ISOLATION OF FUNGAL PIGMENTS AND EVALUATION OF THEIR TEXTILE DYEING EFFICACY

A Thesis

submitted in the partial fulfillment of the requirement for

the award of the degree of

MASTER OF SCIENCE

IN

MICROBIOLOGY

Submitted by

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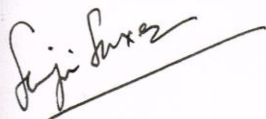

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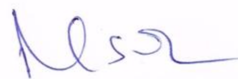

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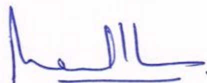

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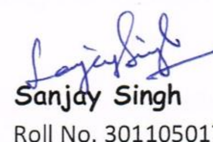

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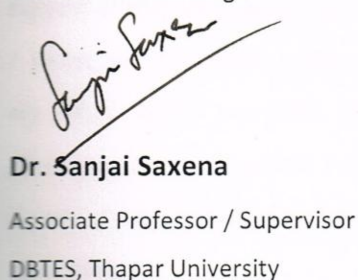

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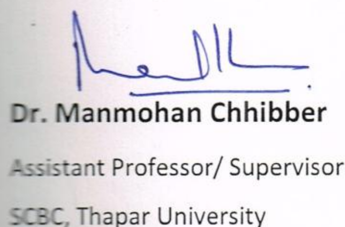

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This thesis is dedicated to my parents

For their endless love, support and encouragement

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ABBREVIATIONS

LD	lethal dose
Kg	kilogram
mg	milligram
PDA	potato dextrose agar
Sp.	species (singular)
Spp.	species (plural)
SSF	solid state fermentation
g	gram
L	liter
mg	milligram
rpm	revolution per minute
hrs	hours
ml	milliliters
i.e	that is
TLC	thin layer chromatography
UV	ultra-violet
R_f	retention factor
Cm	centimeter
min	minutes
pH	power of hydrogen
ISO	international organization for standardization
ITS	inter transcribed region
S	subunit
viz	namely

°C	degree Celsius
Psi	pound per inch square
mm	millimeter
%	percent
CDB	czapak dox broth
μl	microliter
KPa	kilopascal
EDTA	ethylenediaminetetraacetic acid
PCR	polymerase chain reaction
DCM	dichloromethane
NMR	nuclear magnetic resonance

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EXECUTIVE SUMMARY

In the current study, endophytic fungi were screened for the production of pigments and colored compounds exploiting them as textile dyes. The potential of endophytic fungi to produce pigments was assessed by growing them on a solid medium for prolonged duration. Isolates those were capable of growing on such medium with considerable production of pigments were then raised on submerged and solid state medium.

#4RSLBRT demonstrated maximum potential to produce pigments both on submerged and solid substrate. It produced red colored compound. Dyeing potential of the crude dye produced by #4RSLBRT on cotton swatches was assessed by dyeing process as per ISO standard. It gained 2 points on 5 point scale. Further various chromatographic techniques were used to isolate and purify the individual dye component and their tentative structures were deciphered through NMR and GCMS analysis. The ethyl acetate and diethyl ether fraction of the colored compound also possesses antibacterial activity against *Staphylococcus aureus* and *Staphylococcus epidermidis*. #4 RSLBRT was taxonomically identified as *Fusarium solani* using morphotaxonomy and molecular tools

Further studies on optimisation of extraction procedure as well as dyeing process would open the possibilities of using the fungal dyes at industrial scale.

Chapter 1

INTRODUCTION

1. INTRODUCTION

Pigments are coloured, organic or inorganic solid powders used for colouring fibers, plastics and other polymeric materials. They are generally insoluble and are not affected by the substrate/ medium in which they are incorporated. They alter appearance either by selective absorption, interference or scattering of light. Pigments are characterised by high tinting strength, photochemical strength, light and weather resistance, hiding power, flocculation resistance, bleed resistance, brightness and dispersibility, heat and solvent resistance. Flowers, corals, fungi and even animal skin contain pigments which give them their colours. Pigments may be natural, organic (*i.e.* containing carbon) or inorganic.

Natural pigments are produced by living organism like plants, animals, fungi and other microorganism. Pigments are produced by living system because of their metabolic and genetic background. The organic pigments are a group of colorants synthetically produced through complex carbon-containing chemistry involving various materials including petroleum, coal tar and natural gas. Organic pigments are generally brighter, stronger, and more expensive than their inorganic counterparts and range in color over entire visible spectrum. They are also less resistant to sunlight, humidity, and chemicals. Inorganic pigments are those that are found in nature or produced by synthesis. Commercially pigments are available in various forms including dry powders, thick paste, press cakes, granules and resin predispersed. Natural dyes and pigments have been known for their use in food, leather as well as fibres like wool, silk and cotton since prehistoric times.

However with the advent of widely available and cheaper synthetic dyes in 1856 with moderate to excellent color fastness properties, the use natural colorants having poor to moderate fastness properties declined to great extent. The development of synthetic dyes led to a more complete level of quality and more reproducible techniques of application. The new dyes, essentially pure chemical compounds, were more consistent and more brilliant and cheaper than the natural dyes previously used. These synthetic dyes have received faster acceptability due to its ease in dyeing, reproducibility and other factors. Their dominance continued for decades but received a severe blow when toxicological effects of synthetic dyes during use became more and more known and caused a great concern about the use of synthetic dyes. The increased application of synthetic dyes and colorants in textile industry and their inefficiency in dyeing result in large amount of dyestuff being directly lost to wastewater. The wastewater generated by the textile industry is classified as most polluting of all industrial sectors. The textile effluents contain xenobiotic compounds that are very recalcitrant to biodegradation processes and various colon carcinogens. Hence there is a

revival of interest towards the application of natural colorants on textile fibres due to worldwide environmental consciousness.

Natural dyes are non toxic, non-polluting and less health hazardous, moreover their antioxidant and antimicrobial effect adds to their positive effect (Sharma *et al*., 2011). Dyes from plant sources are in use since prehistoric times. Plant parts such as leaves, barks, roots and berries are used for extraction of dyes. Plant based dyes produce very uncommon, smooth and soft shades compared to synthetic dyes. Moreover they had the added advantage of biodegradability and compatibility with skin. Turmeric, the brightest naturally occurring yellow dye is a powerful antiseptic, while indigo gives cooling sensation. However the major disadvantage associated with natural dyes or pigments from plant sources is the magnitude of yield which makes cost of end product too high. Moreover for producing dyes on a large scale a huge number of plant sources will be required which would disturb the ecosystem.

To defeat this constrain, biological sources such as fungi, bacteria are being exploited for potential color production. Microbial dyes have added advantage over plant and animal based dyes as microbes are fast growing and have the potential of being standardised commercially. Fungus has been reported as potent source of pigments. Mushroom and lichens has been source of pigment for textile dyeing since ages. Mycelial extracts of mushrooms such as *Calvatia gigantean* gives red brown, *Bankera violascens* gives greens and *Agaricus arvensi*s gives blues. They have a tremendous potential for dying wool and silk fabrics. *Carvalaria lunata, Alternaria alternata, Trichoderma virens* produces pigments that can be used in textile industry (Sharma *et al*., 2011). Fungal pigments are basically secondary metabolites and therefore play no substantial role in cell growth or development. Secondary metabolites are compounds produced by an organism that are not required for primary metabolic process and involves mainly synthetic processes and is species-, often strain-specific. Secondary metabolites are produced when the cell is not operating under optimum conditions e.g. when primary nutrient source is depleted. In other words, secondary metabolism provides a route for the removal of intermediates which would otherwise accumulate and thus enables the primary processes leading to these intermediates to remain operational during the time of stress. Their activities cover a wide range of possibilities, including antibiotics, ergot alkaloids, naphtalenes, nucleosides, peptides, phenazines, quinolines, terpenoids and some complex growth factors which have attracted a huge commercial interest. The sources of secondary metabolites are plants and microbes. Among microbes, fungi provide a vast myriad of secondary metabolites that have an ecological role in regulating their interactions with the immediate environment.

Endophytic fungi are an important group of microorganisms which reside within the healthy tissue of the plant without providing any obvious clue of their existence and are hypothesized to be providing protection to host plant from the attack of herbivorous insects or vertebrate grazers by production of bioactive compounds (Strobel *et al.,* 2003; Tan and Zou, 2001). Fungal endophytes have been recognized as a repository of novel secondary metabolites, some of which have beneficial biological activities (Bills and Polishook, 1991; Strobel and Daisy, 2003). Endophytic fungi have been isolated and screened for exploration of an array of bioactive compounds like antimicrobials, antivirals, anti-oxidants and immunosuppressive agents. With this background, it can be deduced that endophytic fungi offer themselves to be potential sources of myriad of secondary metabolites. Limited information exists on pigment production from endophytic fungi. Endophytic fungi thus represent an unexplored biological resource for screening pigments for possible use as a textile dye compared to other natural dyes.

Chapter 2

REVIEW OF LITERATURE

2. REVIEW OF LITERATURE

2.1. Synthetic dyes: A global concern

Colorants (dyes and pigments) are important industrial chemicals. According to the technological nomenclature, dyes are colorants which are soluble in medium in which they are added whereas pigments are insoluble in the medium to which they are added. Mauevin, world's first commercially successful synthetic dye was discovered serendipitously in 1856 by William Henry Perkin. Since then, more than 100000 new synthetic dyes have been discovered, textile industries are the biggest consumers of the total dyestuff market (Asad *et al.*, 2007). With the worldwide expansion of the textile industries, there is significant increase in the consumption of the synthetic dyestuff, resulting in rise in environmental pollution due to the contamination of wastewater with these dyestuffs (Pandey *et al.*, 2007; Saratele *et al.*, 2011).

A survey carried out by Ecological and Toxicological Association of the Dyestuffs (ETAD) showed that of a total of approximately 4,000 dyes that had been tested, more than 90% showed LD_{50} values above 2×10^3 mg/kg, the most toxic being in the group of basic and direct diazo dyes (Shore, 1996; Robinson *et al.*, 2001).

2.1.1 Azo dyes

Azo dyes are aromatic compounds with one or more nitrogen-nitrogen (-N=N-) double bonds. Monoazo dyes have only one N=N double bond, whereas diazo and triazo dyes contains two and three N=N double bonds. Azo dyes are diazotised amines linked to benzene and naphthalene rings (Zollinger, 1991). The colour and shades produced by azo dyes is attributed to their side chain (McCurdy, 1992). According to Ngwasiri *et al.*, (2011) azo dyes are xenobiotic and recalcitrant in biodegradation process, causing environmental problems. Azo dyes in the effluent are not degraded by conventional treatment methods (Nam & Reganathan, 2000). Some azo dyes are known to cause bladder cancer, splenic sarcomas and hepatocarcinomas (Chequer *et al.*, 2011). Others induce liver nodules in experimental animals and incidences of bladder cancer have been seen in workers exposed to these dyes. Toxic effluents containing azo dyes discharged from various industries adversely affect water resources, soil fertility, aquatic organism and ecosystem integrity (Puvaneswari *et al.*, 2006).

Al-Sabti (2000) reported that Azo dye has clastogenic activity, a potent risk factor for the development of genetic, teratogenic or carcinogenic diseases in fish populations, which could have disastrous effects on the aquatic ecosystem.

2.1.2 Anthraquinone dyes

Anthraquinone dyes are the second most important class of dyes after Azo dyes, and constitutes a little over 50% of the commercial colorants.. Anthraquinone dyes are extensively used in textile dyeing because of superior brightness, good light-fastness properties, wide array of shades, ease of application and minimal energy consumption (Siddiqui *et al*., 2010). Anthraquinone dyes due to presence of fused aromatic are resistant to degradation (Banat *et al*., 1996). According to Lu *et al.,* (2008) most of these dyes are toxic, Carcinogenic and mutagenic. Pandey *et al.,* (2007) reported that release of dye-containing effluents into the environment is undesirable, not only for affecting the aesthetics, the water transparency and the gas solubility of water bodies, but also because many of these compounds and their breakdown products are toxic, mutagenic or carcinogenic

Carcinogenic effect of the anthraquinone derivative Disperse Blue 1, an agent causing induction of rat bladder tumours, was reported by Atkins, (2000). This compound was also found to be mutagenic in bacteria and thus the possibility of a genotoxic mechanism of cancer induction cannot be excluded.

2.2. Natural colorants:

Natural colorants are the term used for all the dyes and pigments derived from plants, insects, and minerals i.e. derived from natural resources. The dyeing with natural colorants was one of the oldest techniques and is practised since Bronze Age (Swami *et al.*, 2012). Historians believe red iron oxide, from red dirt, was one of the first natural dyes used. Natural dyes are mostly non-substantive and must be applied on textiles by the help of mordants, usually a metallic salt, having an affinity for both the colouring matter and the fibre. The metallic mordants combines with dye in the fibre and forms an insoluble precipitate and thus both the dye and mordant get fixed to become wash fast to a reasonable level. A worldwide interest in textile dyeing using natural dyes is generated as a result of increasing awareness associated with environment and health hazards associated with the synthesis, processing and use of synthetic dyes.

2.2.1. Advantages of natural dyes/colorants

A wide range of soft, lustrous, soothing shades are produced by the natural dyes by small variations in mordant and dyeing conditions. The raw materials for natural dyes are renewable as well as biodegradable thereby reducing consumption of fossil fuel (petroleum) based synthetic dyes. Moreover they have added advantage of having constituents that are

anti-allergens and gives cooling sensation hence prove safe for skin contact and are mostly non-hazardous to human health.

2.2.2 Limitations of natural dyes/ colorants

Reproduction of same shades and process standardisation using natural dyes is nearly impossible. A low yield from agro sources makes the price of end product too high for bulk use. Moreover Lack of precise technical knowledge on extraction and dyeing techniques limits the use natural dyes.

2.3 Dyes/pigments from plant sources:

In the recent past there has been growing interest in revival of the natural dyes due to their wide availability and growing concern among environmentalists in protecting environment from indiscriminate exploitation and pollution by dyeing industries. For the last one decade or more research has been carried out to explore the hidden component found in plants which could be used as dye alternative to synthetic one.

Gulrajani in 2001 demonstrated possibilities of nylon dyeing by using vegetable dyes like Annatto, Ratanjot and Berberine. Evaluation of color fastness of dyed cloths and colour value were also studied. Wash fastness was good for Rajantot, very good for Annatto and poor for Berberine. Light fastness values were included. Nylon could be dyed with these vegetable dyes at pH 4 (Rajanjot), 6 (Annatto) and 9 (Berberine). The results noted the Anlab Colour Space plots for each dye prepared by plotting their a* b* values. K/S values were discussed and the percentage dye exhaustion was also reported.

Gupta *et al*., (2012) showed that the ethanolic extract of *Sesbania aculeata* yields a range of camouflage shades. The dyed samples showed very good fastness properties. The plant of *Sesbania aculeata* is easy to tend and maintain, hence the plant economics is not high. It was thus observed that varied range of camouflage shades were obtained by change of mordants on dyeing with ethanolic extract of *Sesbania aculeata*. Usually tans, dark shades were obtained through sulphates and chromes while chlorides and alum gave lighter tints.

Mongkholrattanasit *et al.,* (2013) described that Silk fabrics dyed with Eucalyptus leaf extract, quercetin, rutin, and tannin using the pad-batch method show higher color strength than those dyed with the pad-dry technique. Tannins are considered as a main colorant in

dyeing processes because of the shade similarities of Eucalyptus leaves. The color fastness to washing showed very good results, whereas the color fastness to rubbing was fair to good, except for silk fabric dyed with tannin mordanted with Eucalyptus with ferrous sulfate, where ratings were poor when subjected to wet rubbing. The light fastness rating of the silk fabric mordanted with ferrous sulfate showed a fair to good result, but in the case of the silk fabric dyed with quercetin without mordant, the fastness rating was poor. The application of natural dyes to silk fabric by the pad-batch technique can be considered to be an effective eco-option; hence this technique could be considered to be the most suitable for small scale industry and cottage dyeing.

Grover *et al.,* (2005) for the first time evaluated dyeing potential of flower of Jatropha plant. The colour shades, mordant wise colour change and fastness to washing, rubbing and perspiration were studied. The results revealed that the flowers of this plant have good dyeing potential with satisfactory fastness to light, washing, crocking and perspiration on silk.

Sl.No	**Source**	**Dye/colorant**	**References**
1.	Safflower flower buds	Red dye	Kusaka *et al*., (1994)
2.	Turmeric	Yellow	Teli *et al*., (1994)
3.	Madder	Red, Brick red	Zou, (1999)
4.	Pomegranate	Yellowish, Brown	Ansari and thakur, (2000)
5.	Catechu	Red	Bhattacharya, (2000)
6.	Eupatorium leaves	Greenish	Bansal and Sood, (2001)
7.	Indian madder	Red , Brick red	Gupta, (2001)
8.	*Eclipta prostrate*	Yellowish green	Devi *et al*., (2002)
9.	Henna	Red	Dweck, (2002)
10.	Safflower	Red	Park *et al*., (2002)
11.	Parthenum leaves	Yellow	Suneta & Mahale, (2002)
12.	Turmeric	Yellow	Tawfik, (2002)
13.	Dyer’s chamomile	Yellow	Bochnaan and Weiser, (2003)
14.	Mango bark	Yellow	Bains *et al*., (2003)
15.	Banana flower petaloide	Yellow	Mathur and Gupta, (2003)
16	Neem	Brilliant Green	Mathur *et al*., (2003)
17.	Red sandalwood	Red	Samanta *et al*. (2003)

18.	Marigold flowers	Yellow	Sarkar *et al*., (2005)
19.	*Rubia tictorium L.*	Red	Goodarzian and Ekrani,(2010)
20.	Walnut	Red , Brick red	Grover and Sharma, (2011)

Table No.1 List of Dyes/colorants produced by plants

2.4 Dyes/pigments from bacterial sources:

Although there are number of natural dyes and pigments are available from plant sources, but only a few can fulfil the need of commercial availability that can be. Inspite of availability of large number of plant pigments, there is growing interest in microbial pigments due to several reasons like their natural character and safety to use, production being independent of seasons and geographical conditions, controllable and predictable yield (Francis, 1987). Bacteria produce pigment as part of their normal metabolism. A wide variety of bacterial taxa, including *Serratia rubidaea, Vibrio gazogenes, Alteromonas rubra, Rugamonas rubra, Streptoverticillium rubrireticuli* and *Streptomyces longisporus ruber* produces prodigiosin (a red color pigment) and/or derivatives of this molecule (Austin and Moss, 1986). According to Joshi *et al.* (2003), bacteria are potent source of pigments, which are mostly Carotenoids, especially β- carotene in nature. Lycopene, zeaxanthine and lutein are reported to be produced by different bacterial species.

Bacteria	Colour	References
Flavobacterium sp.	Yellow	Shepherd *et al*., (1976)
Agrobacterium aurantiacum	pink-red	Yokoyama *et al*., (1994)
Paracoccus carotinifaciens	Pink-red	Tsubokura *et al.,* (1999)
Bacillus species	Brown	Joshi *et al*., (2003)
Brevibacterium sp.	Orange , yellow	Joshi *et al*., (2003)

Rhodococcus maris	Bluish red	Joshi *et al*., (2003)
Pseudomonas sp.	Yellow	Joshi *et al*.,(2003)
Streptomyces sp.	Yellow, Red ,Blue	Joshi *et al*., (2003)
P. tunicata	Yellow	Choi *et al*., (2005)
S. marcescens	Red	Enomoto *et al*., (2011)
Alteromonas luteoviolacea	Violet	Enomoto *et al*., (2011)
Streptomyces shaanxiensis sp	Blue	Fang *et al.,*(2012)

Table No. 2 List of bacteria producing dye/pigment

2.5 Fungus: A potent source of colorants for textile dyeing

Fungus is a significant source of diverse secondary metabolites. Hamlyn (1995) reported the importance of pigments such as anthraquinone, anthraquinone carboxylic acids, pre-anthraquinones extracted from filamentous fungi. These compounds have been identified as their secondary metabolite. Microbes, particularly fungus have recently been in focus as potential source of natural pigments (Mapari *et al*., 2005).

Mapari *et al.*, (2005) described Ascomycetes and bacidiomycetes as a potent source of range of colours that include several chemical classes of pigments. Mushrooms and lichens have a rich history as diverse source of pigments. Mycelial extracts of some mushrooms such as *Chroogomplus vinicolor* gives red tints, *Bankera violascens* gives greens and *Collybia iocephala* gives blues. They have a tremendous potential for dying wool and silk fabrics. However, such fungi are difficult to grow under lab conditions and therefore are not suitable for large scale industrial productions. On the other hand ascomycetous fungi are more suitable for lab scale production because they can be grown in a relatively easier way to give high yields. Food colorants from ascomycetous fungi have been explored with few successful

attempts. Carotenoids such as β-carotene and lycopene have been known to be produced by fungal cell factories.

A fungus producing magenta pigment was isolated from cellulosic material by visual observation on Czapek's agar media. The fungal strain that produced magenta pigment was closely related to *Phoma herbarum*. Mycelia attached to the surface of nylon-6 and excreted magenta pigment into the fibers. The pigment structure was partially determined. This is the first report of the production of magenta pigment by a microorganism specifically in the presence of nylon-6 fibers, via an unknown mechanism. This phenomenon suggests that fabrics can be dyed using microorganisms (Chiba *et al*., 2006)

Sharma *et al*., (2012) found three fungi, namely *Curvalaria lunata, Trichoderma virens* and *Alternaria alternata* that produce pigments that can be used as textile dyes. These dyes gave good rub and wash fastness when applied to wool and silk. Further these dyes have no adverse effect on tensile strength of fabrics. Even pigments are found to be non toxic to human skin.

Eleven fungal strains were tested for their ability to produce brown and reddish brown textile dyes using H-acid (1-naphthol-8-amino-3, 6-disulfonic acid) as a dye precursor in the fermentation medium. All tested fungal strains exhibited high ability to produce dyes varying in both dye colour (brown to reddish brown) and fastness properties to washing, perspiration and UV light. The produced dyes were subjected to further analysis for quantitative determination of dye components for investigation of their inter-relations as well as their role in dye colour and stability (Atalla *et al.*, 2010).

Sl.No.	Fungus	Colour/Appearance	References
1.	*Paecilomyces sinclairii*	Red	Cho *et al*., (2002)
2.	*Mucor circinelloides*	Orange-yellow	Iturriaga *et al*., (2005)
3.	*Ashbya gossipy*	Yellow	Laurent Dufosse., (2006)
4.	*Penicillium. Cyclopium*	Reddish-brown	Mapari *et al*., (2009)
5.	*Fusarium. Venenatum*	Red	Mapari *et al*., (2009)
6.	*Fusarium. Oxysporum*	Orange	Mapari *et al*., (2009)
7.	*Penicillium purpurogenum*	Red	Mendez *et al*., (2011)
8.	*Aspergillus sp.*	Orange-red	Malik *et al*., (2012)

9.	*Aspergillus galucus*	Dark -red	Malik *et al*., (2012)
10.	*Blakeslea trispora*	Cream	Malik *et al*., (2012)
11.	*Helminthosporium catenarium*	Red	Malik *et al*., (2012)
12.	*Helminthosporium avenae*	Bronze	Malik *et al*., (2012)
13.	*Penicillium cyclopium*	Orange	Malik *et al*., (2012)
14.	*Haematococcus Pluvialis*	Red	Malik *et al*., (2012)
15.	*Monascus roseus*	Orange-pink	Malik *et al*., (2012)

Table No. 3 List of colours produced by fungal sources

2.5 Screening of producing fungi

Screening for pigment or colorant producing fungi is done by growing the fungus on PDA medium and looking for potential pigment producers (Velmurugan *et al.,* 2009).Fungi grows luxuriously on the medium until all the nutrients are provided. Once the supply of vital nutrients gets depleted, part of mycelium may switch biochemical activity to pathway of secondary metabolism. Rather than producing new building block materials the fungi starts producing other compounds. Fungal pigments are produced in this way and one species may contain a mixture of several different fungi. *Fusarium spp*. (Red pigment), *Penicillium spp*. (yellow pigment), *Isaria spp*. (reddish brown pigment) produces color on PDA plates (Velmurugan *et al.,* 2009). More recently Sharma *et al*., 2011 screened *Tricoderma virens*, *Curvularia lunata* and *Alternaria alternate* by growing them on PDA plates

2.6. Production of pigment/colorant production via different fermentation procedures

Fermentation is a process very much similar to anaerobic respiration and is carried out in a mixture of nutrients and metabolites essential for the growth and reproduction of the microbe. In the course of this process, microbes also release several additional compounds apart from the usual products of fermentation, such as carbon dioxide and alcohol. These additional compounds are called secondary metabolites. Submerged fermentation utilizes free flowing liquid substrates, such as molasses and broths. The bioactive compounds are secreted into the fermentation broth. The substrates are utilized quite rapidly; hence need to be constantly replaced/supplemented with nutrients. An advantage of this technique is that purification of products is easier. Submerged fermentation is primarily used in the extraction of secondary metabolites that need to be used in liquid form. Solid-state fermentation (SSF) is defined as the fermentation process in which microorganisms grow on solid materials

without the presence of free liquid. The aim of SSF is to bring cultivated fungi or bacteria in tight contact with the insoluble substrate and to achieve the highest nutrient concentration from the substrate for fermentation (Bhargav *et al.*, 2008). Wheat bran amended with 10 mL of mineral salt solution containing (g/L) KH_2PO_4, $MgSO_4$, $NaCl_2$ and $MnSO_4$ has been used by Syed *et al*., (2012) for amylase production using *Aspergillus niger*-ML-17.

Dikshit and Tallapragada in 2011 reported unpolished rice, the best source as solid substrate for natural pigment production from *Monascus. sp***.** Homogenous spore suspension prepared by taking full loop of sporulated (6-day old) agar slope culture diluted in distilled water was used as inoculum. The use of rice for solid state fermentation for production of pigment from *Monascus purpureus* was further mentioned by Kaur *et al*., (2009).

2.7. Pigment/colorant extraction

Solid liquid extraction can be used for extraction of metabolites produced by fungal source on solid substrate. Solid liquid Extraction is withdrawing of active agent from a solid mixture with a liquid solvent. By intensive contact the active agent transfers from solid (raffinate) to solvent (extract). Dikshit and Tallapragad (2011) used this process for extraction of natural pigment from solid substrate. For pigment extraction solid substrate, the culture medium was dried at 50°C for 48 hours. One gram of fermented solid substrate was taken for pigment extraction using 10 ml of 95% ethanol and shaking it on a rotary shaker at 200 rpm for 24 hrs. The extracts were allowed to settle at room temperature and then filtered through Whatmann filter paper.

2.8 Isolation and purification of pure dye compound

Thin layer chromatography and column chromatography is used for identification and purification of pure dye compound.

2.8.1 Thin layer chromatography

TLC is simple, quick, and inexpensive procedure that gives a quick answer as to how many components are in a mixture. TLC is also used to support the identity of a compound in a mixture when the R_f of a compound is compared with the R_f of a known compound (preferably both run on the same TLC plate). A TLC plate is a sheet of glass, metal, or plastic, which is coated with a thin layer of a solid adsorbent (usually silica or alumina). A small amount of the mixture to be analyzed is spotted near the bottom of this plate. The TLC plate is then placed in a shallow pool of a solvent in a developing chamber so that only the very bottom of the plate is in the liquid. This liquid, or the eluent, is the mobile phase, and it

slowly rises up the TLC plate by capillary action. As the solvent moves past the spot that was applied, equilibrium is established for each component of the mixture between the molecules of that component which are adsorbed on the solid and the molecules which are in solution. In principle, the components will differ in solubility and in the strength of their adsorption to the adsorbent and some components will be carried farther up the plate than others. When the solvent has reached the top of the plate, the plate is removed from the developing chamber, dried, and the separated components of the mixture are visualized. If the compounds are colored, visualization is straight forward. Usually the compounds are not colored, so a UV lamp is used to visualize the plates

TLC was used by several workers to identify different colours components in natural dyes to be applied on textiles (Samanta and Agarwal, 2009). Sharma *et al.*, 2011 performed paper chromatography using solvent Butanol: glacial acetic acid: distilled water (12: 3: 5) and reported that all the three fungal pigments are multi-component with different R_f values. Protein and lipid components of the fungal dye were also analysed by using different solvent system using TLC.

TLC of crude pigment isolated from *Rhodotorula glutinis* using petroleum ether: acetone (80:20) as mobile phase revealed the presence of three major pigments. The three fractions were yellow, orange, red in colour respectively (Latha and Jeevaratnam, 2010).

2.8.2 Column Chromatography

Column chromatography is a conventional technique, practised universally due to simplicity of its operation. It is carried out in glass columns packed with a stationary phase like the Silica gel. The packing is carried out by two methods- the slurry packing and dry packing (Salituro and Dufresne, 1998). The sample is applied to the top of the column bed and the components are eluted with a mobile phase, which percolates the column under gravitational influence. Step gradients are generated by preparing a range of mobile phases with varying rations of polar and non-polar solvents. In open column chromatography step gradients are most often used, as they are simplest to generate and produce excellent results if the solvent composition is appropriate. The elutes are collected as fractions for subsequent analysis (Liteanu and Gocan, 1974). Column chromatography remains a method of choice among the natural product chemists or the phytochemist.

The pigments from the red yeast *Rhodotorula glutinis* DFR-PDY were extracted with acetone, transferred to petroleum ether and fractionated on magnesium oxide-Hyflo Super Cel (1:2, w/w). A major red coloured fraction was adsorbed on the column under these conditions, while the other pigments were eluted by the developing solvents (petroleum ether, ethyl ether and methanol). The yellow was eluted first by petroleum ether, followed by orange fraction by ethyl ether and methanol (10:1) (Latha and Jeevaratnam, 2010).

Silica gel column chromatography was selected to purify capsicum red pigment, elution conditions were optimised by TLC. Concentrated extract was injected into the column 100 cm in height and 10 cm diameter and was eluted by petroleum-ether: 90% ethanol (2:1) as developer. Afterward several parts of solution were gained and red part was collected and vacuum concentrated to relatively pure red pigment (Chen and Wu, 2009**)**.

2.9. Dyeing process

Dyeing can be carried out in an alkaline bath, acidic bath or in a neutral bath. There are various reports available on different methods of mordanting on different fibers such as cellulosic, protenic and synthetic for dyeing with different natural dyes. Dyeing of cotton and silk with babool, tesu, manjistha, heena, indigo, mariegold etc is already reported (Gulrajani *et al.*, 1992).Various kinds of shades like black to brown, green to yellow to orange, etc can be obtained by application of different mordants.

Dyeing process using natural dyes varies depending upon the source of dyes. Before dyeing, the cloth needs to be scoured, breached or treated chemically by different methods. Thus source wise and state wise, different dyers are performing natural dyeing of silk, cotton and wool from long time and have derived special technique for obtaining the desired shade (Samanta and Agarwal, 2009).

Atalla *et al.*, (2010) for dyeing using fungal dyes prepared dye bath at a liquor ratio 50:1 and with 1 g/L amphoteric levelling agent pH was adjusted to pH 3.0. Dyeing was started at 50°C for ten min and then the dye bath temperature was raised to boil over 30 min and the dyeing continued for 45 min. After dyeing the temperature was lowered to 60 °C then the dye samples were rinsed and washed off in an aqueous solution 2 g/L non-ionic detergent at 60°C using a liquor ratio 50:1 for 30 min then rinsed and dried.

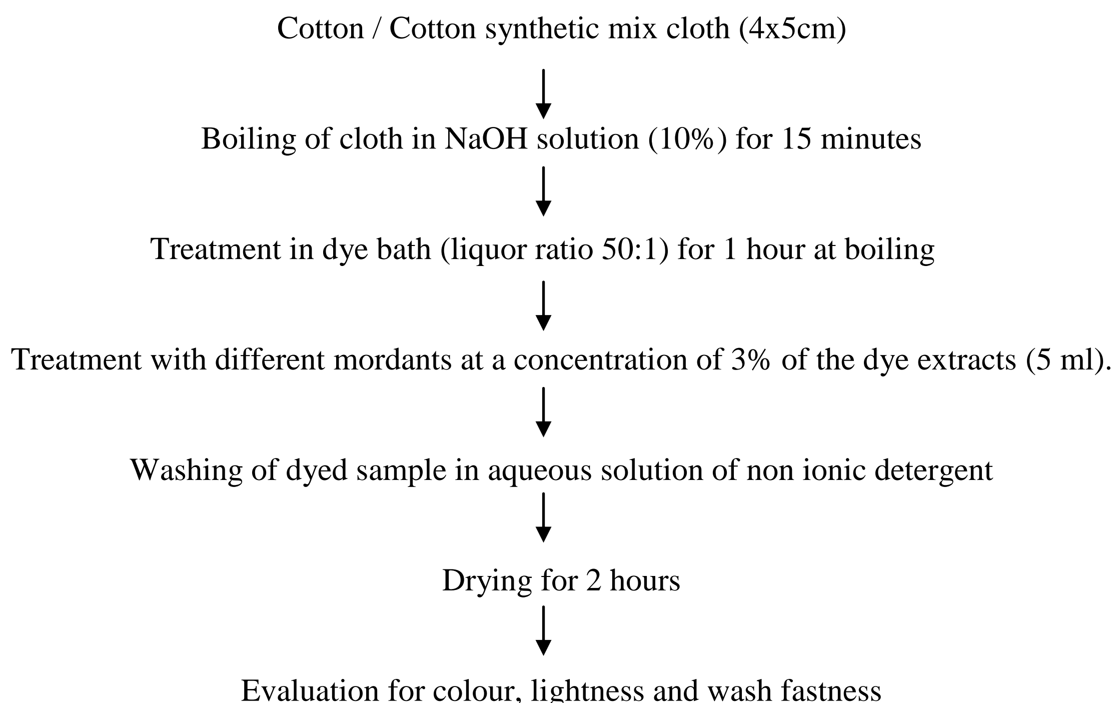

Figure 1: Flow chart showing dyeing procedure

2.10 Colour fastness properties of natural dyed fabrics

Colour fastness refers to the resistance of colour to fade or bleed of a dyed or printed textile materials to various types of influences e.g. water, light, rubbing, washing, perspiration etc. to which they are normally exposed in textile manufacturing and in daily use. The colour fastness is usually rated either by loss of depth of colour in original sample or it is also expressed by staining scale.

2.10.1 Light fastness

Most of the natural dyes have poor light stability and hence the colour of dyed textile is different from original colour (Padfield and Landi, 1966). Light fastness of many natural dyes particularly those extracted from flower petals are found to be poor to medium as reviewed by Samanta and Agarwal, (2009).Most of natural dyes are mordent based dyes and requires mordants to improve the fastness properties. However dyes isolated from fungal sources are found to have good light fastness properties (Atalla *et al.*, 2010)

2.8.2 Wash fastness

Duff *et al*., (1977) with a view to study the wash fastness carried out tests under standard conditions (at 50ºC and 20ºC) with washing formulation used in conservation work for restoration of old textiles. There were marked changes in the hue of some dyes on washing due to the presence of even small amount of alkali in washing solution highlighting the importance to know the pH of alkaline solution used for cleaning of textile dyed with natural dyes. As per ISO-II test natural dyes show moderate wash fastness to wool.

The dyes produced by three fungal isolate viz. *Curvalaria lunata, Trichoderma virens* and *Alternaria alternata* have good wash fastness property (Sharma *et al*., 2012).

2.10.3 Rub fastness

Rub fastness of most of the natural dyes have been found to be moderate to good and does not require any treatment (Samanta anf Agarwal, 2009). Dyes extracted from Jackfruit wood, manjistha, red sandal wood, babool, mariegold etc have good rubfastness (Samanta *et al*., 2006). Sharma *et al*., (2012) found in their study that fungal dyes has excellent rub fastness properties.

2.11 Identification of the fungi

The identification of the fungi which are the potential pigment producers could be done by morphological and molecular techniques. Morphological characterisation is done by microscopic study of the fungal structures such as mycelia, fruiting bodies. Fungal taxonomy is based upon comparative morphological feature (Sette *et al*., 2006) .Various optimization of growth conditions have been used in case of the fungi which are non-sporulating in the culture as they cannot be identified by the conventional techniques, so different media such as Potato dextrose agar (PDA), Water agar (WA), Potato Carrot agar (PCA), Corn meal agar (CMA) have been used in which the fungi sporulate. (Guo *et al*., 2000)

In contrast, molecular techniques exhibit more sensitivity and specificity for identifying microorganisms and can be used to classify the microbes on the diverse hierarchical taxonomic levels (Sette *et al*., 2006). Most of the fungi are being identified and detected by the comparative analysis of ribosomal DNA sequences especially ITS region. Harney *et al*., (1997) identified arbuscular mycorhizzal fungi from *Artemesia californica* using the ITS (internal transcribed site) region. The 5.85 S ribosomal gene flanking region ITS 1 and ITS 4 have also been used extensively to identify the fungi. Ezra *et al*., in 2004 used ITS primers ITS 1 and ITS 4 for identification of a new endophytic isolate of *Muscodor*

albus. Thirty-four representative isolates of various morphological endophytes were identified by DNA sequence analysis using the whole ITS region, including ITS 1, 5.8S, and ITS 2. (Hyde *et al*., 2008).

3.1 Aim of the Study

The current study was subjected towards the isolation of fungal pigments and evaluation of their textile dyeing efficacy.

The objectives of the current study were:

1. Screening of fungi producing extracellular pigments on solid media.
2. Isolation and purification of dyes.
3. Characterization and evaluation of textile dyeing efficacy.

Chapter 4

MATERIALS AND METHODS

4. MATERIALS AND METHODS

4.1 Sub culturing and preservation of endophytic fungi

This involves preparation of media and glasswares, pure culturing of the fungal isolates and preservation of the endophytic fungi.

4.1.1 Preparation of Potato Dextrose Agar (PDA) plates

39.0 g of PDA (Hi Media) was dispensed in liter lukewarm single distilled water and stirred thoroughly. This was then dispensed in 250 ml Erlenmeyer flasks and autoclaved at 121°C, 15 psi for 15 min. Glass Petri plates were sterilized at 121 °C, 15 psi for 20 min. Then under sterile conditions 25 ml of autoclaved PDA was dispensed in sterile 90 mm plates and allowed to solidify at room temperature. The plates were stored in incubator at 26 ± 2°C till further use (Agarwal and Hasija, 1986).

4.1.2 Sub Culturing

The fungi were transferred from the stock culture tube on to fresh PDA plates to get pure isolates. The plates were sealed and incubated at 26 ± 2° C for next 7 days. All procedures were carried out aseptically under laminar air flow hood.

4.1.3 Long term preservation of endophytic fungi

The pure fungal isolates thus obtained were transferred to PDA slants supplemented with 10% glycerol and streptomycin (1 mg/ml). The fungal cultures were successfully maintained as spawn culture and paper culture for 6 months.

4.2 Preliminary screening for fungal pigment production

Preliminary screening for pigment producing fungi was done by growing the fungus on PDA medium and looking for potential pigment producers. 5 mm agar plug of the test culture was placed over PDA plate. The plates were sealed and incubated at 26± 2°C for next 10 days with 12 hours of light period. The plates were then visually observed for pigment production.

4.3 Production of fungal pigments using submerged and solid state fermentation

The fungal isolates which produced pigment during primary screening were then subjected to submerged and solid state fermentation over. The submerged fermentation was carried out in Czapak Dox broth (CDB) (Atalla *et al*., 2010). Mycelia plug of 7 day old fungal culture was inoculated in 100 ml pre sterilized CDB aseptic conditions. The flasks were then incubated over orbital shaker at 120 rpm at 26°± 2°C for 3 weeks for color production (Atalla *et al*., 2010). The mycelia growth was monitored in terms of colour and morphology along with the change of colour of the medium.

Sl.no	Composition	Quantity (g)
1.	Sucrose	30.00
2.	Sodium nitrate	2.00
3.	Dipotassium phosphate	1.00
4.	Magnesium Sulphate	0.50
5.	Potassium chloride	0.50
6.	Ferrous Sulphate	0.01
Final pH (at 25°C)		7.3 ± 0.2

Table No 4. Composition of Czapek Dox broth

Local unpolished rice was chosen as substrate of choice for pigment production using solid state fermentation method. 20 g of substrate was placed in a tissue culture bottle to which distilled water was added and autoclaved at 121°C for 20 minutes. After cooling, the medium was inoculated with 5 ml ($1x10^6$ spores/ ml) of spore suspension and incubated at 28 ± 2°C for 20 days for pigment production.

4.4 Isolation and extraction of fungal pigments

Fungal pigment produced by solid state fermentation was isolated by drying the medium at 50°C for 2 hours. Then 200 ml of ethanol was poured and the tissue culture bottles were kept at 150 rpm for 30 mins at 28°C. The extracts were allowed to settle at room temperature and then filtered through Whatmann filter paper 1. The crude pigment was then stored at 4°C till further use.

Fungal culture broth showing color production after 3 weeks of incubation were filtered out in order to separate biomass from the broth using Whatmann filter paper and further centrifuged at 10000 rpm for 15 min to obtain cell free extract which was kept for further testing.

4.5 Isolation of colored compound

The crude ethanol extract containing colored compounds was concentrated in hot oven to remove organic solvent. The crude dye was dissolved in water and pH was adjusted to 7 and then extracted three times with ethyl acetate in the ration 1:3 (v/v) by using separating funnel. The organic layer was collected by dehydration of water by using anhydrous sodium sulphate. The filtrate was then placed in preweighed crucible and left for drying at 37°C for 24 hours.

4.6 Isolation and purification of single dye compound

4.6.1 Optimization of mobile phase for TLC

Optimization of mobile phase for TLC was done using trial and error method using various binary mobile phases. Three different binary solvent systems were used for TLC optimization. In the beginning petroleum ether: methanol in varying ratios (50:50, 60:40, 70:30, 80:20, 90:10, 40:60, 30:70, 20:80, 10:90) was used. This was followed by use of petroleum ether: ethyl acetate as solvent system in varying ratios. Finally chloroform: methanol was chosen as mobile phase. The optimal ratio of solvents for separation was achieved by trial and error method. The crude dye was run in pure chloroform followed by increase in polarity till best separation was achieved.

This was then visualized under visible and UV light. The resolution of the colored compound was achieved by using TLC over glass slides. The glass slides were washed with water, dried in oven. The dried plates were then swabbed with acetone soaked cotton in order to remove any fatty residue. These were then coated to 0.5 mm thickness with silica gel (Merck) and kept for activation at 100°C for 2 hours. 20 µl of the crude colour compound (dissolved in ethyl acetate) was spotted over the silica. Developing chamber was saturated with chloroform/methanol in the ratio of 93:7 for minimum of 20 mins. The TLC plates were developed till the solvent front reached the distance 2 cm from top. TLC chromatogram was observed under natural light and short UV light for separation of different dye components.

Specific colours relative to different dye components were developed. Retention factor (R_f) was calculated for every band of each spot.

$$R_f = \frac{\text{Distance moved by solute}}{\text{Distance moved by solvent front}}$$

4.6.2 Column chromatography

The column was packed with silica gel (Kieselgel 60, mesh 60-120). Slurry of silica gel was prepared by dissolving silica in pure chloroform and added into a glass column (Length: 55 cm and 1.1 cm diameter). Column length was kept to 45 cm and when desired height of the adsorbent bed was obtained, a few hundred millilitre of chloroform was run through the column for proper packing of the column. The sample was prepared by adsorbing crude dye extract onto silica gel (Kieselgel 60, mesh 60-120), allowed to dry and subsequently applied on top of the adsorbent layer. The column was then eluted with chloroform, followed by mixtures of chloroform and methanol of increasing polarity. To avoid variations with temperature fluctuations the column chromatography was carried out at 26± 2°C.

All the column fractions were screened by TLC and visualised under natural and UV light for determination of pure dye compounds. The TLC plates were then compared with standard/reference TLC plate for detection of pure compounds. Fraction which showed interference were then clubbed together and rechromatographed to achieve better separation of different dye components.

4.7 Dyeing of fabrics

Dyeing was done on cotton fabrics purchased from local market. The cotton cloth was cut into 4 cm X 5 cm size for dyeing. The cloth was then washed in distilled water and dried for 1 hour prior to dyeing. The dye bath was prepared by dissolving the fungal dye in distilled water. The liquor ratio was set fixed to 1:50. Initially dyeing was done for 10 minutes at 45°C and then dye bath temperature was raised to 100°C and dyeing was continued for 45 minutes. The dyed cloth was then dried in sunlight for 2 hours and then evaluate for colour fastness properties.

4.8 Colour fastness properties of dyed fabrics

Colour fastness properties of the dyed fabrics were tested by as per IS/ISO test standards.

4.8.1 Wash fastness

The colour fastness to washing was determined according to ISO 105 part C10.The dyed cloth was sewed between two pieces of undyed white washed cotton and wool fabrics. Then washing was done in a laundering device. A soap solution (5gm/l) was prepared and M: L ratio was fixed at 1:50. Washing was done at 40±2°C for 45 minutes. After that the sample was washed in cold water and then the stitching was removed and cloth was dried in air at room temperature.

4.8.2Light fastness

The colour fastness to light was determined according to IS 686. The dyed cloth and a standard blue wool pattern were mounted on exposure rack. One–third of both specimens was covered and was exposed to sunlight from 9 pm to 4 pm for 48 hours. The fastness was assessed by comparing the fading of specimen with that of blue wool pattern.

4.8.3 Rub fatness

The rub fastness properties of the dyed cloth were determined according to IS 766 test methods. The test specimen was placed on the flat surface of the crock meter. An undyed cloth (4 cm x 5 cm) was covered on the finger of sliding arm. The finger was covered onto the test specimen and crocked back and forth for 20 times making 10 complete turns. The undyed cloth was removed and evaluated.

4.8.4 Perspiration fastness

The colour fastness to perspiration was assessed according to IS-971- method. The test specimen was placed between two pieces of white fabrics and stitched. The composite sample was soaked in alkaline and acidic solution separately for 30 minutes. The samples were then placed in between glass plates of perspiration, under load of 4.5 Kg.The apparatus was then kept in the oven for 4 hours at 37±2°C. After this, the specimen was removed and dried in oven at a temperature 60°C for one hour.

4.9 Chemical characterization

^{1}H NMR and ^{13}C NMR were performed on BRUKER AVANCE II and JOEL 400 MHz spectrometer. GCMS was done in the Shimadzu QP 2010 plus Gas chromatograph with thermal desorption system TD 20. A RTX column (diphenyl 95%, dimethyl polysiloxane 5%) with 30 m × 0.25 mm ID and 0.25 mm DF was used for separating of the fungal volatiles. The column was programmed at 100 °C for 2 minutes followed by an increase in the temperature to 250°C for 2 minutes and finally to 300°C for 13 minutes. The carrier gas was Helium and

the initial column head pressure was 94.4 KPa. Data acquisition and processing was done on GCMS solution software. The obtained compounds were then tentatively identified based on their high quality matching with database of National Institute of Standard and Technology (NIST) compounds (NIST05).

4.9.1 Spectroscopic analysis

Fraction F1:

^{1}H NMR (400MHz, $CDCl_3$): δ 0.8 (m, 8H), 1.2 (s, 12H), 2.1 (s, 1H), 2.1 (s, 1H), 2.2 (s, 1H), 3.9 (d, J= 9.6 Hz, 2H);

^{13}C NMR ($CDCl_3$) : δ 12.8, 14.2, 22.8, 29.8 (Intense peak for four methyl carbon atoms), 30.1, 32.0, 41.3, 56.9, 77.3 (-**C**H_2-OH) (Falling in chloroform region confirmed with DEPT);

DEPT : Carbons with odd no. of protons (-**C**H-, -**C**H_3-) : - 22.8 (-**C**H-), 29.8 (Intense peak for four methyl carbon atoms), 32.0 (-**C**H-), 41.3 (–**C**H-),Carbons with even no. of protons (-**C**H_2-): - 12.9, 14.2, 30.1, 56.8, 77.3 (-**C**H_2-OH).

Fraction F2:

^{1}H NMR (400MHz, $CDCl_3$): 0.8 (m, 8H), 1.2 (s, 24H), 1.2 (m, 12H), 1.3 (d, J= 9.04 Hz, 2H), 1.6 (m, 7H), 2.0 (m, 3H), 2.3 (q, J=10.56 Hz, 2H), 2.8 (t, J=5.96 Hz, 1H), 5.3 (m, 2H);

^{13}C NMR ($CDCl_3$): δ 14.1, 22.6, 22.7, 24.7, 25.6, 27.2, 29.0. 29.1, 29.13, 29.35, 29.4, 29.5 (quaternary carbons) 29.6, 29.7 (Intense peak for methyl protons), 31.4, 31.5, 31.9, 33.6, 33.8, 77.2, 127.8, 128.0, 130.0, 130.2;

DEPT: Carbons with odd no. of protons (-**C**H-, -**C**H_3-): - δ 22.7, 22.8, 24.8, 25.7, 27.2, 29.1. 29.2, 29.3, 29.4, 29.7, 29.8 (Intense peak for methyl protons), 31.6, 32.0, 33.8, and 33.9; Carbons with even no. of protons (-**C**H_2-): - δ 14.2, 30.2, 77.3, 127.97, 128.1, 130.1, 130.3.

Fraction F3:

^{1}H NMR (400MHz, $CDCl_3$): δ 0.9 (d, J= 6.64 Hz, 6H), 2.0 (m, 1H), 4.0 (d, J= 6.84 Hz, 2H), 7.4 (dd, J= 5.48 Hz, 1H), 7.6 (dd, J= 5.92 Hz, 1H);

^{13}C NMR ($CDCl_3$) : δ 19.3 (Intense peak for two carbons), 27.8 (-**C**H-), 71.8 (-**C**H_2), 128.9, 131.0, 132.4, 167.7 (Carbonyl carbon atom);

DEPT : Carbons with odd no. of protons (-**C**H-, -**C**H_3-): - δ 19.3 (Intense peak –**C**H_3), 27.8 (-**C**H-), 128.9 (Ar. –**C**H-), 131.0 (Ar. –**C**H-). Carbons with even no. of protons (-**C**H_2-): - δ 71.8;

GC-MS: m/z 178(M+1) $^{\circ+}$ (Molecular ion peak), 149 (M) $^{\circ+}$ (Base peak).

4.10 Identification of the fungus producing colored compound

Fungal isolate that produces colored compounds was identified using morphotaxonomy (Classical approach) and molecular taxonomy.

4.10.1 Morphotaxonomy

The fungal isolate which produces colored compound were examined under the microscope to characterize the isolates on the basis of their morphological and microscopic characters. The cultures were grown on PDA. Glass slides were cleaned with alcohol and dried. Drop of water was put on glass slide, upon which the mycelial mass placed and teased properly. It was then stained with Lactophenol cotton blue (Hi Media). The slide was covered with 18 X 10 mm coverslip avoiding the formation of air bubble and mounted with DPX. The slide was microscopically observed at 10X, 40X and 100X using Nikon Stereozoom microscope (Nikon SMZ 745T) coupled with NIS element D 3.2 software and Nikon Eclipse Compound microscope (E100). Micrometry was carried out using Image J software with at least 30 observations per structure. The fungi were identified based upon their spore structure and other morphological characteristics.

4.10.2 DNA Isolation

The fungal genomic DNA was isolated from 3-4 day old culture grown on PDA. 2-3 mycelia plug of 5mm diameter was grounded into very fine powder by crushing with liquid nitrogen. 660-750 µl of the extraction buffer was added and the biomass was crushed again. The contents were transferred to a 1.5 ml microcentrifuge tube and 10 µl of β-mercaptoethanol and 4 µl of Proteinase K was added to each tube. The contents were vortexed and incubated at 65°C in water bath for 1 hour, they were mixed after every 15 minutes. After the incubation was over, the microcentrifuge tubes were centrifuged at 10,000 rpm for 15 minutes so as to remove cell debris. Further 6 µl of RNAase was added to each tube and incubated at 37°C for 30 minutes. For the removal of protein contents, Equal volume of Phenol: Chloroform (1:1) solution was added to each tube and mixed properly for 15 mins and centrifuged at 12,000 rpm for 10 minutes, this step was repeated three times. Transfer the aqueous layer containing DNA to the fresh microcentrifuge tube carefully avoiding the inclusion of debris and other impurities along with it. Then added 20 µl of 3M sodium acetate and the contents of each microcentrifuge tube was top up with absolute ethanol and incubated at -4° C overnight. Mix the contents by swiftly inverting the tubes so as to observe the white

threads of precipitating DNA. On the next day, the microcentifuge tubes were centrifuged at 12,000 rpm for 10 minutes; the pellet was washed again with 70% ethanol, and centrifuged at 12,000 rpm for 5 minutes. The pellet was air dried and dissolved in 30 µl of Tris EDTA buffer (pH=8).

The qualitative estimation of the DNA isolated was done by agarose gel electrophoresis.

4.10.3 Agarose gel electrophoresis

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

4.10.4 PCR amplification, sequencing and BLAST analysis

PCR is a rapid process for *in vitro* amplification of desired DNA sequence by using specific primer so as to produce a large amount of desired DNA fragment of defined sequence length. The PCR reaction mixture methodology is as follows. ITS 1, 5.8S, ITS 2 rDNA sequence was amplified using ITS 1 and ITS 4 primers, synthesized by Integrated DNA Technologies (IDT), USA, in a Thermocycler (My Cycler, Bio-Rad Laboratories, Inc.). PCR reaction was carried out by using the primers ITS1 (5’ TCC GTA GGT GAA CCT GCG G 3’) AND ITS 4 (5’ TCC TCC GCT TAT TGA TAT GC 3’) (White *et al.*, 1990). Amplification was performed in 25 µl reaction mixture containing 1 µl of extracted fungal DNA, 10 µM of each primer (ITS 1 and ITS 4), 2.5 mM of dNTP (Bangalore GeNei), 25 mM $MgCl_2$ (Bangalore GeNei), 1.5 U of Taq DNA Polymerase (Bangalore GeNei) in 10X Taq buffer (Bangalore GeNei). The PCR cycling conditions consisted of initial denaturation at 96ºC for 5 min followed by 39 cycles of 95ºC for 45 sec, 60ºC for 45 sec, 72ºC for 45 sec followed by final extension at 72ºC for 5 mins. The PCR products were examined using gel electrophoresis in a 1.5% agarose gel dissolved in 1X TAE buffer at 40V for 1.30 hr. Gel imaging was performed under UV light in Bio- Rad Gel documentation System. Purified ITS rDNA amplicons were sent for direct PCR sequencing to Chromus Biotech Laboratory, Bangalore. The obtained

sequence was then subjected to sequence similarity search by using BLAST software at NCBI server.

Sl.No.	Reagent	Stock Conc.	Quantity	Final Conc.
1.	Sterile double distilled water	_	15 µl	_
2.	PCR Buffer	10X	2.0 µl	1X
3.	$MgCl_2$	50 mM	1.0 µl	0.4 mM
4.	dNTPs	2.5 mM	1.5 µl	0.2 mM
5.	Primer	5 µM	2.0 µl	0.5 µM
6.	Taq Polymerase	3 U/µl	0.5 µl	1 U
7.	DNA Template	25 ng/µl	2.0 µl	25-50 ng

Table No. 5 Reagents used during PCR reaction

The amplification of DNA was done, using the temperature profile given in Table 5

Temperature profile

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

Table No.6:Temperature profile of PCR reaction

4.11 Antimicrobial activity of the colored compound

This involves three steps; Production of secondary metabolites, solvent extraction and evaluation of the antimicrobial potential using agar well diffusion assay

4.11.1 Production of secondary metabolites

Mycelial plug of 5mm diameter of 7-days old fungal culture was inoculated in 100 ml pre sterilized Richard's broth. The flasks were kept at shaker at 120 rpm and 26°± 2°C for 10

days for production of secondary metabolites. After 15 days broth was separated from mycelia by filtration. Filtration is carried out aseptically through muslin cloth and subsequently through Whatman paper 4.

4.11.2 Solvent Extraction

Liquid-liquid extraction procedure was adopted to extract the fermented broth of endophytic fungal isolates. The aqueous layer was extracted using four different solvents: ethyl acetate, diethyl ether, dichloromethane and chloroform (Merck GR) in the ratio 1:2 twice. The solvent layer was pooled off. Then organic layer containing compounds of interest was dehydrated with anhydrous sodium sulphate. The organic layer is then collected in a pre-weighed crucible and the solvent is removed. After removal of solvent, stock solutions of extracts were prepared in methanol and ethyl acetate in the ratio 3:2 (Merck GR).The reconstituted residue were then stored at –20°C till further use (Raviraja *et al*., 2006).

4.11.3 Antimicrobial activity of the colored compound using agar well Diffusion Assay

Wells of 5 mm were scooped with the help of pre sterilized cork borer in MH agar (Hi Media) plates to provide a depth of 4 mm. 30 µl of the test extract was dispensed in the wells and allowed to diffuse for 15 mins. 30 µl of streptomycin and methanol was also loaded in the control wells as positive and negative control. The wells were sealed with molten MH agar and allowed to solidify. After 15 min the plate was swabbed with 18-24 hrs old, 0.5 McFarland adjusted culture of the test isolate. Antibacterial activity was determined by measuring the zone of inhibition formation. All the tests were performed in triplicates. Antibacterial activity was checked against two pathogenic bacterial cultures: *Staphylococcus aureus* (MTCC 96) and *Staphylococcus epidermidis* (MTCC 2639) (Goomber and Saxena, 2007)

Chapter 5

RESULTS AND DISCUSSIONS

5. RESULTS AND DISCUSSIONS

5.1 Preliminary screening for pigment producing fungi

Cultures of endophytic fungi were screened for their pigment producing potential on PDA plates. 35 cultures were screened on PDA. Out of the 35 culture screened, 6 fungal isolates were from *Aegle marmelos* (AM), 2 were from *Cinnamonum camphora* (CC), 2 were from *Rauwolfia serpentina* (RS), 7 isolates were from *Cinnamomum malabaricum* (CM), 10 isolates were from *Taxus baccata* (TB), 1 was from *Jatropha curcas* (JT), 2 were from *Cimmelia sinensis* (CS), 4 from *Jatropha species* and 1was from *Citrus limon* (*CR*).

S.No	Culture codes	Plant Part	Host plant	Pigment production
1.	#1CCSTITD	Stem, Internal tissue	*Cinnamonum camphora*	-
2.	#61TBBALM	Bark	*Taxus baccata*	+++
3.	#61AMLWLS	Leaves	*Taxus baccata*	-
4.	#27CMLBRT	Leaf	*Cinnamomum malabaricum*	+++
5.	#12CMBABRT	Bark	*Cinnamomum malabaricum*	+
6..	#6AMLWLS	Leaf	*Aegle marmelos*	-
7.	#12BJSS	Stem	*Jatropha species*	+
8.	#44CMSTNEY	Stem	*Cinnamomum malabaricum*	-
9.	#1RSBANEY	Bark	*Rauwolfia serpentina*	-
10.	#44TBBALM	Bark	*Taxus baccata*	++
11.	#3BJSS	Stem	*Jatropha species*	-
12.	#20TBBALM	Bark	*Taxus baccata*	-
13.	#2CCSTITD	Stem, Internal tissue	*Cinnamonum camphora*	-
14.	#1CSSTOT	Stem	*Cimmelia sinensis*	-
15.	#7AMSTYEL	Stem	*Aegle marmelos*	++
16.	#4RSLBRT	Leaf	*Rauwolfia serpentina*	+++
17.	#14TBBALM	Bark	*Taxus baccata*	-
18.	#23AMSTYEL	Stem	*Aegle marmelos*	-
19.	#57TBBALM	Bark	*Taxus baccata*	++
20.	#97TBBALM	Bark	*Taxus baccata*	++
21.	#13CRLPAL	Leaf	*Citrus limon*	+
22.	#43TBBALM	Bark	*Taxus baccata*	-

23.	#42TBBALM	Bark	*Taxus baccata*	-
24	#33TBBALM	Bark	*Taxus baccata*	-
25.	#53CMSTNEY	Stem	*Cinnamomum malabaricum*	+
26.	#37(b)AMSTWLS	Stem	*Aegle marmelos*	-
27.	#42AMSTWLS	Stem	*Aegle marmelos*	-
29.	# 2(b)JTLSVNP	Leaf	*Jatropha curcas*	-
30.	#6(b)CSSTOT	Stem	*Cimmelia sinensis*	-
31.	#17BJSS	Stem	*Jatropha species*	-
32.	#29BJSS	Stem	*Jatropha species*	-
33.	#5CMSTNEY	Stem	*Cinnamomum malabaricum*	+++
34.	#1CMSTNEY	Stem	*Cinnamomum malabaricum*	++
35.	#4CMSTNEY	Stem	*Cinnamomum malabaricum*	+++

(-) no pigmentation, (+) average, (++) good, (+++) very good

Table No.6 Screening for pigment/color production on PDA plates

Out of these 35 cultures screened, 5 cultures showed very good pigment production on PDA plates. Another set of 5 cultures i.e. #44TBBALM, #57TBBALM, #7AMSTYEL, #97TBBALM, #1CMSTNEY showed acceptable pigment production on PDA plates. The production of pigment on PDA plates is attributed to depletion of essential nutrient on plate as a result of which the fungus shifts its biochemical activity towards the pathway of secondary metabolism. Rather than producing new fungal building material, this shift gives rise to other compounds (secondary metabolite). The coloration of the fungi is due to inclusion of the pigment at various points within the structure of the fungus during growth.

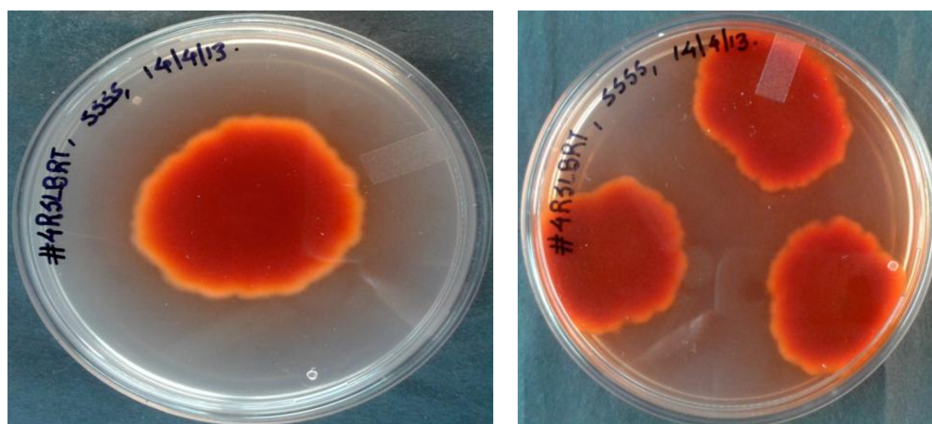

Figure 2 PDA plates showing colored colonies of #4RSLBRT

5.2 Production of fungal pigments using submerged and solid state fermentation

CZD (Czapak Dox broth) was used as fermentation media for the production of pigments (secondary metabolites) for the 10 fungal strains selected for liquid culture fermentation (Table No. 7). Out of the 10 fungal cultures tested for their ability to grow on CZD (Czapak Dox broth), 5 cultures showed fastest ability to degrade carbon source and utilize for the mycelia growth. The sooner the fungus utilizes the carbon source the faster it will start producing secondary metabolites. There was considerable change in the colour of medium of 5 fungal strains i.e. #4RSLBRT, #27CMLBRT, #7AMSTYEL, #44TBBALM,#61TBBALM. The remaining five cultures had little or negligible change in the medium colour.

Sl.No	Culture Code	Pigment Production
1.	#61TBBALM	+++
2.	#27CMLBRT	+++
3.	#4RSLBRT	+++
4.	#5CMSTNEY	+++
5.	#4CMSTNEY	+
6.	#44TBBALM	+++
7.	#57TBBALM	+
8.	#1CMSTNEY	-
9.	#9AMSTYEL	+
10.	#97TBBALM	++

Table No. 7 Culture showing growth on CZD

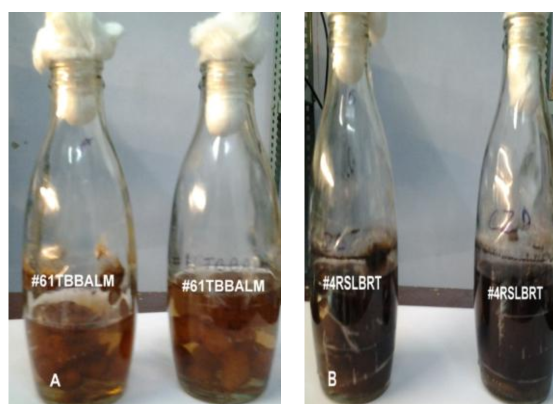

Figure 3 Liquid culture fermentation broth of; (A) #61TBBALM, (B) #4RSLBRT, showing change in color of medium.

Rice was used as solid substrate for the production of pigments. The above cultures were tested for their ability to use rice as a carbon source. Rice is very cheap source of fermentation medium and the colour change due production of pigment was easily visualized. Out of 10 cultures, #61TBBALM, #44TBBALM showed significant pigment production while #4RSLBRT showed maximum pigment production on solid substrate. Pigment production was observed on 4th day after incubation and continued to accumulate throughout the fermentation period. Maximum pigmentation was observed on the 10th day after incubation. Fungal growth and pigment production occurred at or near the surface of the solid substrate particle having low moisture contents. Thus, it was crucial to provide optimized water content, and control the water activity of the fermenting substrate for; the availability of water in lower or higher

concentrations affects microbial activity adversely. Moreover, water has profound impact on the physico-chemical properties of the solids and this, in turn, affects the overall process productivity.

The fungal isolate #4RSLBRT showed the maximum pigment production on both submerged and solid state fermentation and was selected for further study.

5.3 Isolation and extraction of fungal pigments

The fungal culture #4RSLBRT showing maximum pigment production was subjected to solid state fermentation for 10 days and isolated using hydrophilic solvent ethanol. The culture bottles were kept for shaking at 150 rpm for 30 minutes for maximum recovery of pigment from the surface and within the matrix of solid substrate. Rice particles from the crude dye were removed from the ethanol extract using filtration through Whatmann filter paper 1.

5.4 Isolation of crude pigments

Ethanol extract of the pigment isolated from fungal culture #4RSLBRT was evaporated to get red colored sticky solid. This was dissolved in water and extraction done using relatively less polar than methanol, ethyl acetate. Ethyl acetate being moderately polar can extract many non-polar components from the crude. The upper ethyl acetate layer was separated from aqueous one using separatory. The traces of water in organic layer were removed using anhydrous sodium sulphate. Anhydrous sodium sulphate can absorb residual moisture due to its hygroscopic nature. Evaporation of solvent after removal of solid sodium sulphate through filtration gave crude yellow paste (402 mg).

5.5 Isolation and purification of individual dye compounds

The crude dye compound was obtained as orange colored solid after extraction with ethyl acetate and later evaporation of solvent. The crude natural product was tested for its purity using thin layer chromatography (TLC). Initial results indicated presence of three compounds in the obtained solid. A detailed optimization using different combinations of the solvents had to be carried out using TLC.

5.5.1 Optimization of mobile phase for TLC

A combination of polar and non-polar compounds was used in different ratios to separate the crude compound on TLC plate. Initial solvent system of petroleum ether: ethyl acetate (90: 10) and petroleum ether: methanol (90:10, 80: 20, 70: 30) did not give any separation (Figure 4). Therefore, a different solvent system consisting chloroform and methanol, with a range of

solvent ratios, was tried in different combinations with and without a drop of acetic acid. The results obtained were better than previous solvent systems. Figure 5 below shows the results for 88: 12 (without acetic acid), 88: 12 (with acetic acid) and 90: 10 (with acetic acid) for chloroform and methanol solvent system. However, separation of the compound to obtain pure ones was to be carried out using column chromatography. To avoid the difficulty in evaporating high boiling acetic acid the solvent ratio was polish up to 93: 7.

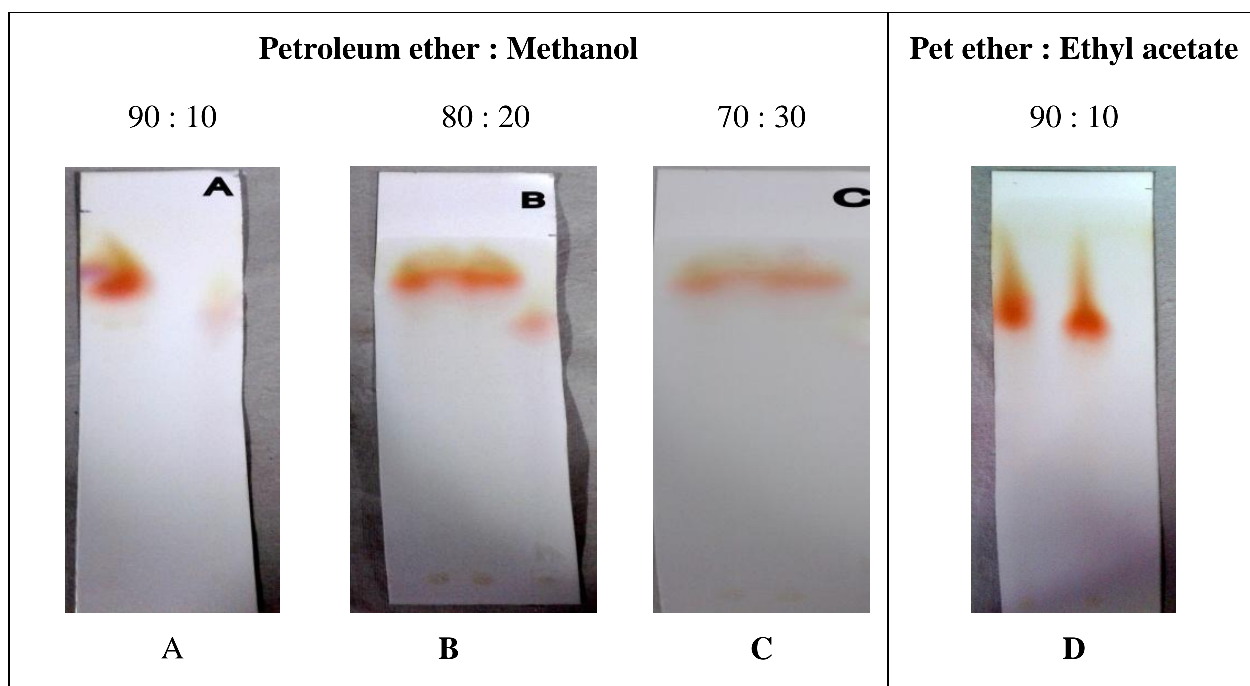

Figure 4 Thin layer chromatography pictures of the solvent systems petroleum ether: ethyl acetate and petroleum ether: methanol tried in different ratios for the separation of the compound.

The finalized solvent system was allowed to rise on TLC plate, due to capillary action, after taking it out of solvent chamber and evaporating the solvent to get better resolution. This gave as many as four compounds out of which three were prominent (Figure 3). The R_f value of all the three compounds was calculated in solvent system chloroform: methanol (93:7).

Spots	R_f value	Colour
Spot 1	0.95	Red
Spot 2	0.80	Red
Spot 3	0.47	Yellow

Table No.8 R_f value and colour of spots.

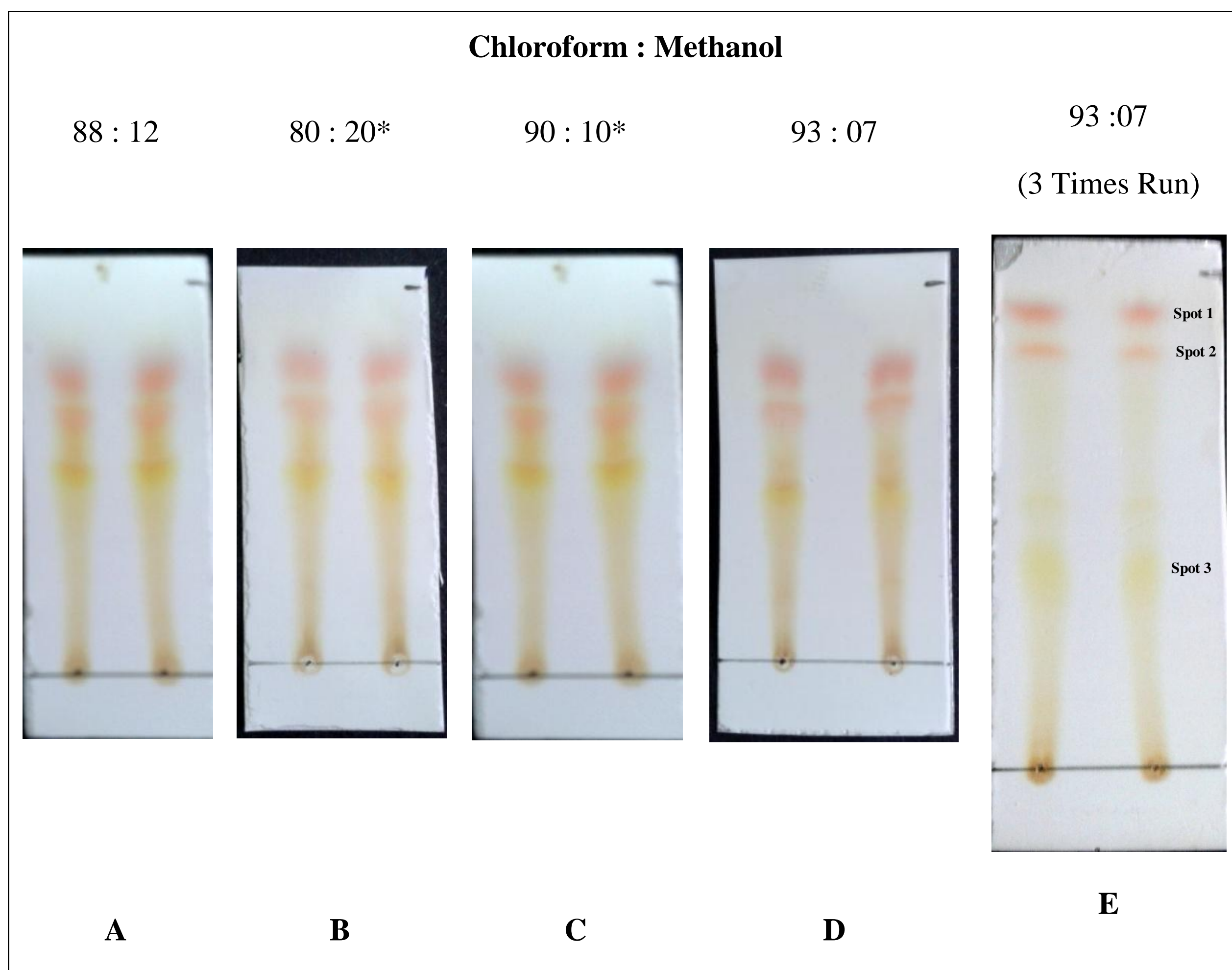

Figure 5 Thin layer chromatography pictures of the solvent systems chloroform and methanol in ratios 88: 12 (without acetic acid), 88: 12 (with acetic acid) and 90: 10 (with acetic acid). The best separation was obtained in ratio 93: 07.

5.5.2 Purification by Column Chromatography

Optimization of solvent system for separation of crude natural product was useful to isolate pure compounds by column chromatography. Using silicon dioxide (SiO_2) as stationary and above optimized solvent system as mobile phases, fractions of 15 ml each was collected from the eluent. Adsorption of crude product was carried out on top of silica column prior to elution with solvent system.

Thin layer chromatographic analysis was carried for all the fractions collected and the ones appearing on same height were pooled together. Thus, three fractions were obtained corresponding to Spot-1, Spot-2 and Spot-3 and named as F-1, F-2 and F-3 respectively (Figure 5E). The R_f values of the compounds obtained were same as those during TLC optimization. The quantity of fractions (F-1 and F-2) obtained was around 2 mg each. However, F-3 was

obtained in sufficient amount (15 mg).

5.6 Dyeing of fabrics

In the present study the dyeing experiment was made with cotton fabrics. The fabrics was well washed in distilled water, so that all lose fibers and stains of dust and grease if any, may be removed. Proper washing of fabrics facilitates the fixation of dye of dye in uniform manner.

Figure 6 (A) Control white cloth (B) Dyed cloth

5.7 Colour fastness properties of dyed fabrics

During the present study colour fastness properties (wash fastness, rub fastness, Light Fastness, and perspiration fastness) of dyed cloth was evaluated. Fastness properties of dyed fabrics are shown in Table No. 10. Analysis of colour fastness showed that washing fastness grade of the fungal dye is 2, i.e. considerable change was observed on the dyed cloth which means the dye has fair fastness. The rub fastness grade of the dyed cloth is 2, which indicate fair fastness of the dyed cloth on rubbing. The fungal dye has poor light fastness property which is revealed by the fastness grade of the cloth which was found to be 2. In perspiration fastness test considerable change was observed in the dyed cloth. The perspiration fastness grade of dyed cloth is 2. Poor color fastness properties of the dye could be attributed to non usage of mordants. The use of mordants for textile dyeing using plant dyes and pigments have been suggested by several authors, however for fungal dyes use of mordants is not reported

Sl .No.	Colour fastness parameter	Fastness grades
1.	Washing Fastness	2
2.	Rubbing Fastness	2
3.	Light Fastness	2
4.	Perspiration Fastness	2

Table No.10 Colour fastness grades of dyed cloth on fastness grade scale of 5

5.8 Analysis of the isolated fractions

The fractions F-1, F-2 and F-3 obtained after column chromatography were subjected to GCMS, ^{1}H and ^{13}C NMR analyses for their respective structure determination.

The GC of fractions F-1, unexpectedly, contained multiple components due to presence of impurities in the small quantity (2 mg) obtained via column chromatography. Prominent peak (18.45%) was appearing at 41.3 min. However ^{1}H and ^{13} C NMR confirmed the compound to be aliphatic due to absence of signal in the aromatic region which usually appears at 6-8 ppm in case of ^{1}H and at 125 -133 ppm in case of ^{13}C NMR. Fraction F-1 appeared to be a saturated hydrocarbon due to appearance of all the signals in 0.8 to 4.0 ppm. A broad singlet at 1.6 ppm indicates protons attached to electronegative group, probably oxygen (-OH). Saturated aliphatic hydrocarbon was also confirmed by ^{13}C where all signals appeared between 12 to 56 ppm.

The GC of fractions F-2, like F-1, also contained multiple components. For this compound also, the quantity obtained via column chromatography was very little (2 mg). Prominent peak (12%) appeared at 41.3 min. The ^{1}H NMR of the compound indicated presence of hydrocarbon because all the peaks appeared between 0.8 ppm and 2.8 ppm. A multiplet at 5.3 ppm indicated the presence of double bond. The ^{1}H NMR data was also complimented by ^{13}C NMR where all the signals appeared between 27.1 ppm to 31.9 ppm. However four carbons appeared between 127-130 ppm confirming the presence of double bond.

A confirmed structure could not be predicted both in case of F-1 and F-2 fractions due to presence of impurities in GC analysis. Work is underway to purify larger samples of both F-1 and F-2 so that pure sample could be obtained for further GCMS.

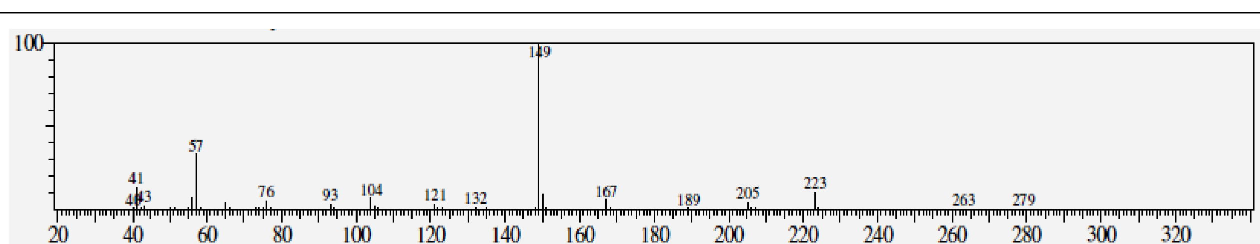

Figure 7 Mass Spectra of F-3 showing molecular ion peak at 279 and Base peak at 149.

The fraction F-3 that was obtained in larger quantities (15 mg) after column chromatography was analyzed and gave satisfactory results. GCMS analysis of the fraction gave a prominent peak (82.06%) at 19.75 min. The MS data (Figure 7) and the NIS library proposed the

compound to be Phthalic acid diisobutyl ester namely Bis(2-methylpropyl)1,2-benzenedicarboxylic acid that gave molecular ion peak (M+1) corresponding to 279 appeared for molecular mass of 278.15. The base peak at 149 corresponded to 2-formylbenzoic acid radical as shown in Figure 8.

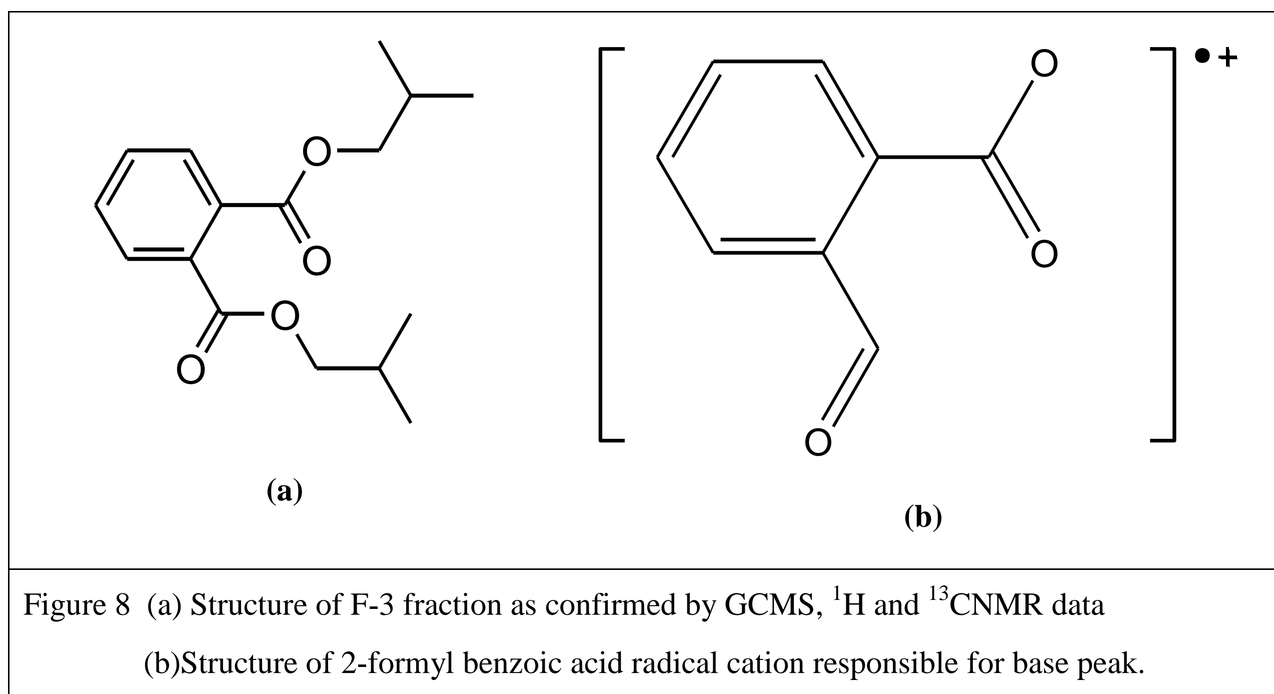

Figure 8 (a) Structure of F-3 fraction as confirmed by GCMS, ^{1}H and ^{13}CNMR data
(b)Structure of 2-formyl benzoic acid radical cation responsible for base peak.

^{1}H, ^{13}C NMR data was compatible with above proposed structure. ^{1}H NMR indicated two aromatic protons corresponding to four protons at 7.4 and 7.6 ppm and the corresponding carbons appeared in ^{13}C at 128.9 and 131 ppm respectively. The two quaternary aromatic carbons attached to 2-methylpropyl group appeared at 132.4 ppm at a height than the former ones. The carbon due to carbonyl group appeared at 167.7 ppm way downfield as expected. The isopropyl group appeared as doublet at 0.9 ppm for 12 protons and as intense peak at 19.2 ppm due to 4 carbons in ^{1}H and ^{13}C NMR correspondingly. The doublet was observed due to splitting by neighboring single proton (-C**H**-C_2) which itself appeared at as multiplet at 1.9 -2.0 ppm and at 27.8 ppm in ^{1}H and ^{13} C NMR respectively. The doublet at 4.0 ppm was downfield due to its attachment with oxygen and corresponded to (-C**H**$_{2-}$) group. Its equivalent carbon also appeared downfield at 71.8 ppm due to same reason. Figure 9, 10, 11 shows ^{1}H, ^{13}C and DEPT spectra of the compound respectively.

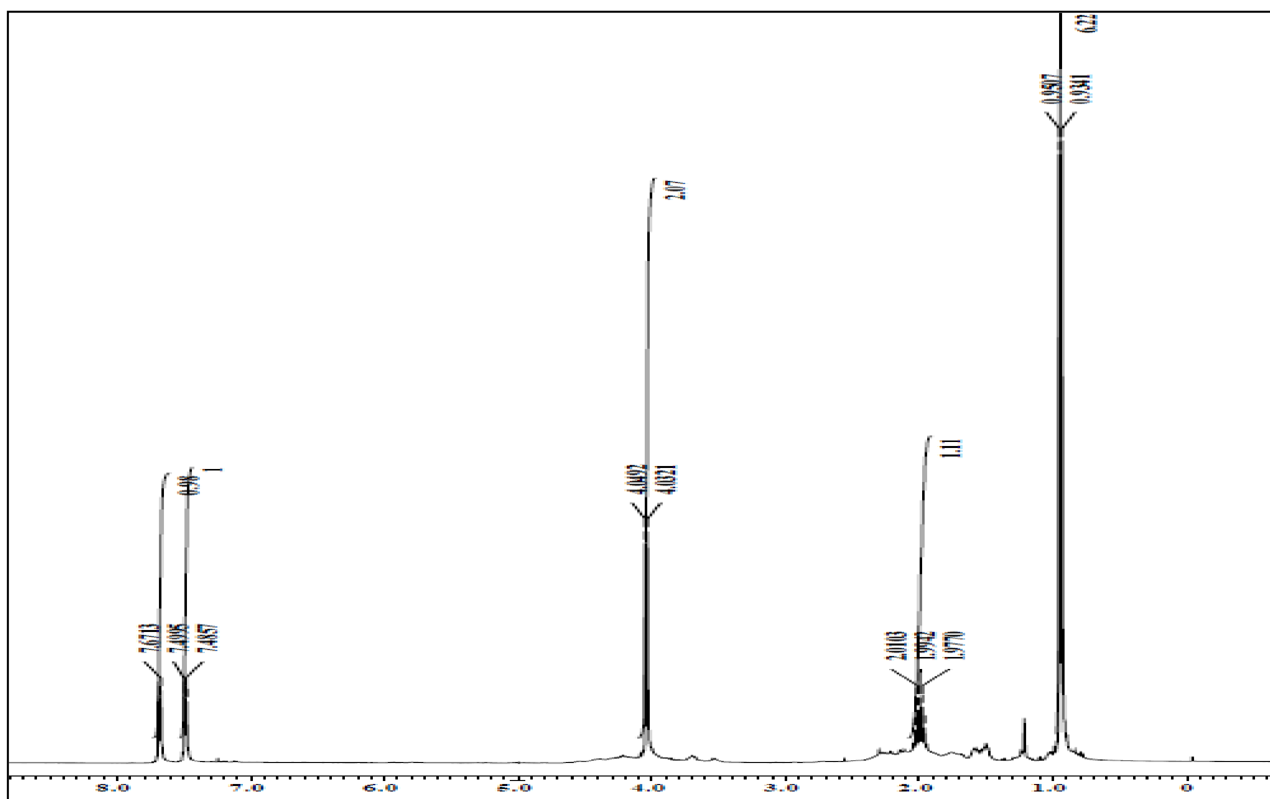

Figure 9 ^{1}H spectra of fraction of F3 fraction

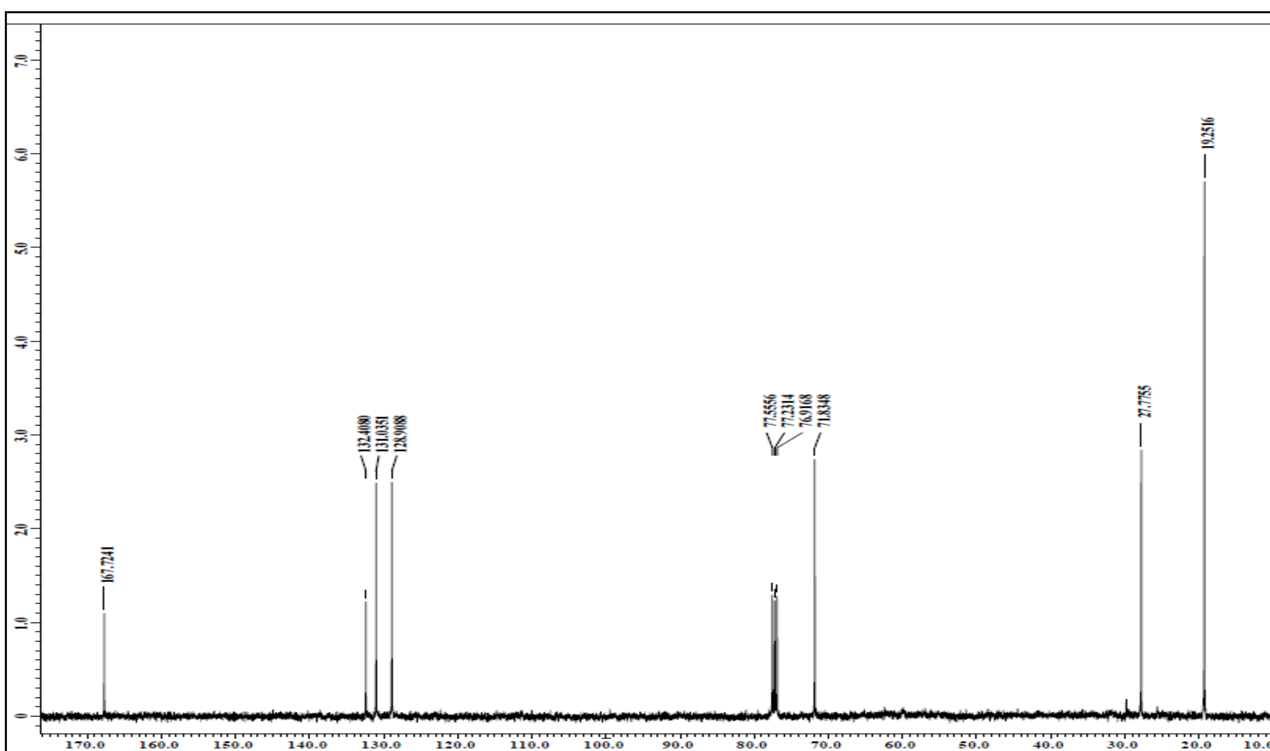

Figure 10 ^{13}C spectra of fraction of F3 fraction

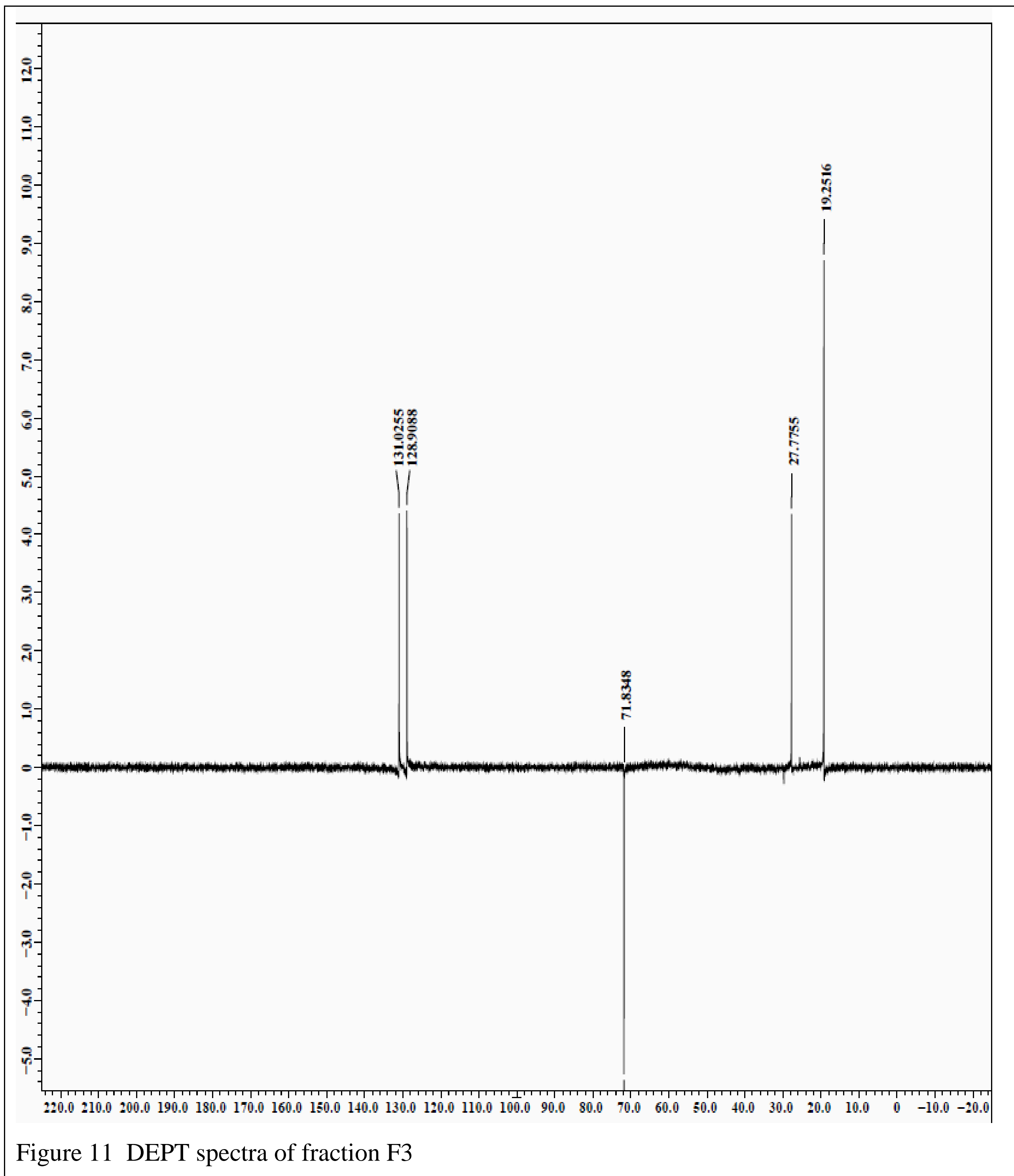

Figure 11 DEPT spectra of fraction F3

5.9. Identification of #4 RSLBRT

5.9.1. Morphotaxonomy

Over PDA it forms moderately growing white to white pink colored floccose aerial mycelium which produces orange red soluble pigment on the reverse side. The colony diameter was 45-52 mm on 7th days of incubation at 26°C. Hyphae coenocytic, thick branched. Two types of conidia

were borne. Macroconidia large in size usually 3-4 septate (27-39 µm), moderately curved, with short blunt apical cell where as microconidia small in size (5-12 µm), abundant, two celled, comma shaped.

Thus based on the following features the fungus was identifies as ***Fusarium solani***

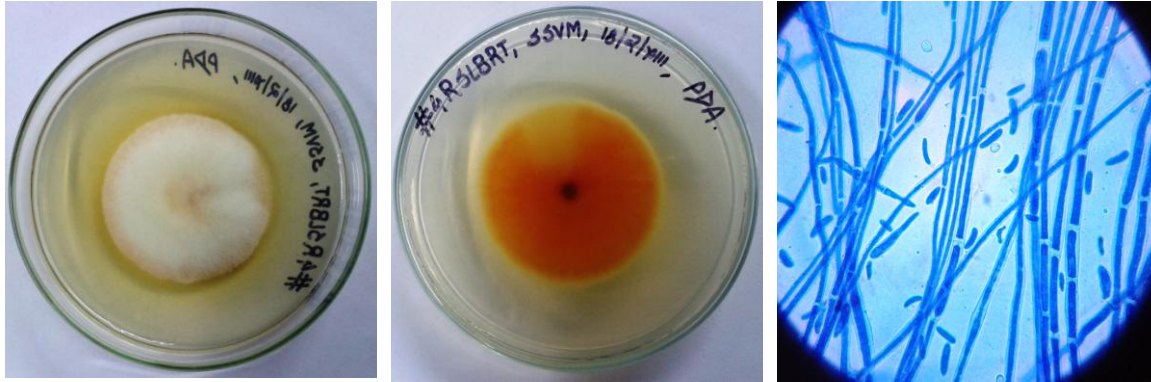

Figure 12 Morphotaxonomy of #4RSLBRT (A; Front View on PDA, B; Reverse view, C; Hyphae with microconidia)

5.9.2 Genomic DNA Isolation and PCR Amplification

The genomic DNA isolation of #4RSLBRT showing the highest pigment producing potential of was done. The DNA was qualitatively estimated using Agarose gel electrophoresis and the size of the genomic DNA was deciphered by comparing its mobility in the gel with the 1 kb DNA ladder which ranges from 1kb to 10 kb. There was no RNA bands seen, hence the RNase treatment was successful. The size of the genomic DNA was found to be approximately 10 kb.

Lane 1: Ladder
Lane 3 and 4: #4RSLBRT
Figure – 13**:** Agarose gel electrophoresis of genomic DNA isolated from #4RSLBRT

The PCR amplication of the genomic DNA was carried out; the amplicons obtained were resolved on 1.5% Agarose in order to check the size on the basis of the mobility and comparison with the 500 bp ladder. The size of the amplicon was found to be approximately 500 bp to

550 bp. This size can be easily compared to the ITS region, which was amplified in order to characterize the fungi at molecular level.

Figure – 14: Shows ladder in lane 1 and PCR product of #4RSLBRT in lane 4.

5.10 Sequencing and BLAST analysis

The sequences for ITS 1 and ITS 4 received from Chromus Biotech, Bangalore were made into a final nucleotide sequence of 564 bp using online software tools BLAST (http://blast.ncbi.nlm.nih.gov/) and Multalin (http://multalin.toulouse.inra.fr/multalin/). The FASTA format of the final nucleotide sequence of #4RSLBRT obtained after sequencing from Chromus Biotech was

>#4RSLBRT

```
TTGAACCTGCGGAGGGATCATTCATGACTTATACAAATCATCAATCCTGTGAAAGGACCTAAACGTTGCCTCGGCGG
GAACAGACGGCCCCGTGAAAAAGAAAAAGCCCCCGCCAGAGGACCCCCTAACTCTGTTTCTATAATGTTTCTTCTGA
GTAAAACAAGCAAATAAATTAAAACTTTCAACAACGGATCTCTTGGCTCTGGCATCGATGAAGAACGCAGCGAAATG
CGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCGCCAGTATTCTGGC
GGGCATGCCTGTTCGAGCGTCATTACAACCCTCAGGCCCCCGGGCCTGGCGTTGGGGATCGGCGGAGGCCCTCCGTG
GGCACACGCCGTCCCCCAAATACAGTGGCGGTCCCGCCGCAGCTTCCATCGCGTAGTAGCTAACACCTCGCGACTGG
AGAGCGGCGCGGCCACGCCGTAAAACCCCCAACTCTTCTGAAGTTGACCTCGAATCAGGTAGGAATACCCGCTGAAC
TTAAGCATATCAATAAGACGGAGGA
```

Sl.No.	Description	Query cover	E value	Maximum Identity	Accession No.
1	*Fusarium solani* isolate MZ02 18S ribosomal RNA gene	99%	0.0	97%	FJ874633.1
2.	*Fusarium solani* isolate MZ01 18S ribosomal RNA gene	99%	0.0	97%	FJ886799.1
3.	Uncultured *Ascomycota* clone ALBM1 18S ribosomal RNA gene	99%	0.0	97%	EU862818.1
4.	*Fusarium solani* isolate CL1 internal transcribed spacer 1	98%	0.0	97%	JQ771182.1
5.	*Fusarium solani* strain ATCC MYA-4552 18S ribosomal RNA gene	98%	0.0	97%	FJ914886.1
6.	*Fusarium sp*. NRRL 44892 internal transcribed spacer 1	97%	0.0	97%	GU170638.1
7.	*Fusarium solani* isolate T03 18S ribosomal RNA gene	99%	0.0	96%	FJ459973.1
8.	*Chloridium sp.* AzR-0142/07 18S ribosomal RNA gene	96%	0.0	97%	EU394444.1
9.	*Ascomycete sp*. SV13F8 18S ribosomal RNA gene	96%	0.0	97%	AY770412.1
10.	*Fusarium solani* isolate S19 18S ribosomal RNA gene	99%	0.0	96%	EF062312.1

Table No.11: Showing first 10 results of BLAST analysis of query sequence with database

Hence from this data we analyzed that as the obtained sequence shows 97% identity with *Fusarium solani,* #4RSLBRT was confirmed to be *Fusarium solani*.

5.11 Antimicrobial Activity of the colored compound

The ethyl acetate fraction was active against *Staphylococcus epidermidis* (MTCC 2639). It forms a zone of inhibition whose mean diameter was 11±0.57 mm where as the diethyl ether fraction was active against Staphylococcus aureus (MTCC 96). The zone of inhibition measures 14± 0.57 mm. No antibacterial activity was observed in hexane and chloroform fraction.

Test Culture	Mean zone if inhibition (in mm)			
	Ethyl acetate	Diethyl ether	Chloroform	Hexane
Staphylococcus epidermidis MTCC 2639	11±0.57	-	-	-
Staphylococcus aureus MTCC 96	-	14± 0.57	-	-

Table No.11 Antimicrobial activity of crude colored compound via agar well diffusion assay.

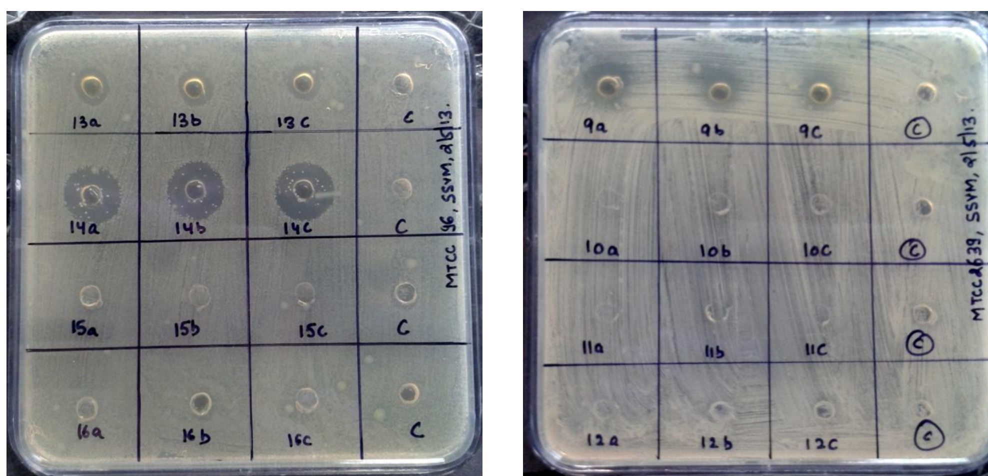

Figure 15 Antimicrobial activity of the colored compound in agar well diffusion assay

Chapter 6

CONCLUSION

CONCLUSION

The current study reveals that besides plants, bacteria and other known microbial sources, endophytic fungi are capable of producing pigments which could be used as textile dyes. Out of 35 endophytic fungi screened, only five isolates were found capable of producing pigments extracellularly on solid media. These five cultures were then tested for their ability to produce pigments through liquid culture fermentation and solid substrate fermentation. Fungal isolates #44TBBALM and #61TBBALM showed considerable pigment production whereas #4RSLBRT showed maximum production of pigment in both liquid and solid substrate fermentation. Morphotaxonomy of the culture #4RSLBRT was relatively similar to that of *Fusarium sp.* and molecular approaches and sequence similarity search using BLAST software at NCBI server confirmed species to be *Fusarium solani*.

Dyeing potential of the crude compound was tested on cotton swatches which showed fair colour fastness properties. Further isolation and purification of the crude compound from #4RSLBRT showed that it is composed of three individual coloured compounds. NMR and GC-MS analysis of purified compounds revealed the complete structure determination of one compound i.e. F3 and tentative structures of two individual compounds viz F1 and F2. An area of concern seems to be the large scale production & extraction of this pigment. Hopefully pigment from #4RSLBRT would be marketed as textile dyeing agent in India in few years from now.

Chapter 7

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