Isolation and screening of Endophytic Fungi For oil production

Dissertation

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in
BIOTECHNOLOGY

Submitted
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Declaration

I hereby declared that work being presented in the thesis entitled '**Isolation and screening of Endophytic Fungi for oil production**' in the partial fulfilment of requirements for the awards of degree of Masters of Science in Biotechnology, Department of Biotechnology, Thapar university, Patiala is my own laboratory work during period of January 2015 to June 2015 under the conception and supervision of Dr. Sanjai Saxena, [redacted] Professor, Department of Biotechnology, Patiala. I have not submitted the matter embodied in this thesis for the award of any other degree.

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CERTIFICATE

This is to certify that thesis entitled, "**Isolation and screening of Endophytic Fungi for oil production**" submitted by **Ms. Simranjit Kaur (Roll no- 301301016)** in partial fulfilment of the requirements for the award degree of Master of Science Degree in Biotechnology at Thapar University, Patiala is a bonafied work carried out under the esteemed supervision and conception of Dr. Sanjai Saxena and that no part of thesis has been submitted in the award of any other degree.

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ABBREVIATIONS

Abbreviation	**Full form**
J.curcus	*Jatropha curcus*
SNA	Synthetischer Nahrtoffarmer Agar
PDA	Potato Dextrose Agar
WA	Water Agar
DMSO	DimethylSulphoxide
DNA	Deoxyribonucleic acid
EDTA	Ehtylenediaminetetra acetic acid
TAE	Tris Acetate EDTA
EtBr	Ethidium bromide
UV	Ultraviolet
ITS	Internal transcribed spacer
DNTPs	Deoxynucleotide Triphosphate
U/µl	Units per micro litre
ng/µl	Nanogram per micro litre
ng	Nanogram
mM	Mili Molar
bp	Base pair
PCR	Polymerase Chain Reaction
Min	Minutes
ml	Millilitre
µM	Micro molar
mg/kg	Milligram per kilogram

Executive Summary

There is an urgent call for bio-fuels which are highly effective and can be used as an alternative to petro-diesel. Popularly known for its potential as bio-fuel, *Jatropha curcus* is a perennial plant which can be grown in marginal or poor soil. The seeds of *Jatropha curcus* are reported to contain 27-40% oil content that can be converted into superior kind of biodiesel. Besides Jatropha, many other plants and microbes are being explored for oil production.

Endophytes are microbes which resides intercellularly within the host for at least once in their lifetime without showing any significant clue of their existence. *Jatropha curcus* was exploited its endophytic fungi for oil production. Unlike other edible crops, endophytic fungi can accumulate high levels of lipids, do not require land for cultivation and they do not compete in food production. A total of 26 fungal endophytes were isolated from leaves, stem and mid rib of *Jatropha curcus*, collected from Thapar University and outskirts of Patiala in month of January 2015.

The fungal endophytes having potential to produce oil were primarily grown on Potato Dextrose Broth medium. The culture filtrate was screened for their potential to produce oil extracellularly. The secondary screening was carried out the basis of biochemical testing. Out of 26 cultures, #33JCLTU produced detectable amount of oil (4.7 mg). Microscopic identification showed that #33JCLTU belongs to *Alternaria* sp. Further for molecular identification, genomic DNA of the #33JCLTU isolate was amplified using universal ITS primers. They showed an amplicon in size between 550-600bp. The isolate was identified as *Alternaria macrospora*. Further characterization is warranted of the endophytic isolates to develop them as a source of oil for biodiesel production.

1. Introduction

"Some of the best things in life are free", the pleasure we enjoy from favor provided by nature is incalculable .The depletion of fossil fuels is a hindrance which is fencing our nation. Also a wide set of environmental problems is associated with burning fossil fuels (coal, petroleum, natural gas). As the outcome, now noticeable attention has been made to biodiesel production as an alternative to petro-diesel. Bio-diesel is defined as mono-alkyl esters of fatty acids derived from vegetable oil and animal fat, which has evidenced a number of assuring characteristics including minimization of exhaust emission (Altin et *al*., 2001) .The main preference of practicing bio-diesel is that it is biodegradable and can be used without customizing existing engines (Parajuli, 2009).

Jatropha curcus is the most promising candidate for a cheaper source of oil for producing biodiesel. It is a perennial renewable plant which is reported to withstand varying environmental conditions requires very little irrigation (Banapurmath, 2008). Jatropha oil can be extracted at a cheaper cost than coconut oil, resulting in low-cost biodiesel production. From jatropha seeds jatropha oil can be obtained which exhibit similar properties analogous to diesel (Pandey *et al*., 2012). It is widely being studied due to its potential as excellent source of biodiesel fuel and it also possess many medicinal properties (Parajuli, 2009). Jatropha oil has higher oil content than other oils and 27-40% of oil is contained within the seed (Achten *et al*., 2008).

Renewable fuel termed as biodiesel is derived from natural oils like castor, cannola, jatropha, sunflower, coconut, mustard, rapeseed, soybean etc (Jaeger, 2008). Biodiesel from coconut is a costly affair, hence there is demand for cheaper biodiesel feed stocks. Noticeable efforts have been made to progress vegetable derivatives that have approximate characteristics and performance of hydrocarbons-based diesel fuels. The hindrance which is stopping the substitution of triglycerides for diesel fuel is mainly related with high viscosity, low volatility and poly-unsaturated properties.

Various varieties of plants such as Soybean, canola are also being explored as a source of oil (Sensoj *et al*., 2006; Leung *et al*., 2006). Extracting oil only from plants is becoming a major threat to plant biodiversity on earth. Hence, there exists an urgent demand of novel alternative sources of oil that are cheaper and eco-friendly. Nature is a never ending source of novel bioactive metabolites with pharmaceutical applications. Another source of oil other than plants includes animal fat wastes (Feddern *et al*., 2011; Daraunde *et al*., 2012), bacteria, yeast and fungi. Many Bacteria viz. *Escherichia coli* (Lu *et al*., 2008), *Bacillus subtilis* (Ying *et al*., 2007) are being used as oil sources. Plants and microbes are extensively being studied for production of biodiesel, in which jatropha and endophytes have gained most noticeable interest because of bioactive metabolites produced by them (Cai *et al*., 2004; Strobel *et al*., 2004; Newman and Cragg, 2007).

Endophytes are microbes that live within the living internal tissue of host for at least a period of their life cycle without showing any clue of their existence. Endophytes have been analyzed to be a rich source of novel biological active secondary metabolite (Zhang *et al*., 2006). One of the most important advancement via endophytic fungi in medical science is Paclitaxel (Taxol) which is world's first billion-dollar anticancer drug is produced by endophytic fungi namely *Pestalotiopsis* sp. residing in the *Taxus* tree (Gangadevi *et al*., 2008). Camptothecin which is a potent anti-neoplastic agent has been contributed by endophytic fungi of the inner bark of *Campthotheca acuminata*, which is a known source of camptothecin (Kusari *et al*., 2009).Endophytic fungi also contribute to crop protection by controlling pests. *Guignardia camelliae* is reported to show activity against *Sclerotinia sclerotiorum,* which is the first report of antifungal activity of fungus(Kumar & Kaushik, 2013). *Alternaria* sp. have isolated as an endophyte from several host plants including medicinal plants of Western ghats from India .

Endophytic fungi are yet unexplored source of volatile low molecular mass hydrocarbons and lipids for biodiesel production. The need of hour is to produce biofuels more efficiently and with less number of biosynthetic steps directly from microbes and which can be immediately used without modifying engine design. Researchers are constantly reporting isolation of endophytes which are having optimum lipid profiles for biodiesel production. Endophytic fungi have also been reported to produce volatile organic compounds similar to petro diesel. Hence based on above reports, the present study was undertaken to assess the endophytic fungi from *Jatropha curcus* for oil production.

2. Review of Literature

2.1 Biodiesel

The depletion of fossil fuels and environmental problems is associated with burning fossil fuels are growing at an alarming rate. As the outcome, now noticeable attention has been made to biodiesel production as an alternative to petro-diesel. Biodiesel is an environmental-friendly substitute for petro-diesel which is prepared from renewable sources like vegetable oils and animal fats (Antony *et al*., 2011). The main advantage of exploiting biodiesel instead of petro-diesel is that it is biodegradable and can be used without customizing existing engines (Parajuli, 2009). Bio-diesel have the potential to solve number of concerns like air pollution and global warming. It can be produced from edible oil seed crops like like castor, cannola, jatropha, sunflower, coconut, mustard, rapeseed, soybean etc (Jaeger, 2008). But by using edible oil feedstock there will be large choas in human nutrition versus fuel also as compared to petrofuel, production of biofuel from edible feedstock will sound economically unfeasible. So need of hour is conversion of in-edible seed oil feedstock like jatropha into biofuel (Sruthi, 2013).

2.2 *Jatropha curcus*

Jatropha curcus (Linnaeus) belongs to family Euphorbiaceae, a perennial plant growing in marginal or poor soil. *Jatropha curcus* is a drought resistant plant which can produce seeds for approximately 50 years and can be raised easily (Thomas *et al*., 2008). It is most commonly grown in tropics and subtropics. *Jatropha curcus* being the potent source of oil, can be commercially exploited as an economical substitute for diesel.

Jatropha curcus was first time classified by Carl Von Linne in 1753. Its botanical name *Jatropha curcus* came from the greek word “Jatros” which means a “Doctor” and “trophe” means “nutrition” (Gubitz *et al.,* 1999)**.** Arabian Researchers explored the plant for its medicinal bioactivities. There are other 200 names different names for its significant importance to man and various prospects of its uses.

2.2.1 Jatropha as a potent source of biodiesel

The seeds of “Biodiesel plant” Jatropha, has been reported for production of anti-nutrional factors such as phorbol esters due to which it is non-edible (Gübitz *et al.,* 1997). Utilization of this non-edible crop into seed oil will not only minimize this problem but also will prove a helping hand for nation to fight against energy crises by production of biodiesel, an

alternative to petro-diesel. A number of engine test runs have been done across the world, which has proven jatropha as one of the best alternative source to petrodiesel (Parajuli, 2009).

Jatropha oil extraction can be carried out either by extracting oil with chemicals i.e Hexane or by using a manual ram press or an engine derived expeller. It has been reported that solvent extraction with n-hexane could produce about 41% yield by weight of oil per kg of the jatropha seed. In addition to this, it has been reported that the dry seed of *J. curcas* would yield about 30–38% of crude oil using an engine driven – expeller (Forson *et al.*, 2004).

J. curcas seed oil used in a direct injection diesel engine showed lower emissions of hydrocarbons and oxides of nitrogen compared to those from mineral diesel About 40-50% of *J. curcas* seed oil can substitute for diesel without any engine modification or preheating of the blends (Pramanik, 2003) and can be used as a fuel in diesel engines by blending it with methanol (Gubitz *et al*., 1999).

2.2.2 Applications of *Jatropha curcus*

Jatropha curcus is reported to contain moisture 6.62%, protein 18.2%, fat 38.0%, carbohydrates 17.30%, fibre 15.50% and ash 4.5%. The oil contains 21% saturated fatty acids and 79% unsaturated fatty acids. There are many applications of *J. curcus* other than oil production. It is being extensively used for soap making due to its high saponification value. At present *Jatropha curcus* oil is being imported to meet the demand of cosmetic industry. In china, a varnish is prepared by boiling the oil with iron oxide. In village it is used as an illuminant as it burns bricants and candles as in case of castor oil. It is used for wool spinning in England. The protein content Jatropha oil cake may be used as raw material for plastics and synthetics fibres (Gubitz *et al.*, 1999).

“Jatrophine” which is reported to be having anti-cancerous properties is an alkaloid which is present in *J. curcus*. Its oil possess purgative properties so is used as an external application for skin diseases and rheumatism, it is also reported to be abortificient and also efficacious in dropsy, sciatic and paralysis. Tender twigs of the plant are used for cleaning teeth. The juice is reported to relive toothache and strengthen gums. The leaf juice is used as an external application for piles. It is also applied for inflammations of the tongue in babies. The twig sap is considered stypic and used for dressing wounds and ulcers. An emulsion of the sap with benzyl benzoate is said to be effective against scabies, wet eczema and dermaties. A decoction of leaves and roots is given for diarrhea. The root is reported to contain yellow oil with strong antelmintic action. The root bank is used to external

application for sores. A decoction of the bark is given for rheumatism and leprosy. Similarly, roots are also reported to be used as antidote for snakebite (Gubitz *et al.,* 1999). Leaves of *J. curcus* have reported to possess insecticidal properties and are also used for fumigating houses against bed bugs. The ether extract shows antibiotic activity against *Staphylococcus aureus* and *Escherichia coli.*

2.3 Natural sources of Biodiesel

Natural products are those which are obtained from living organisms like plants, microorganisms (bacteria, fungi) and marine sources etc. Throughout the ages, natural products are being emplored for various medicinal properties, as a therapeutic entity in industries. Natural products continue to provide distinctive structural diversity in contrast to combinatorial chemistry, which is a novel platform for discovering mainly novel low molecular weight lead compounds.

The plant, *Alhagi maurorum* secretes a sweet, sticky material known as “manna” from the stems and leaves during hot days and it aids in the treatment of anorexia, constipation, dermatosis, epistaxis, fever, leprosy, and obesity (Duke *et al.,* 2007). One of the most known natural product discoveries derived from a fungus is that of penicillin from the fungus, *Penicillium notatum* discovered by Fleming in 1929. The exploration of secondary metabolites of endophytic fungi has marked its era after the discovery of taxol producing enophytic fungi. Ergoflavin, isolated from an endophytic fungi growing in leaves of medicinal plant *Mimusops elengi,* is considered as a novel anticancer agent (Deshmukh *et al*., 2009).

There exist many other natural sources of oil inspite to that of few plants. Various classes of plants, Bacteria such as *E. coli, B. subtilis* are found to be potent oil producer (Table 1).

2.4 Endophytic Fungi

Endophytic fungi are the power house of novel bioactive compounds possessing anticancer, antifungal, insecticidal properties (Strobel., 2004) Endophytic fungi spend the whole or part of their life cycle colonizing inter or intra-cellularly inside the healthy tissues of the host plant. A variety of secondary metabolites have been isolated & characterized from endophytic fungi (Zhang *et al*., 2006). One of the most important advancement via endophytic fungi in medical

SOURCE		REFERENCE
P**lant**		
	Brassica napus	Issariyakul *et al*., 2008
	Glysine max	Kouzu *et al*., 2008
	Cocus nucifera	Hossain *et al*., 2012
	Jatropha curcus	Kumar and Kaushik, 2013
Bacteria		
	Escherichia coli	Huffer *et al.,* 2012
	Bacillus subtilis	Pereira *et al*., 2013
	Staphylococcus sp.	Kim *et al*., 2013
Yeast		
	Saccharomyces cerevisiae	Shi *et al*., 2012
	Candida freyschussii	Amaretti *et al*., 2012
	Yarrowia lipolytica	Tai *et al*., 2013
	Cryptococcus terricola	Tanimura *et al*., 2014
Microalgae		
	Chlorella protothecoides	Gao *et al*., 2010
	Tribonema minus	Wang *et al*., 2013
	Nannochloropsis	Moazami *et al*., 2013
	Chlorella pyrenoidosa	Wu *et al*., 2014
Fungi		
	Gliocladium sp.	Strobel *et al.,* 2008
	Hypoxylon sp.	Tomsheck *et al*., 2010
	Pseudomonas sp.	Ji et *al*., 2010
	Mucor circinelloides	Mitra *et al*., 2012
	Aspergillus sp.	Subhash *et al*., 2014
	Fusarium sp.	Facchini *et al*., 2014
	Colletotrichum truncatum	Ellison *et al*., 2015

Table 1: Different natural sources of Oil

science is Paclitaxel (Taxol) which is world's first billion-dollar anticancer drug is produced by endophytic fungi namely *Taxus andreanae* residing in the *Taxus* tree (Gangadevi *et al*.,2008).

Name of compound	**Source organism**	**Host plant**	**Application**
Paclitaxel	*Taxus andreanae*	*Taxus brevifolia*	Anti-cancerous
Penicillin	*Penicillium* sp.	*Diphylleia sinensis*	Antibacterial
Lovastatin	*Aspergillus terreus*	*Podocarpus sp.*	lowers Cholesterol
Campothecin	*Entrophospora infrequens*	*Camptotheca acuminata*	Anti-neoplastic
Vancomycin	*Nocardia orientalis*	*Wollemia nobilis*	Antibiotic
Diosgenin	*Cephalosporium* sp.	*Paris polyphylla var. yunnanensis*	Reduces serum cholesterol
Pestacin	*Pestalotiopsis microspora*	*Pestalotiopsis microspora*	Anti-oxidant
Cytochalasins	*Rhinocladiella* sp.	*Tripterygium wilfordii*	Antitumor activity
Reserpine	*Alternaria* sp.	*Rauwolfia serpentina*	Antihypertensive
Vinblastine	*Fusarium* sp.	*Madagascar periwinkle*	Renal and testicular cancer

Table 2: Bioactive compounds from endophytic fungi

There are some reports that unravel the potential of microbial endophytes to mimic the bioactive compounds as produced by the plant itself thus making them a capable source of novel compound. Paclitaxel, a diterpene compound, either used alone or in combination with other chemotherapeutic agents for the treatment of a various types of cancers (Croom, 1995). Earlier, it was isolated from the bark of the Pacific yew tree, *Taxus brevifolia* (Wani *et al.*, 1971), because of the limited supply of the drugs alternative sources of these drugs were explored by researchers. As the endophytic fungi can copy the medicinal properties of the host plant so exploitation of such medicinal plants for the isolation of endophytic fungi having important biological activities can be done (Table 2).

2.5 Biochemical screening of endophytic fungi

Biochemical assays are important and less time consuming to detect oil accumulation in internal

cells is done to measure the presence of oil in a given culture. For oil detection, sudan dye is being used. The sudan dye reacts with hydrocarbon chain of triacylglycerol and results in formation of blue coloured complex. This test can be performed with culture filtrate as well as directly staining the mycelium (Xie *et al.,* 2013). Burdon confirmed that the significance of Sudan black and modified the procedure to determine intra-cellular fatty material (Burdon 1946).Observation of blue oil droplets intra-cellularly confirms the presence of oil.

2.6 Thin Layer Chromatography

Thin-layer chromatography (TLC) is a chromatography technique used to separate mixture of compounds on basis of their partition coefficient. It is a very commonly used technique in synthetic chemistry for identifying compounds, determining their purity and following the progress of a reaction. Joseph *et al* separated methyl esters by TLC using hexane/ethyl acetate/ acetic acid in the ratio of 90:10:1 as solvent system. The spot were detected by spraying 50 % alcoholic sulphuric acid and retention factor was calculated (Joseph *et al.*, 2011).

2.7 High-performance liquid chromatography method (HPLC)

High-performance liquid chromatography (HPLC) is a sensitive liquid chromatography technique used to quantify and determine purity of compounds from the mixture .In HPLC and liquid chromatography, where the sample solution is in contact with a second solid or liquid phase, the different solutes in the sample solution will interact with the stationary phase. The differences in interaction with the column can help separate different sample components from each other. Researchers are continuously employing HPLC for seperation of mixture of compounds. The phorbol esters of jatropha were seperated using HPLC. The mobile phase used was a mixture of acetone: acetonitrile (63.5:36.5).The highest peak was shown at 11 min (Ahmed and Salimon, 2009). Makkr *et al.*,carried out HPLC for quantification of phorbol esters. (Makkr *et al.,* 1997)

2.8 Taxonomic Identification of endophytic fungi

Identification of endophytic fungi which are potent of producing oil was done by morphological and molecular techniques. For microscopic study of fungal structures such as mycelia, spores etc identification is done. Different media such as PDA, WA, SNA are used to optimize growth conditions of fungi sporulate.

2.8.1 Morphological Identification

For microscopic study of fungal structures such as mycelia, spores etc identification is done. Kumar and Kaushik identified endophytic fungi by slide preparation. Then Fungal mycelium was stained with cotton blue and mounted in polyvinyl lactic acid glycerol (PVLG) by heating at 65uC for 2–3 days and observed under light microscope (Kumar and Kaushik, 2013)

2.8.2 Molecular Identification

Molecular identification techniques are more sensitive and particular to identify and classify microbes (Sette *et al.,* 2006). There is rapid or high-throughput identification of microorganisms.

Endophytic fungus was identified on the basis of microscopic morphology and nucleotide sequence of ribosomal RNA. Many fungal species such as *Alternaria marmelos* (Meshram *et al.*, 2012), *Penicillium* sp. (Gupta et al., 2014) have been identified on the basis of ITS region amplification.

3. Aim of Study

Isolation and screening of endophytic fungi for oil production

Objective

1. Isolation, screening and identification of oil producing endophytic fungi from *Jatropha curcus*.
2. Biochemical and Analytical analysis of oil producing endophytic fungi.

4.1 Plant Sample collection

Healthy plant samples (Stem and leaves) of *Jatropha curcus* were collected in month of January 2015 from the Thapar University campus, Patiala, Punjab. The samples were placed in sterile Zip packets and stored at 4 ºC till further use.

4.2 Isolation of endophytic fungi

The collected plant samples were wahed under running tap water for 10 min, air dried and then surface sterilized by dipping in 0.1% (v/v) sO.D.ium hypochlorite solution for 3 min followed by 70% ethanol and 30% ethanol for 1 min and 45sec respectively. The surface sterilized samples were cut into 1-2 mm pieces with sterile blade and inoculated onto the PDA plates. The inoculated plates were incubated at 26 ± 2 °C for maximum of 5 days or till fungal growth was seen (Agarwal and Hasija, 1986). The fungal hyphae emerging out from the inoculated samples were picked up and transferred to fresh PDA plates so as to obtain pure culture.

4.3 Maintenance and sub-culturing of endophytic fungi

The maintenance of the cultures involved regular sub culturing on to PDA plates.

4.3.1 Sub culturing of Endophytic fungi

A total of 26 endophytic fungi isolated from *Jatropha curcus* were aseptically sub cultured on to PDA plates and incubated at 26 ºC with 12h light/dark periO.D. for 7-10 days till profuse fungal growth was seen. The pure culture was aseptically transferred on to PDA slants containing 10% glycerol for long term preservation at 26 ±2 ºC.

4.4 Preliminary screening of oil producing endophytic fungi

4.4.1 Lipid Content Test

All the 26 endophytic fungi during the study were screened for oil accumulation by using the lipid content assay as described by Burdon 1946. Briefly, mycelium of each isolate was placed on to clean glass slide and stained by immersing in Sudan Black dye for 5-10 min followed by immediate washing with 70% ethanol for 5-7 s. Then it was visualized under Nikon binocular microscope for dark blue lipid particles in the mycelial tissue.

4.5 Production of culture filtrate

The 26 endophytic isolates were subjected to submerged fermentation in Potato Dextrose Broth (PDB) medium containing pieces of *J. curcus* leaves. 5 mm mycelial disc of 7 day old culture was inoculated into 100 ml pre-sterilized PDB broth in Erlenmeyer flasks under aseptic conditions and were incubated in shaker-incubator at 120 rpm, 28 ℃ for 10 days. After the incubation, the fungal mycelium was separated from broth through filtration using Whatman filter paper followed by centrifugation at 10,000 rpm for 10 min to get cell free culture filtrate.

4.6 Liquid-Liquid Extraction

The isolation of bioactive residue from the cell free filtrate of selected endophytic fungi was achieved by Liquid–Liquid extraction. The culture filtrate was extracted with hexane in the ratio of 1:2 as the solvent system. The organic layer so obtained was collected and the extraction procedure was repeated thrice. Subsequently, the obtained organic layer was dehydrated using anhydrous sodium sulphate and evaporated to dryness to get crude bioactive residue. The fraction so obtained was weighed, reconstituted in hexane and stored at −20 ºC until further use.

4.7 Secondary Screening with Sudan dye test

The Hexane fractions of selected cultures were again tested for the presence of oil by using sudan dye test. Appearance of blue color confirms the presence of oil. Briefly describing, to 2 ml of crude hexane fraction, few drops of sudan black dye was added and observed for the blue coloration. Hexane was used as negative control and Jatropha oil was chosen as positive control. The intensity of blue coloration was used as criteria to distinguish potent oil producing endophytic fungi.

4.8 Thin Layer Chromatography (TLC)

The crude hexane fraction of the positive isolate was subjected to thin layer chromatography (TLC). TLC plates of 0.5 mm thickness were prepared on to 20 x 15 x 5 mm glass plates and were activated by keeping at 100 ºC for 3 h prior to use. The sample and positive control (Jatropha oil) were spotted on to activated TLC plate just 1cm above the lower edge of TLC plate with the help of capillary and air dried. On the other hand, the TLC chamber was saturated with different combinations of solvents mixed in different ratios for 20 min. The TLC plate was allowed to develop in saturated TLC chamber. When the solvent front reaches

upto the desired level, the TLC plate was taken out and air dried. The Chromatogram was observed by keeping the TLC plate in iodine chamber. Jatropha oil (Mirado) was used as standard for the comparison of R_f value. Retention factor (R_f) value of each band was obtained as the ratio of distance move by solute to that of solvent front

$$\text{Retention Factor } (R_f) = \frac{\text{Distance moved by solute}}{\text{Distance moved by solvent}}$$

4.9 High Performance Liquid Chromatography (HPLC)

The purity and concentration of crude hexane fraction of selected isolate was deduced by HPLC (Perkin Elmer- 200 series pump). 40 µg of hexane fraction was dissolved in $CdCl_3$, injected into the C18 (5 µm) reverse phase discovery column (Sigma Aldrich) column and gradient elution was carried out. Gradient elution of orthophosphoric acid (0.1%) and acetonitrile ranging from 10% to 70% was used as mobile phase with a flow rate of 1 ml/min. Jatropha oil was used as standard and 20 µl of 1% Jatropha oil was injected into HPLC column and data of peak area, Retention time was compared with crude Hexane fraction so as to deduce purity and presence of oil in sample.

4.10 Identification of potential endophytic fungi

4.10.1 Morphotaxonomy

The oil producing isolate was identified under the microscope on the basis of morphological and microscopic characteristics. The culture was grown over Potato dextrose agar medium (PDA) for 7 days at 26 °C. Morphological features such as colony growth rate, color, appearance were critically observed and noted. For Microscopic examination, the glass slide was cleaned with alcohol and air dried. A drop of water was put on to glass slide, upon which the mycelial mass was placed and teased properly with the help of sterile needles and then stained with lactophenol cotton blue (Hi Media). The slide was covered with 18 x 10 mm cover slip avoiding the formation of air bubble and mounted with DPX. The microscopic features were observed at 10X, 40X and 100X using Nikon binocular microscope.

4.10.2 Molecular identification of endophytic fungi

4.10.2.1 Genomic DNA isolation

The genomic DNA isolation of selected oil producing fungal isolate was carried out from 3-4 days old culture grown on PDA plates using the Wizard® Genomic DNA purification kit (Promega, USA). Briefly, 0.5 -1 g of fungal mycelia was grounded to powder in mortar and pestle using liquid nitrogen. 660-750 µl of the Nuclei lysis buffer was added to it and was again crushed to get very fine powder. The contents were transferred to microcentrifuge tube, vortexed followed by incubation at 65 °C in water bath for 15 min. After that, the micro centrifuge tubes were centrifuged at 13,000 rpm for 5 min to remove cell debris. Further, 5 µl of RNase was added to each micro-centrifuge tube and incubated at 37 °C for 15 min followed by addition of 200 µl of protein precipitation solution. After this, the micro centrifuge tubes were centrifuged at 13,000 rpm for 3 min to remove contaminating proteins. The aqueous phase containing DNA was transferred to isopropanol containing micro-centrifuge tube and centrifuged at 13,000 rpm for 3 min. The DNA pellet was rinsed with 70% ethanol followed by centrifugation at 13,000 rpm for 1 min. Then pellet was air dried and dissolved in 50 µl of DNA Rehydration buffer (Tris EDTA buffer (pH=8)). The qualitative estimation of the DNA isolated was done by agarose gel electrophoresis

4.10.2.2 Agarose Gel Electrophoresis

Agarose gel (0.8%) was prepared in 1X TAE (Tris Acetate EDTA) buffer and 0.5 µg/ml of ethidium bromide (EtBr) was added and then gel was casted in the electrophoretic apparatus. The gel was allowed to solidify and the comb was carefully removed. The running buffer (1X TAE) was poured into the electrophoretic tank so that the gel is fully immersed into the buffer. The DNA samples mixed with the 6X loading dye were loaded into wells and allowed to run at 50 V for 1 h. The DNA fragments were visualized under UV transilluminator. Gel imaging was performed under UV light in Bio-Rad Gel documentation System using Quantity-1-D analysis software.

Quantitative estimation of the genomic DNA was done by spectrophotometric analysis of the sample. The absorbance of the sample was taken at 260 nm and 280 nm to determine the concentration and purity of the sample. 1 O.D. is equivalent to 50 µg/ml DNA sample. The concentration of the DNA sample was calculated using following formula.

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

The purity of the DNA sample was determined by taking the ratio of absorbance at 260 nm and at 280 nm. If the ratio is less than 1.6, then there is RNA contamination, if the ratio lies

between 1.6-1.8, then DNA sample is pure. If the ratio is more than 1.8, the DNA might be contaminated with protein.

4.8.2.3 PCR amplification, sequencing and Phylogenetic identification

Internal transcribed spacer (ITS) rDNA region is universally accepted for molecular taxonomic identification of fungi. ITS1-5.8S-ITS2 region was amplified by employing primers ITS1 (5'TCCGTAGGTGAACCTGCGG3') and ITS4 (5'TCCTCCGCT TATTGATATGC3') as described by White *et al.*,1990. The primers were synthesized by Xcelris Labs Ltd., Ahmadabad, Gujarat.

S.no	Reagents	Stock concentration	Quantity	Final concentration/ 25µl
1.	Autoclaved double distilled water	_	15 µl	_
2.	Taq buffer	10X	2.5 µl	1X
4.	dNTPs	2.5 mM	2.0 µl	0.2 mM
5.	Forward Primer (ITS1)	10 µM	2.0 µl	0.8 µM
6.	Reverse Primer (ITS4)	10 µM	2.0 µl	0.8 µM
7.	Taq DNA Polymerase	5 U/µl	1.0 µl	2.5 U
8.	Template DNA	50 ng/µl	1.0 µl	50 ng

Table 3 Different reagents used during PCR reactions.

Amplification was carried out in 25 µl reaction mixture (Table no. 1) containing 50 ng of extracted fungal genomic DNA, 0.8 µM of each primer (ITS1 and ITS4), 0.2 mM of dNTP (Bangalore GeNei), 1.5 U of Taq DNA Polymerase (Bangalore GeNei) in 10X Taq buffer (Bangalore GeNei) in a Thermocycler (My Cycler, Bio-Rad Laboratories, Inc). The PCR cyclic conditions consisted of initial denaturation at 96 ºC for 5 min followed by 39 cycles of 95 ºC for 1 min, 58 ºC for 60 sec, 72 ºC for 45 sec followed by final extension at 72 ºC for 7 min. The PCR amplicons were resolved by using agarose gel electrophoresis (1.5 % agarose

gel dissolved in 1X TAE buffer) at 50 V for 1.30 h. Gel imaging was performed under UV light in Bio-Rad Gel documentation System using Quantity-1-D analysis software.

An approximate 550- 600 bp PCR prO.D.uct was purified by using the Wizard® SV Gel and PCR clean up system kit (Promega, USA). The purified amplicon was sent for direct sequencing to Xcelris Labs Ltd., Ahmadabad, Gujarat.

The obtained chromatograms were manually edited and final sequence was subjected to BLAST analysis to ascertain the positional homology with closely related micro-organism and identification of the potential isolate.

5.1 Re-culturing

A total of 26 Endophytic fungi used in the present study were sub cultured regularly on PDA plates and maintained at 26 ˚C. For their long term preservation, all the isolates were transferred on to PDA slants containing 10% glycerol and maintained at 26˚C. The endophytic fungi in the present study were isolated from medicinally important plant *Jatropha curcus* belonging to family Euphorbiaceae collected from Patiala city and adjoining areas. Out of 26 endophytic fungi, 15 isolates were isolated from leaf and 6 from stem and 5 from mid rib of *Jatropha curcus* (Figure 1, Table 3).

S. No	Culture Code	Plant part	Samling location
1.	#18JCLTU	Leaf	Thapar university ,Patiala
2.	#32JCLTU	Leaf	Thapar university ,Patiala
3.	#33JCLTU	Leaf	Thapar university, Patiala
4.	#36JCLTU	Leaf	Thapar university ,Patiala
5.	#48JCLTU	Leaf	Thapar university, Patiala
6.	#75JCLTUC	Leaf	Sirhind Road ,Patiala
7.	#78JCLTUC	Leaf	Sirhind Road ,Patiala
8.	#79JCLTUC	Leaf	Sirhind Road, Patiala
9.	#85JCLTUC	Leaf	Sirhind Road, Patiala
10.	#166JCLB	Leaf	Near Bus Stand ,Patiala
11.	#257JCLB	Leaf	Near Bus Stand ,Patiala
12.	#258JCLB	Leaf	Near Bus Stand ,Patiala
13.	#271JCLB	Leaf	Near Bus Stand ,Patiala
14.	#273JCLB	Leaf	Near Bus Stand ,Patiala
15.	#276JCLB	Leaf	Near Bus Stand, Patiala
16.	#2JCMRTU	Mid rib	Thapar university, Patiala
17.	#4JCMRTU	Mid rib	Thapar university, Patiala
18.	#5JCMRTU	Mid rib	Thapar university, Patiala
19.	#38JCMRTU	Mid rib	Thapar university, Patiala
20.	#93JCMRTU	Mid rib	Thapar university, Patiala
21.	#1JCSTU	Stem	Thapar university ,Patiala
22.	#2JCSTU	Stem	Thapar university, Patiala
23.	#6JCSTU	Stem	Thapar university ,Patiala
24.	#10JCSTU	Stem	Thapar university ,Patiala

25.	#11JCSTU	Stem	Thapar university, Patiala
26.	#52JCSTU	Stem	Thapar university, Patiala

Table 4.: Cultures isolated from *Jatropha curcus* from present study

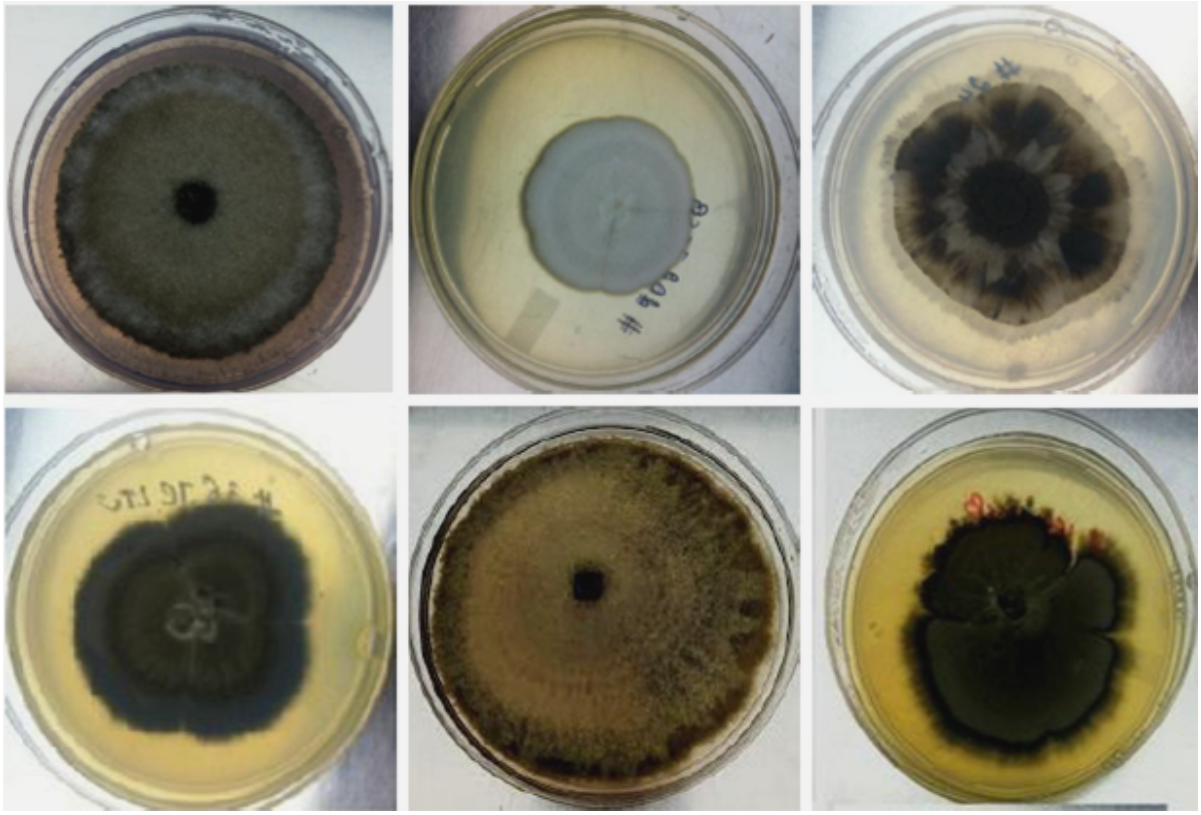

Figure 1 Pure cultures of different endophytic fungi on PDA plates

5.2 Preliminary screening for oil producing fungi by Lipid content test

All the 26 cultures were screened for oil deposition inside mycelium of each fungal isolate by staining with sudan black dye and visualized under microscope for the presence of blue colored lipid particles accumulation in the hyphal mass (Table 4). Out of the 26 cultures, 11 cultures were found to be positive for oil accumulation. #33JCLTU were showing maximum accumulation (> 90%) of blue colored lipid particles (Fig 2a) followed by #18JCLTU, #32JCLTU, #36JCLTU, #48JCLTU and #78JCLTU which were exhibiting 80-90%

S.NO	Culture code	Plant part	Sampling location	Lipid content test
1.	#18JCLTU	Leaf	Thapar university ,Patiala	++
2.	#32JCLTU	Leaf	Thapar university ,Patiala	++
3.	#33JCLTU	Leaf	Thapar university, Patiala	+++
4.	#36JCLTU	Leaf	Thapar university ,Patiala	++
5.	#48JCLTU	Leaf	Thapar university, Patiala	++
6.	#75JCLTUC	Leaf	Sirhind Road ,Patiala	+
7.	#78JCLTUC	Leaf	Sirhind Road ,Patiala	++
8.	#79JCLTUC	Leaf	Sirhind Road, Patiala	+
9.	#85JCLTUC	Leaf	Sirhind Road, Patiala	-
10.	#166JCLB	Leaf	Near Bus Stand ,Patiala	-
11.	#257JCLB	Leaf	Near Bus Stand ,Patiala	-
12.	#258JCLB	Leaf	Near Bus Stand ,Patiala	-
13.	#271JCLB	Leaf	Near Bus Stand ,Patiala	-
14.	#273JCLB	Leaf	Near Bus Stand ,Patiala	-
15.	#276JCLB	Leaf	Near Bus Stand, Patiala	-
16.	#2JCMRTU	Mid rib	Thapar university, Patiala	-
17.	#4JCMRTU	Mid rib	Thapar university, Patiala	-
18.	#5JCMRTU	Mid rib	Thapar university, Patiala	-
19.	#38JCMRTU	Mid rib	Thapar university, Patiala	-
20.	#93JCMRTU	Mid rib	Thapar university, Patiala	-
21.	#1JCSTU	Stem	Thapar university ,Patiala	+
22.	#2JCSTU	Stem	Thapar university, Patiala	+
23.	#6JCSTU	Stem	Thapar university ,Patiala	-
24.	#10JCSTU	Stem	Thapar university ,Patiala	-
25.	#11JCSTU	Stem	Thapar university, Patiala	+
26.	#52JCSTU	Stem	Thapar university, Patiala	-

(+) indicate 60-70%, (++) 70-80%, (+++) >90%, (-) 0% oil accumulation

Table 5: Endophytic isolates screened for Oil production.

of oil accumulation and #1JCSTU, #2JCSTU, #11JCSTU, #38JCMTU and #79JCLTUC were exhibiting 70-80% lipid accumulation.

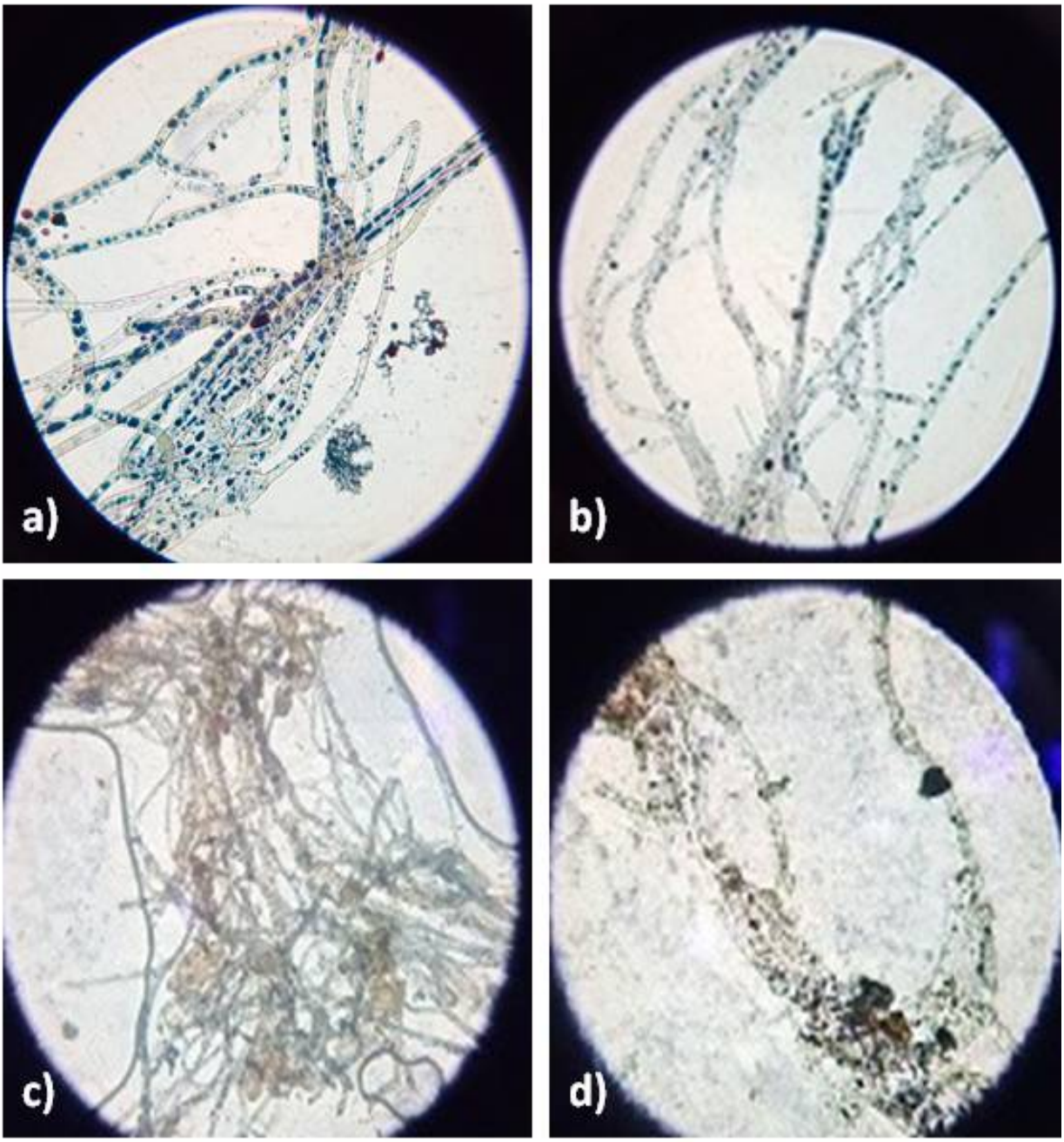

Figure 2: Lipid contest test with sudan Black dye a-c: blue colored lipid particles observed a) #33JCLTU b) #48JCLTU c) #36JCLTU d) #2JCMRTU – No blue colored lipid particles observed

5.3 Production of culture filtrate and solvent extraction

The selected 11 isolates based on preliminary screening by Lipid content test were subjected to secondary metabolites production in PDB medium. Biomass production was considered as a parameter to infer the growth rate of fungal cultures. #36JCLTU was showing the highest and #48JCLTU showing the lowest biomass production. Biomass production of some of fungal cultures ranging from highest to lowest is listed in Table 3. The pH of culture filtrates of most of the isolates were found to be acidic except that of #36JCLTU and #11JCLTU whose pH value rises up to 8.9.

Culture Code	Fresh biomass Weight (g)	pH
#36JCLTU	8.39	8.46
#33JCLTU	7.53	7.2
#11JCSTU	6.21	8.91
#78JCLTUC	4.92	6.23
#18JCLTU	4.15	5.92
#2JCMRTU	3.87	5.04
#48JCLTU	2.89	6.59

Table 6: Biomass production of different cultures under study

The cell free filtrates of selected isolates were subjected to solvent extraction by Hexane. The bioactive residue was reconstituted in Hexane. The maximum yield of the bioactive residue was obtained from #33JCLTU (4.09 mg) and minimum yield from #2JCMRTU (1.71mg) (Table 4).

Culture code	Yield of crude bioactive residue (mg)
#33JCLTU	4.09
#36JCLTU	2.94
#78JCLTUC	2.63
#48JCLTU	2.08
#2JCMRTU	1.71

Table 7: Yield of bioactive residue of different cultures under study

5.4 Sudan Dye test

Biochemical Test specific for the detection of Oil in sample viz. Sudan Dye test was employed. This test was based upon the formation of Blue colored complex due to interaction of dye functional groups with hydrocarbon chain of triacylglycerols. All selected isolates gave positive results for oil production except that of #79JCLTU. But, #33JCLTU was found to be potent producer (>90% oil production) of oil as inferred by the intensity of blue colored complex formation (Figure 3) followed by #48JCLTU, #2JCSTU exhibiting 70-80% oil production. Rest of the isolates were showing <60% of oil production. Hence, #33JCLTU was selected for further analysis. Negative Control i.e. Hexane did not produced blue color and became violet in color.

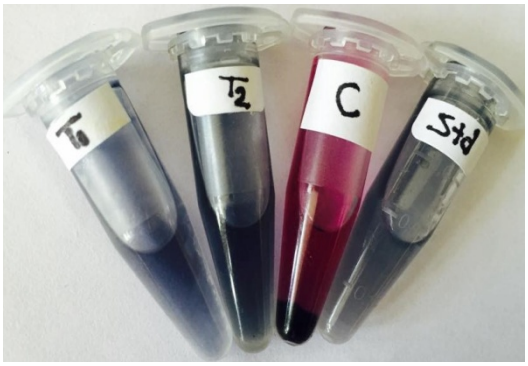

Figure 3 Sudan Black dye test for oil detection. T1- T2 stands for crude hexane fraction of #33JCLTU, C- negative Control i.e. Hexane, Std- Positive Control i.e. Jatropha oil

5.5 Thin Layer Chromatography (TLC)

The crude Hexane fraction of #33JCLTU was subjected to different combinations of solvents (Table 5) to achieve good separation. The best separation was achieved in Hexane: Ethyl acetate in the ratio of 90:10 which resolved the crude fraction into separate bands with R_f value 0.87 and 0.82 (Figure 4). Pure Jatropha oil was also subjected to TLC along with sample extract. Band no 1 was having same R_f value i.e. 0.87 similar to that of pure Jatropha oil.

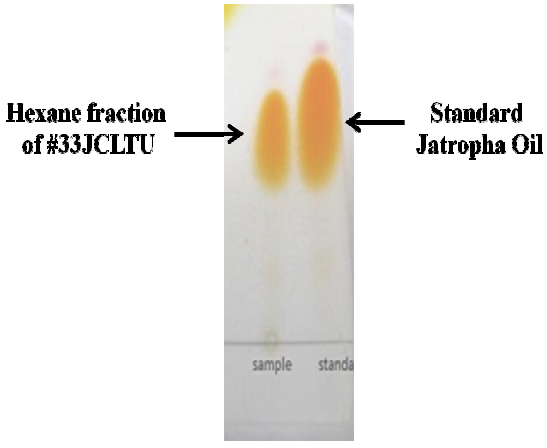

Figure 4 TLC of crude hexane fraction of #33JCLTU along with pure Jatropha oil

Solvent system	Ratio	Bands observed
Hexane: Ethyl Acetate: Glacial Acetic Acid	90:10:1	No Separation
Hexane: Ethyl Acetate	90:10	Two bands
Hexane: Ethyl Acetate	95:5	No Separation
Hexane: Chloroform	99:1	No Separation
Hexane : Chloroform	95:5	No Separation
Hexane : Chloroform	90:10	No Separation

Table 8: Different mobile phase used for TLC of crude Hexane fraction of #33JCLTU

5.6 HPLC of crude Hexane extract of #33JCLTU

The presence of oil in crude hexane fraction was further confirmed by comparing its HPLC spectra to that of HPLC spectra of pure Jatropha oil. In case of Jatropha oil, a single symmetrical peak at retention time of 35 min was observed. The crude hexane extract of #33JCLTU yielded many small peaks at different retention times but a single peak at retention time of 35 min similar to that of single peak in case of Jatropha oil at same retention time. Thereby confirming the presence of oil in the hexane extract of #33JCLTU. But as the peak height in case of fungal oil extract was very short indicating towards lower concentration of oil in the sample (Figure 5).

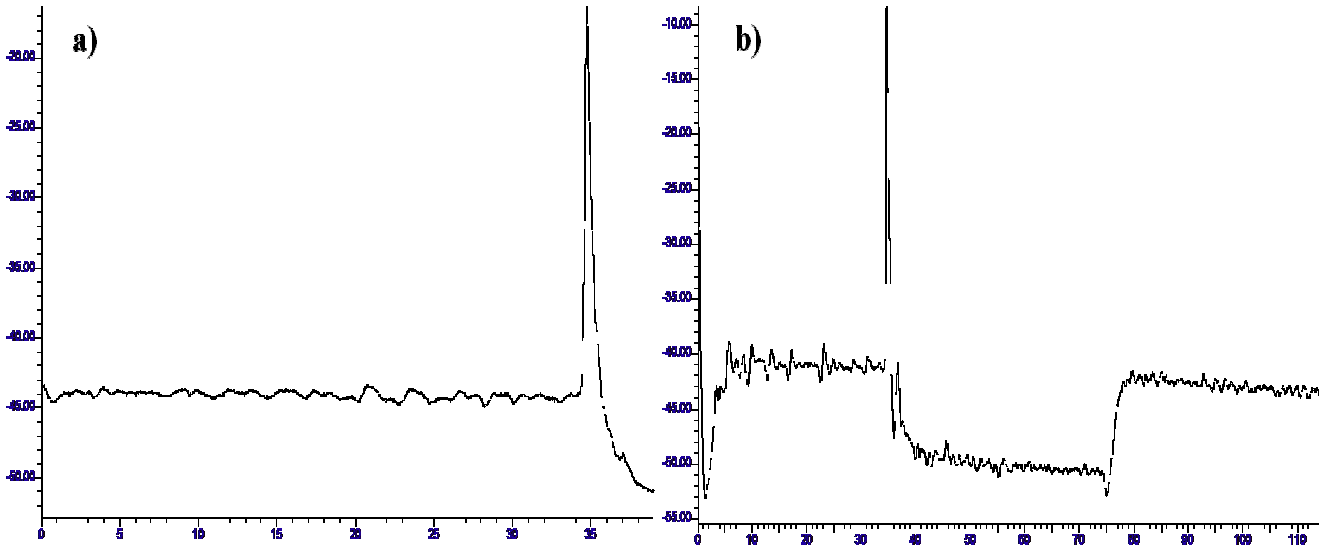

Figure 5: HPLC spectra of a) Standard Jatropha oil l b) crude hexane fraction of #33JCLTU

5.7 Identification of Endophytic fungi

5.7.1 Morphotaxonomy

Colonies over PDA medium fast gwowing, floccose, initially white laer turning to brown black colored (Figure 6a). Hyphae hyaline to brown, thick walled, septate, multinucleate and branched (Figure 6b). Conidia were borne over this branched hyphae. Conidia golden brown in color, large in size, thick walled, 6-8 septate, transverse and longitudinally septate with a prominent beak at the apex and tail at the end (Figure 6c-d). Based on these characters, the oil producing endophytic isolate was identified as *Alternaria* sp.

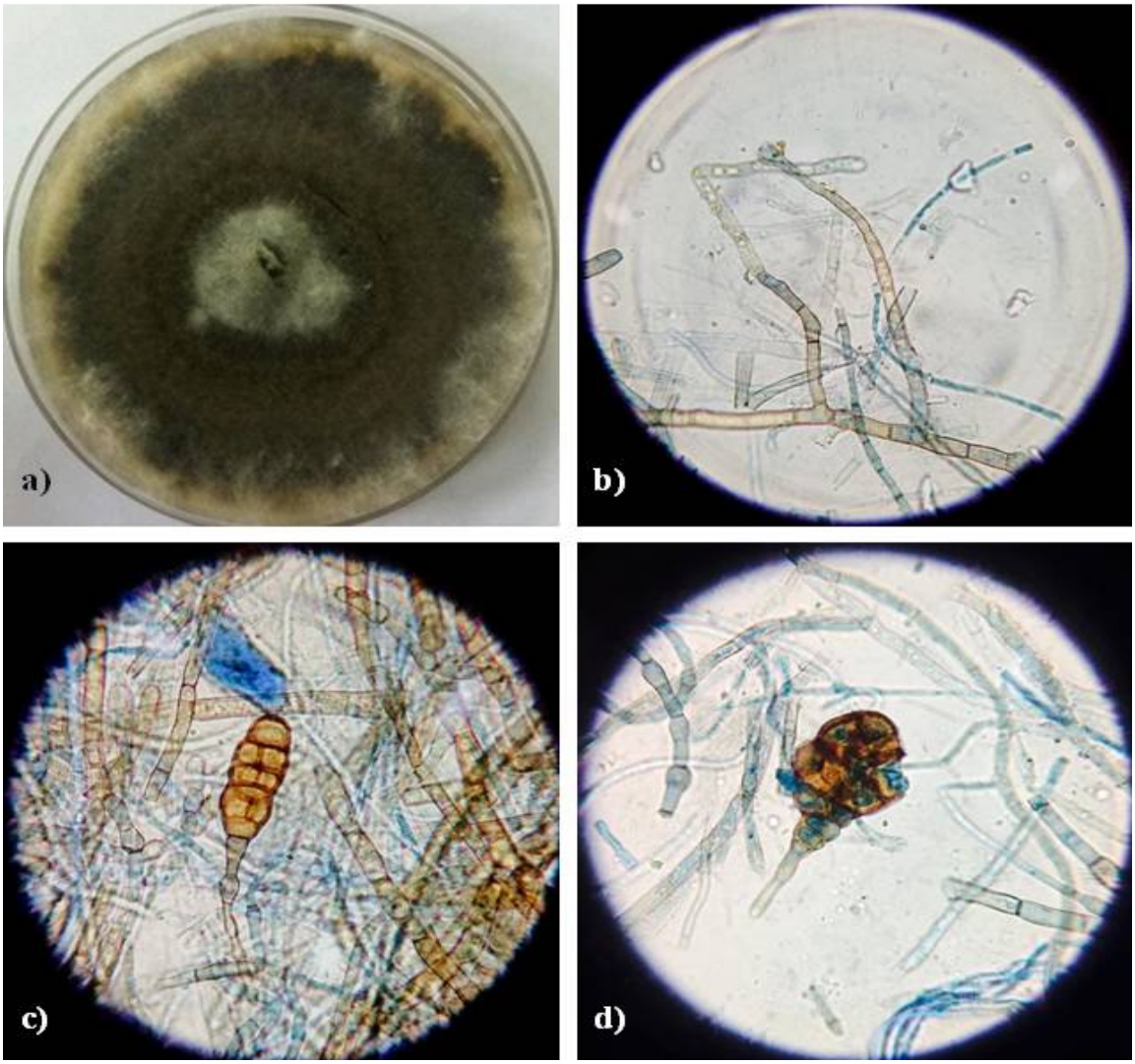

Figure 6: Morphological and Microscopic features of *Alternaria sp.* (#33JCLTU) a) colony characters over PDA medium b- d: Microscopic features over PDA b) hyphae c)-d) conidia produced over PDA medium.

5.8.2 Molecular identification

5.8.2.1 Genomic DNA isolation and PCR amplification

The concentration of genomic DNA (Figure 7a) oil producing endophytic fungi, #33JCLTU was estimated by taking the absorbance at 260 nm and the amount was 50ng/µl by using formula

$$\text{Concentration } (\mu g/ml) = O.D_{260\ nm} \times 50\ \mu g/ml \times \text{Dilution factor}$$

The PCR amplicon was resolved on 1.5 % agarose gel in order to check the size on the basis of the mobility and comparison with the 500 bp ladder. The size of the amplicon was found to be approximately 550 bp to 600 bp (Figure 7b). This size can be easily compared to the ITS region, which was amplified in order to characterize the fungi at molecular level.

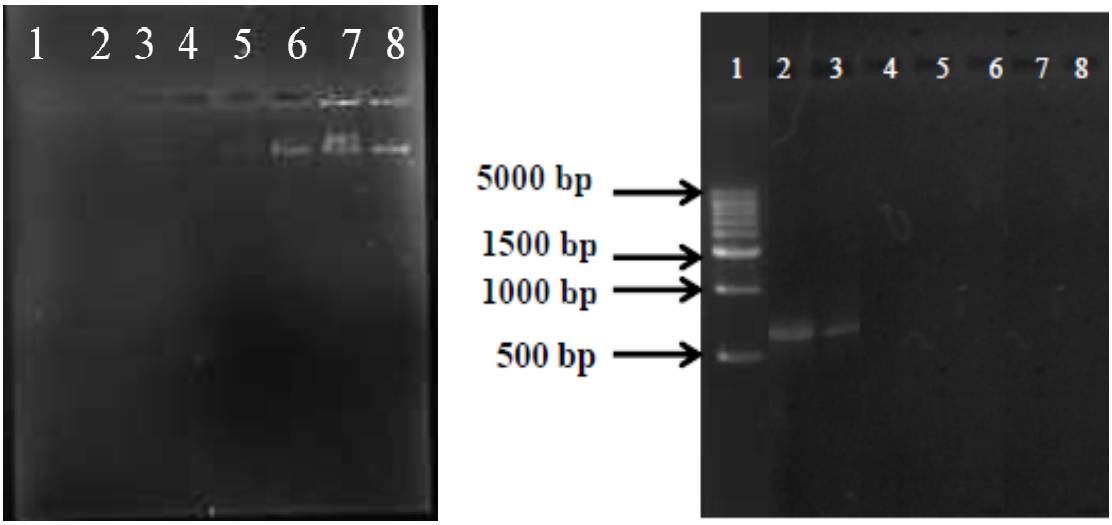

Figure 7: a) Genomic DNA isolation of #33JCLTU; Lane 6-8: Genomic DNA of #33JCLTU b) PCR amplicon of ITS region of #33JCLTU, Lane1- 500bp DNA ladder Lane 2: ITS amplicon, Lane 3-8: Blank

5.8.2.2 Sequencing and BLAST analysis

The PCR amplicon of ITS region of #33JCLTU was sent for sequencing and final sequence was subjected to homology analysis by subjecting the final consensus sequence to similarty search by using BLAST. The BLAST analysis showed 98 % sequence similarity with *Alternaria macrospora* followed by 97 % with *A. sesami, A. porri* and 96 % with *A. dauci, A. alli, A.zinniae* & *A. solani* (Table 6).

On the basis of morphological as well as molecular taxonomy, the potential isolate #33JCLTU was identified as *Alternaria macrospora*

S. no.	Name of Species	Accession Number	Query Coverage (%)	Sequence Similarity (%)
1	*Alternaria macrospora* B	DQ156342	99	98
2	*Alternaria macrospora* MKP3	KM514668	96	98
3	*Alternaria macrospora* MKP4	KM514669	95	98
4	*Alternaria dauci* C76.1	JQ936188	99	96
5	*Alternaria dauci*	EU781950	99	96
6	*Alternaria macrospora* C102	JQ936189	99	95
7	*Alternaria sesami* CNU104026	JF780940	95	97
8	*Alternaria sesami* EGS13.027	JF780939	97	96
9	*Alternaria porri* 00A14	JF710489	92	97
10	*Alternaria porri* 00A12	JF710497	93	97
11	*Alternaria alli* ALT35	DQ323683	95	96
12	*Alternaria alli* ALT34	DQ3223705	95	96
13	*Alternaria zinniae* AZ33	AY372682	96	96
14	*Alternaria zinniae*	AY445813	94	97
15	*Alternaria solani* RJT1	JQ625586	99	96
16	*Alternaria solani* MR12	JQ652580	99	96

Table 9: BLAST analysis of #33JCLTU

Conclusion

From the present study we conclude that endophytic fungi, *Alternaria macrospora* (#33JCLTU) isolated from *Jatropha curcus* produces oil similar to that of its host *J.curcus* as confirmed by biochemical and analytical techniques.

Further studies are warranted on mass production, purification and structure elucidation of oil producing endophytic fungi for their probable role in agrochemical Industries.

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