrobial activity does not exhibit reduction of MTT into formazan. In the screening of antimicrobial compounds, the microplate method provides a potentially useful technique for determining antimicrobial activity of large

numbers of test samples, requiring small amounts of substances; this can be particularly important if the antimicrobial is scarce as is the case for many natural products. This method can also be used for a wide variety of microorganisms, is not expensive and presents reproducible results. The antimicrobial activity of the EPS extract was studied against various bacterial strains like *Pseudomonas aeruginosa, Staphylococcus aureus and Bacillus megaterium,* using ampicillin as positive control. Only 8% antibacterial activity was recorded against *Bacillus megaterium*, whereas no antibacterial activity was recorded against *Pseudomonas aeruginosa and Staphylococcus aureus* (Fig. 18). Similar test performed on agar plate showed no significant antibacterial properties (Fig. 19).

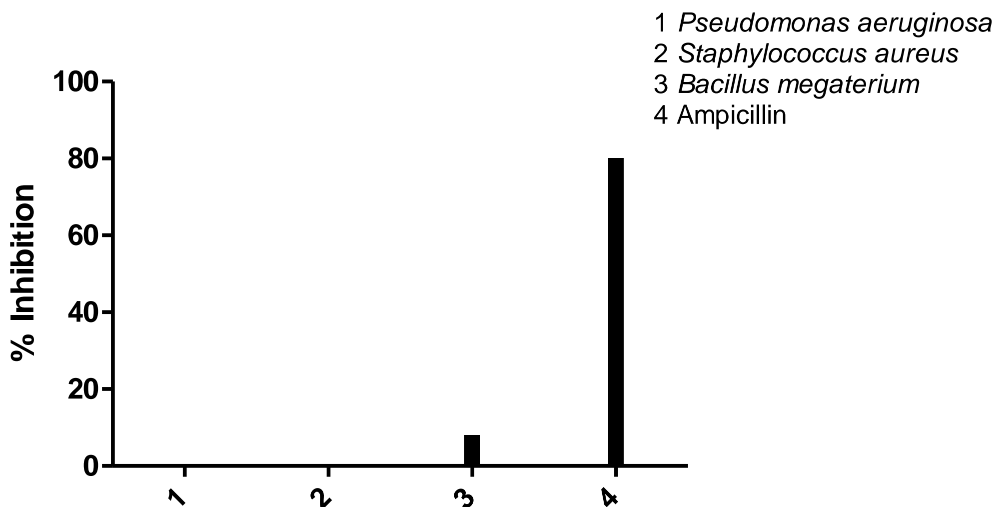

Fig.18 Antimicrobial activity of 500 µg/mL IN2 extract against different bacterial strains.

Agar diffusion method

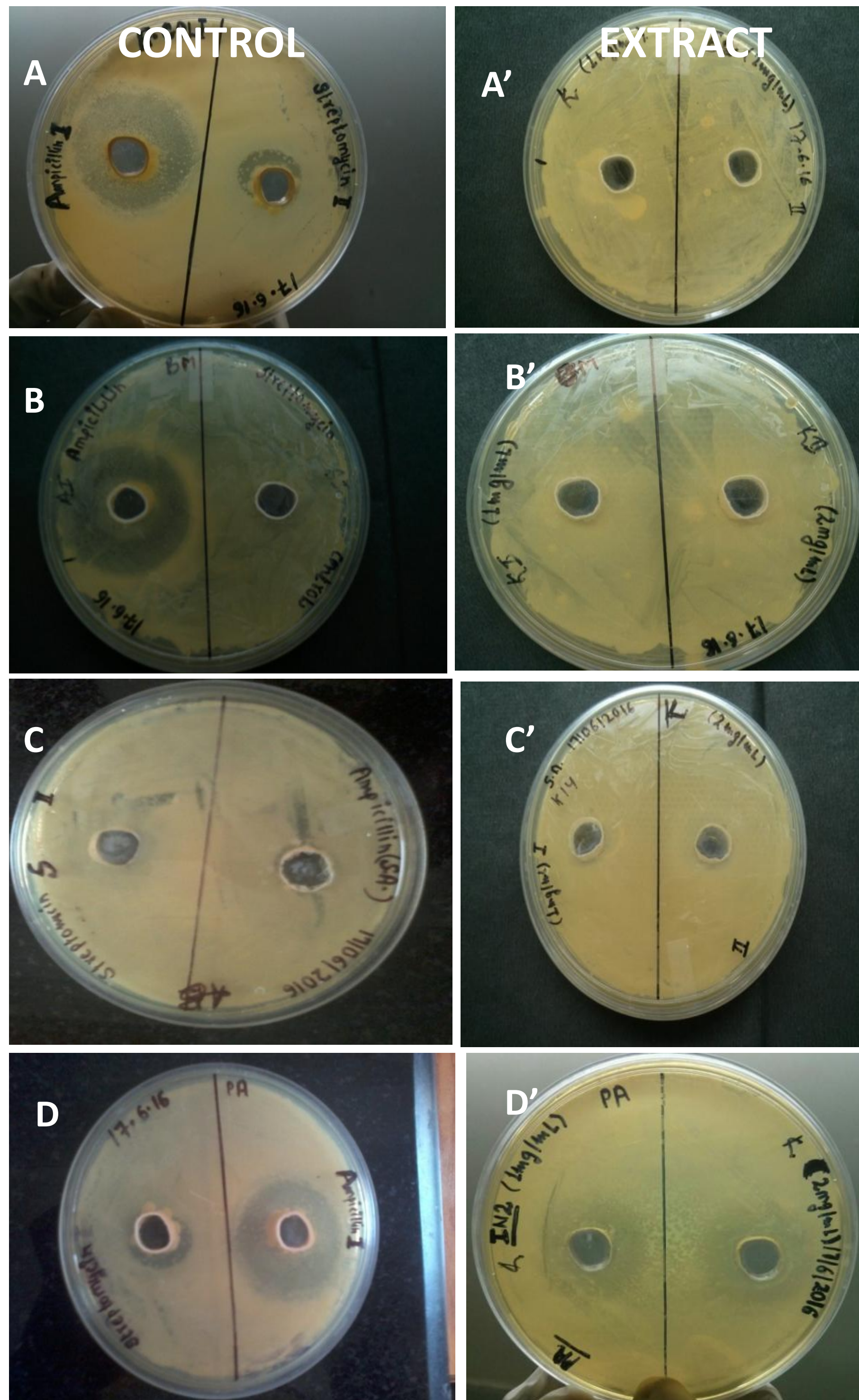

Fig. 19 Agar plate method of antimicrobial assay-at the concentration of 1000µg/mL 2000µg/mLor IN2 showed no inhibition against A and A'-*Bacillus megaterium*, B and B'-*Escherechia coli*, C and C'-*Staphylococcus aureus*, D and D'-*Pseudomonas Aeruginosa*.

5.5 Microscopic examination of fungus using lactophenol cotton blue solution

For microscopic examination of fungus, Lactophenol Cotton Blue Stain is used. Lactophenol Cotton Blue Stain is a staining agent which mainly contains three components phenol, lactic acid, cotton blue.

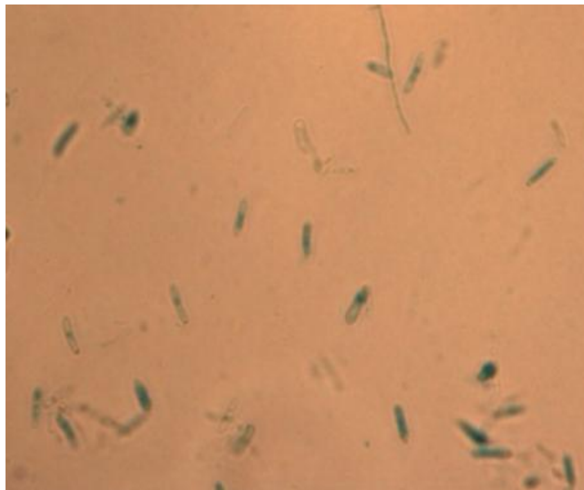

Fig.20 Microscopic image of IN2 endophytic fungus showing spore formation.

The morphological studies show ascospore formation in the IN2 cultures. The usual pattern of spores is one per ascus. The spores are spherical with warty walls. The spores were dense with dark brown colour, which could also be observed on the culture plate (Fig. 20). The morphological studies clearly depicts the significant resemblance of IN2 culture with that of *Debaryomyces hansenii* or *fabryi* . Further confirmation to the identitiy of IN2 with *Debaryomyces* was confirmed by molecular analysis.

5.6 Isolation of genomic DNA

The genomic DNA was isolated from the crushed mycelium as described in section 4.2.9. The isolated DNA was run on agarose gel electrophoresis and visualized by gel documentation syatem (Fig. 21). The genomic DNA was quantified using nanodrop.

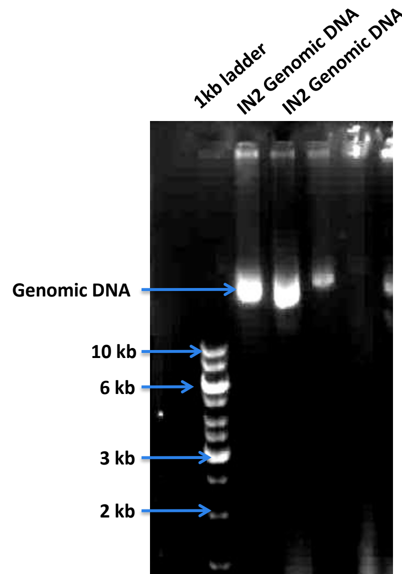

Fig.21 Gel picture of genomic DNA isolated from IN2 endophytic fungus.

5.6 Gene amplification from genomic DNA

The genomic DNA amplified using ITS1 and ITS4 primers was run on 1% agarose gel. The amplified product obtained was approximately 600bp (Fig. 22).

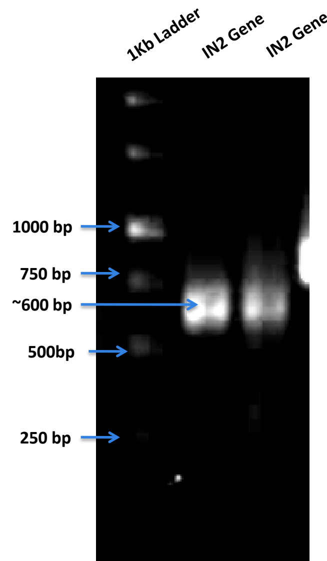

Fig.22 Gene amplification of IN2 genomic DNA using ITS1 and ITS4 primers, showing ~600bp band

5.6 Bioinformatic analysis for identification of IN2

The ITS amplified sequence obtained was run through BLASTn (Fig. 23) and the homologous sequences of different strains were recorded in FASTA format.

>IN2
GCCCATACCCCGATTATTTTTCCGTAGGTGAACTTGCGGAAGGAAGGTTCCGAAGGGAACCCGGGGGC
TTTTCAATAACCGGAGAAAGGTTCCGAAGGGAAACCGCCGGCAAATCAAAAAACGGAGAAAGGTTCC
CAAGGGGAACCTCTTATTTAATTGTAAATTTGGGGATTATCTTCAAAAAATCTTCCTGCTTTCAACAAC
GGATCTCTTGGTTCTCGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATATGAATTGCAGATTT
TCGTGAATCATCGAATCTTTGAACGCACATTGCGCCCTCTGGTATTCCACAGGGCATGCCTGTTTGAGC
GTCATTTCTCTCTCAAACCTTCGGGTTTGGTATTGAGTGATACTCTTACTCCAACTAGGCGTTTGCTTGA
AATGTATTGGCATGAGTGGTACTGGATAGTGCTATATGACTTTCAATGTATTAGGTTTATCCAACTCGT
TGAATAGTTTAATGGTATATTTCTCGGTATTCTAGGCTCGGCCTTACAATATAACAAACAAGTTTGACC
TCAAATCAGGTAGGATTACCCGCTGAACTTAAGCATATCAATAAGCGGAGGAATAT

Description	Max score	Total score	Query cover	E value	Ident	Accession
Debaryomyces hansenii gene for 18S rRNA, ITS1, 5.8S rRNA, ITS2, 28S rRNA, partial and complete sequence, strain: IG 01	282	282	100%	1e-72	99%	LC164190.1
Debaryomyces hansenii gene for 18S rRNA, ITS1, 5.8S rRNA, ITS2, 28S rRNA, partial and complete sequence, strain: IG II	282	282	100%	1e-72	99%	LC164187.1
Debaryomyces sp. strain CSHP5 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence	282	282	100%	1e-72	99%	KU350401.1
Debaryomyces sp. strain CarHP5 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence	282	282	100%	1e-72	99%	KU350313.1
Debaryomyces sp. MYf166 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence	282	282	100%	1e-72	99%	KX079875.1
Uncultured fungus clone S23T_61 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence	282	282	100%	1e-72	99%	KU164261.1
Uncultured fungus clone S23T_07 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal	282	282	100%	1e-72	99%	KU164224.1

Fig. 23Blastn analysis showing homologous sequences.

Different sequences obtained in FASTA format were aligned using multiple alignment tool-MultAlin (Fig. 24) and the slignment was analyzed.

Fig.24 Multiple sequence alignment of homologous sequences retrieved through BLASTn analysis.

The multiple sequence alignment show conserved regions of ribosomal RNA with various endophytic ascomyctes. Further the evolutionary relationship between different homologous sequences was found by Maximum parsimony phylogenetic tree constructed by MEGA7 software with 500 bootstraps (Fig. 25).

26 IN2
24 Debaryomyces hansenii
23 Debaryomyces fabryi
23 Debaryomyces subglobosus
97 Torulaspora
Candida famata
53 24 Candida psychrophila
27 Schwanniomyces capriottii
Pichia guilliermondii
99 Meyerozyma caribbica
54 Wickerhamomyces anomalus
Candida multigemmis
Candida ergatensis
90 Scheffersomyces parashehatae

Fig.25 Maximum parsimony tree showing evolutionary relationship of IN2 with different fungal species.

The evolutionary studies using maximum parsimony phylogenetic tree clearly depicts the evolutionary relationship of IN2 with Debaryomyces hansenii. Hence the unknown endophytic fungal (IN2) Isolate is identified as Debaryomyces hanseni.

6. SUMMARY

Fungal endophytes are microorganisms that dwell inside the living plant tissues without causing any harm. They are defensive mutualists that protect plant from biotic or abiotic stresses. Endophytes acquire nutrients from host plant and in return protect the plant from infections or diseases. A number of compounds are produced by fungal endophytes that are not only important for plant but also have economical advantage to human life. These compounds are found to be important in pharmaceutical and agricultural industries. In the present investigation *Camellia sinensis* leaves procured from palampur were used to isolate endophytic fungi. Ten fungal isolates (IN1 to IN10) were obtained from the *Camellia sinensis* leaves, among which IN2 was selected in the present study. The isolated fungus was cultured in 500 ml PDB medium and incubated at 28°C for 14 days. After 14 days, the PDB broth was filtered and the biomass procured was dried and weighed. Total mycelium dry weight was 42 mg. The PBD broth was used further for the estimation of extracellular polymeric substances having bioactive properties. Firstly, the 500 ml PDB broth was first concentrated to 30 ml lypholized and purified by using dialysis membrane. Then the exopolysaccharides were extracted from extract (30mg/ml stock) by using phenol sulphuric acid method. The results showed that 300 µg/ml contain 258 µg/ml glucose concentration. Similariliy the protein concentration in extract was estimated by Bradford method. The results showed that 20µg/ml protein content in 5 mg/ml extract. Further the antioxidant activity was performed by DPPH assay. 53.2% scavenging activity was observed in 500µg/ml extract. This showed that IN2 produce significant amount of antioxidants. Also the antimicrobial assay was performed against various microorganisms. Amongst which 500µg/mL of extract showed 8 % inhibition against *Bacillus megaterium.* Not much antimicrobial activity was observed in IN2 extracellular extract. Further the IN2 endophytic fungus was identified on the basis of both morphology and genetic makeup. In the morphological studies of the fungus, ascospore formation in the IN2 cultures was studied. The usual pattern of spores is one per ascus. The spores are spherical with warty walls. The spores were dense with dark brown colour, which could also be observed on the culture plate. The morphological studies clearly depicts the significant resemblance of IN2 culture with that of *Debaryomyces hansenii* or *fabryi* . Further confirmation to the identitiy of IN2 with *Debaryomyces* was confirmed by molecular analysis, where the genomic DNA of IN2 was isolated and amplified using ITS1 and ITS4 primers. The

~600 bp sequence so obtained was purified and sequenced. The sequence so obtained was searched for homologous sequences in the NCBI database using BLASTn tool. The homologous sequences so obtained were aligned using multiple sequence alignment tool- MultAlin. This showed that IN2 was best aligned with *Debaryomyces hansenii*. Further the Maximum Parsimony phylogenetic tree constructed through MEGA7 software showed that IN2 share maximum evolutionary relationship with *Debaryomyces hansenii* or *fabryi.* Hence we can conclude that the unknown fungus IN2 isolated from *Camellia sinensis* leaves is identified as *Debaryomyces hansenii,* having high antioxidant activity.

7. CONCLUSION

In the present study , endophytic fungus (IN2) isolated from *Camellia sinensis* leaves was identified as *Debaryomyces hansenii* . The exopolymeric substances isolated from this endophytic fungus showed high EPS content. The fungus also showed high antioxidant activity against free radicals and little antimicrobial activity against *Bacillus megaterium* and no activity against *Pseudomonas aeruginosa, Staphylococcus aureus*.,Hence it can be concluded that this endophytic fungus (*Debaryomyces hansenii*) is a good reservoir of bioactive compounds having wide applications in pharmaceutical, agricultural and industrial areas.

REFERENCES

Amna T, Puri SC, Verma V, Sharma JP, Khajuria RK, Musarrat J, Spiteller M, Qazi GN. Bioreactor studies on the endophytic fungus *Entrophospora* infrequens for the production of an anticancer alkaloid camptothecin. Canadian journal of microbiology. 2006 Mar 1;52(3):189-96.

Bacon CW, White Jr JF. Biotechnology of endophytic fungi of grasses. CRC Press Inc.; 1994.

Bacon CW, White JF. An overview of endophytic microbes: endophytism defined. Microbial endophytes. 2000 Feb 25;3:29-33.

Bae H, Sicher RC, Kim MS, Kim SH, Strem MD, Melnick RL, Bailey BA. The beneficial endophyte *Trichoderma hamatum* isolate DIS 219b promotes growth and delays the onset of the drought response in Theobroma cacao. Journal of Experimental Botany. 2009 Jul 1;60(11):3279-95.

Brakhage AA, Langfelder K, Streibel M, Jahn B, Haase G. Biosynthesis of fungal melanins and their importance for human pathogenic fungi. Fungal Genetics and Biology. 2003 Mar 31;38(2):143-58.

Bernays EA. Aversion learning and feeding. InInsect learning 1993 (pp. 1-17). Springer US.

Bolwerk A, Lagopodi AL, Lugtenberg BJ, Bloemberg GV. Visualization of interactions between a pathogenic and a beneficial *Fusarium* strain during biocontrol of tomato foot and root rot. Molecular Plant-Microbe Interactions. 2005 Jul;18(7):710-21.

Brady SF, Singh MP, Janso JE, Clardy J. Cytoskyrins A and B, new BIA active bisanthraquinones isolated from an endophytic fungus. Organic Letters. 2000 Dec 14;2(25):4047-9.

Bhagat J, Kaur A, Sharma M, Saxena AK, Chadha BS. Molecular and functional characterization of endophytic fungi from traditional medicinal plants. World Journal of Microbiology and Biotechnology. 2012 Mar 1;28(3):963-71.

Clay K. Fungal endophytes of grasses. Annual Review of Ecology and Systematics. 1990 Jan 1:275-97.

Debbab A, Proksch P. Fungal endophytes: unique plant inhabitants with great promises. Applied Microbiology and Biotechnology. 2011 Jun 1;90(6):1829-45.

Duarte K, Rocha-Santos TA, Freitas AC, Duarte AC. Analytical techniques for discovery of bioactive compounds from marine fungi. Trends in Analytical Chemistry. 2012 Apr 30;34:97-110.

Engelhard M, Hurek T, Reinhold-Hurek B. Preferential occurrence of diazotrophic endophytes, *Azoarcus* spp., in wild rice species and land races of Oryza sativa in comparison with modern races. Environmental Microbiology. 2000 Apr 1;2(2):131-41.

Firáková S, Šturdíková M, Múčková M. Bioactive secondary metabolites produced by microorganisms associated with plants. Biologia. 2007 Jun 1;62(3):251-7.

Firdaus M, Prihanto AA, Nurdiani R. Antioxidant and cytotoxic activity of *Acanthus ilicifolius* flower. Asian Pacific journal of tropical biomedicine. 2013 Jan 31;3(1):17-21.

Gehring CA, Whitham TG. Mycorrhizae-herbivore interactions: population and community consequences. In Mycorrhizal ecology 2002 (pp. 295-320). Springer Berlin Heidelberg.

Guo B, Wang Y, Sun X, Tang K. Bioactive natural products from endophytes: a review. Applied Biochemistry and Microbiology. 2008 Mar 1;44(2):136-42.

Hormazabal E, Astudillo L, Schmeda-Hirschmann G, Rodríguez J, Theoduloz C. Metabolites from Microsphaeropsis olivacea, an endophytic fungus of Pilgerodendron uviferum. Zeitschrift für Naturforschung C. 2005 Feb 1;60(1-2):11-21

Hardoim PR, van Overbeek LS, van Elsas JD. Properties of bacterial endophytes and their proposed role in plant growth. Trends in microbiology. 2008 Oct 31;16(10):463-71.

Huang WY, Cai YZ, Hyde KD, Corke H, Sun M. Biodiversity of endophytic fungi associated with 29 traditional Chinese medicinal plants. Fungal diversity. 2008.

Jones CG, Firn RD, Malcolm SB. On the evolution of plant secondary chemical diversity [and discussion]. Philosophical Transactions of the Royal Society of London B: Biological Sciences. 1991 Aug 29;333(1267):273-80.

Kageyama SA, Mandyam KG, Jumpponen A. Diversity, function and potential applications of the root-associated endophytes. InMycorrhiza 2008 (pp. 29-57). Springer Berlin Heidelberg.

Kumar DS, Lau CS, Wan JM, Yang D, Hyde KD. Immunomodulatory compounds from Pestalotiopsis leucothes, an endophytic fungus from Tripterygium wilfordii. Life Sciences. 2005 Nov 26;78(2):147-56.

Kumar S, Aharwal RP, Shukla H, Rajak RC, Sandhu SS. Endophytic fungi: as a source of antimicrobials Bioactive compounds. World journal of pharmacy and pharmaceutical sciences. 2014;3(2):1179-97

Kumaran RS, Muthumary J, Hur BK. Isolation and identification of an anticancer drug, taxol from Phyllosticta tabernaemontanae, a leaf spot fungus of an angiosperm, Wrightia tinctoria. The Journal of Microbiology. 2009 Feb 1;47(1):40-9.

Lee JC, Strobel GA, Lobkovsky E, Clardy J. Torreyanic acid: a selectively cytotoxic quinone dimer from the endophytic fungus Pestalotiopsis microspora. The Journal of Organic Chemistry. 1996 May 17;61(10):3232-3.

Lockwood JL. Exploitation competition. The fungal community: its organization and role in the ecosystem, 2nd Edn.(Carroll, GC and Wicklow, DT, Eds.). 1992 Jun 26:243-63.

Malinowski DP, Belesky DP. Adaptations of endophyte-infected cool-season grasses to environmental stresses: mechanisms of drought and mineral stress tolerance. Crop Science. 2000 Jul 1;40(4):923-40.

Mandyam K, Loughin T, Jumpponen A. Isolation and morphological and metabolic characterization of common endophytes in annually burned tallgrass prairie. Mycologia. 2010 Jul 1;102(4):813-21.

Mahapatra S, Banerjee D. Evaluation of in vitro antioxidant potency of exopolysaccharide from endophytic Fusarium solani SD5. International journal of biological macromolecules. 2013 Feb 28;53:62-6.

Mirjalili MH, Farzaneh M, Bonfill M, Rezadoost H, Ghassempour A. Isolation and characterization of Stemphylium sedicola SBU-16 as a new endophytic taxol-producing fungus from Taxus baccata grown in Iran. FEMS microbiology letters. 2012 Mar 1;328(2):122-9.

Park JH, Choi GJ, Lee HB, Kim KM, Jung HS, Lee SW, Jang KS, Cho KY, Kim JC. Griseofulvin from Xylaria sp. strain F0010, an endophytic fungus of Abies holophylla and its antifungal activity against plant pathogenic fungi. J Microbiol Biotechnol. 2005 Feb 1;15(1):112-7.

Pimentel MR, MOLINA G, DIONISIO A, JUNIOR M, PASTORE G. The use of endophytes to obtain bioactive compounds and their application in biotransformation process. Biotechnology Research International, 1, 11.

Pandey PK, Kass PH, Soupir ML, Biswas S, Singh VP. Contamination of water resources by pathogenic bacteria. AMB Express. 2014 Jun 28;4(1):1.

Qadri M, Johri S, Shah BA, Khajuria A, Sidiq T, Lattoo SK, Abdin MZ, Riyaz-Ul-Hassan S. Identification and bioactive potential of endophytic fungi isolated from selected plants of the Western Himalayas. SpringerPlus. 2013 Jan 11;2(1):1.

Rai MK, Tiwari VV, Irinyi L, Kövics GJ. Advances in taxonomy of genus Phoma: polyphyletic nature and role of phenotypic traits and molecular systematics. Indian journal of microbiology. 2014 Jun 1;54(2):123-8.

Singh SB, Ondeyka JG, Tsipouras N, Ruby C, Sardana V, Schulman M, Sanchez M, Pelaez F, Stahlhut MW, Munshi S, Olsen DB. Hinnuliquinone, a C 2-symmetric dimeric non-peptide fungal metabolite inhibitor of HIV-1 protease. Biochemical and biophysical research communications. 2004 Nov 5;324(1):108-13.

Schulz B, Boyle C, Draeger S, Römmert AK, Krohn K. Endophytic fungi: a source of novel biologically active secondary metabolites. Mycological Research. 2002 Sep 1;106(09):996-1004.

Specian V, Sarragiotto MH, Pamphile JA, Clemente E. Chemical characterization of bioactive compounds from the endophytic fungus Diaporthe helianthi isolated from Luehea divaricata. Brazilian Journal of Microbiology. 2012 Sep;43(3):1174-82.

Strobel G, Daisy B. Bioprospecting for microbial endophytes and their natural products. Microbiology and molecular biology reviews. 2003 Dec 1;67(4):491-502.

Strobel GA. Endophytes as sources of bioactive products. Microbes and infection. 2003 May 31;5(6):535-44.

Tan RX, Zou WX. Endophytes: a rich source of functional metabolites. Natural product reports. 2001;18(4):448-59.

Valko M, Leibfritz D, Moncol J, Cronin MT, Mazur M, Telser J. Free radicals and antioxidants in normal physiological functions and human disease. The international journal of biochemistry & cell biology. 2007 Dec 31;39(1):44-84.

Wagenaar MM, Corwin J, Strobel G, Clardy J. Three new cytochalasins produced by an endophytic fungus in the genus Rhinocladiella. Journal of natural products. 2000 Dec 27;63(12):1692-5.

Yu H, Zhang L, Li L, Zheng C, Guo L, Li W, Sun P, Qin L. Recent developments and future prospects of antimicrobial metabolites produced by endophytes. Microbiological research. 2010 Aug 20;165(6):437-49.

Zhang HW, Song YC, Tan RX. Biology and chemistry of endophytes. Natural product reports. 2006;23(5):753-71.

APPENDIX

Genomic DNA extraction buffer	Sodium acetate 100Mm
	Na_2EDTA 50mM
	NaCl 500mM
	SDS 1%
10x TBE Buffer	Tris-HCL 0.09 M (pH 8)
	Boric acid 0.9 M
	EDTA 0.02 M (pH 8)
DNA loading dye (6 X)	Bromophenol blue 0.25 %
	Xylene cyanol 0.25 %
	Glycerol 30%
Ethidium bromide	Ethidium bromide 30 mL
	Distilled water 30 mL
Potato dextrose media	Potatos, infusion form 200 g/l
	Dextrose 20 g/l
	pH at 25°C = 5.1±0.2