STUDIES ON PIGMENT PRODUCING FUNGI STRAINS

A Dissertation-II Report of BTY-731 submitted in the Partial Fulfilment of the Requirements for the Degree
of

MASTERS OF THE SCIENCES

In

(Honours) MICROBIOLOGY

By

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UNDER THE SUPERVISION OF

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TOPIC APPROVAL PERFORMA

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PROPOSED TOPIC : Studies on pigment producing pigment strains

Qualitative Assessment of Proposed Topic by PAC		
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2	Project Feasibility: Project can be timely carried out in-house with low-cost and available resources in the University by the students.	7.50
3	Project Academic Inputs: Project topic is relevant and makes extensive use of academic inputs in UG program and serves as a culminating effort for core study area of the degree program.	8.00
4	Project Supervision: Project supervisor's is technically competent to guide students, resolve any issues, and impart necessary skills.	7.50
5	Social Applicability: Project work intends to solve a practical problem.	8.00
6	Future Scope: Project has potential to become basis of future research work, publication or patent.	7.50

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Final Topic Approved by PAC: Studies on pigment producing pigment strains

Overall Remarks: Approved

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CERTIFICATE

This is to certify that work embodied in the Dissertation-II report entitled "**STUDIES ON PIGMENT PRODUCING FUNGI STRAINS"** and has been carried out by **Diksha koul (11203735)** under my guidance and supervision**.** To the best of my knowledge, the present work is the result of his original investigation and study. No part of the dissertation has ever been submitted for any other degree or diploma. The work has been carried out by them at the School of Biotechnology & Biosciences, Lovely Professional University, Phagwara, and Punjab, India. They fulfilled the requirement for the award of the degree MSc. (Honours) Microbiology.

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DECLARATION

I here to declare that the work presented in DISSERTATION-II entitled "**STUDIES ON PIGMENT PRODUCING FUNGI STRAINS"** is my own and original. The work has been carried out by me at School of Biotechnology & Biosciences, Lovely Professional University, Phagwara, Punjab, India under the guidance of **Dr. ASHISH VYAS,** Assistant Professor and Coordinator, Lovely Professional University, Phagwara, Punjab, India, for the award of the degree Masters in Science in Microbiology.

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Chapter1

Introduction

Many pigments produce colors that can be observed in our day to day life. Every organisms produce different types of pigments in the world some plants are the natural producers of pigments. Pigments can be found everywhere like leaves, fruits, vegetables, and flowers. Also they are present in skin of the organisms and in their eyes and also in fungi and bacteria. In the food industry these pigments can be used as color intensifiers, additives, antioxidants, etc. Pigments are available in many different varieties of color and some are soluble in water. For these reasons, pigment compounds have been produced and isolated. (Duran *et al*., 2002).

Different pigments have importance in various fields like in cosmetic, food stuff and in pharmaceutical processes (Kim *et al*., 1995). Fungi are the best source of pigments and some fungal strains are rich in stable colorants. For the isolation of fungi that influence the vegetative growth and colony morphology, pigmentation and sporulation different types of media are used. Temperature, light, pH, and surrounding atmospheric gas mixture are important for the fungi. (Kumara and Rawal, 2008).

Most of the fungi have been reported that they are producing non-carotenoid pigments but only few numbers of fungi species used as food colorants (Sameer et al., 2006). Many types micro-organisms have the capability to produce different pigments in high amount examples are- *Paecilomyces, Monascus, Serratia, Streptomyces, Cordyceps* and blue and yellow-red compounds produced by *Penicillium atrovenetum* and *Penicillium herquei.*

Pigments can be classified on the basis of structural affinities and natural occurrence. These are the some examples of pigments which are naturally occurring.

Photosynthetic Pigments These are the chemical compounds that absorb light in different wavelength in the range of visible region. Pigments interact with light to absorb only certain wavelengths and these pigments give color to flowers, coral, and also to animal skin. Pigments are used where different autotrophic organisms make their own food by the process of photosynthesis. Colored pigments are present in the plants (leaves) that are chlorophyll and carotenoids.

Canthaxanthin**:** Orange and dark pink pigmented bacteriochlorophyll is produced by *Bradyrizobium* (photosynthetic) strain found in nature.

Some of the potential source of beta-carotene is **Beta-**carotene, Phycomyces and Mucor circinelloides which is wild type (Hari *et al*., 1994).

Phycobilins are found in the stroma of the chloroplast or in the cytoplasm and they are water soluble pigment. They occur only in the Cyanobacteria and Rhodophyta. (Bartley *et al*., 1991).

Riboflavin is a yellow colored vitamin which is water-soluble and produced by many micro-organisms. It is commercially synthesized by ascomycetes species like *Ashbya gossypii*, filamentous fungi like *Candida famata* and bacterium like *Bacillus subtilis* (Stahmann *et al*., 2000).

PIGMENTS IN GENERAL:-

Definition: These are chemical compounds which absorb some light in the wavelength range of the visible region. The color which is producing from pigments is due to chromophore which is a molecule specific structure.

Classification: These pigments can be classified on the basis of their origin; these can be divided into three types; natural pigments, inorganic pigments and synthetic pigments. Natural pigments are those which can be produced naturally by some living organisms like animals, plants, and some microorganisms like molds, bacteria, yeasts, algae etc.

Inorganic pigments are made up of mineral compounds like sulphides, oxides etc.

Examples of inorganic pigments are azurite, red and yellow earths.

Synthetic pigments are those pigments which are made in the laboratories and these pigments are organic compounds. (Delgado-Vargas *et al*., 2000).

Structural characteristics: Apart from origin, naturally producing pigments can be classified on the basis of structural characteristics these are;

- **Derivative of Tetrapyrrole**: these are heme colors and chlorophylls.
- **Derivative of Isoprenoid**: these are iridoids and carotenoids.
- **Compounds of *N*-heterocyclic:** these are totally different from derivative of tetrapyrrole compounds. These compounds are pterins, betalains, purines, phenazines and phenoxazines.
- **Derivatives of Benzopyran**: these are oxygenated heterocyclic compounds like flavonoid and anthocyanins compounds.
- **Quinones**: these are anthraquinone, benzoquinone etc
- **Melanins.**

Natural pigments: (Distribution)

Derivative of Tetrapyrrole: This type of compound having a pyrrole ring in cyclic or linear arrays in their structure. The compound phytochrome is common in different types of algae. Examples are- *Cryptophyta* and *Rhodophyta*.

The bilin compound is basic structure in linear arrays. In cyclic compounds the heme group is present in which the ring porphyrin is attached to the atom of iron. Heme group is present in myoglobin and haemoglobin and present in cytochromes, catalases, peroxidases etc and also in animals.

Chlorophyll is the most important subgroup as a pigment in the derivatives of tetrapyrrole. The pigment chlorophyll is present in the chloroplasts of plants (higher) and in algae also.

Chlorophylls ("a" and "b") are present in higher plants, mosses, ferns and in green algae and rest of chlorophyll have been found in other groups like bacteria and algae.

Derivative of Isoprenoid: Isoprenoid represents a big family of natural compounds and they are called as terpenoids. They found almost in all kingdoms and they carry multiple functions such as hormones, phtyoalexins, and pigments.

On the basis of their structure and abundance, two main subgroups of compounds are taken as pigments which are- carotenoids and quinones.

In quinones not all produced biosynthetic pathway and these compounds (quinones) are considered as another group.

Carotenoids (iridoids) are present in 70 families such as Rubiaceae, Capriofilaceae, Cornaceae and in among others grouped in 13 orders. Cape jasmine fruit and saffron are known as best iridoid plants and their colors are influenced by the pigment which is carotenoid (Delgado-Vargas *et al*., 2000).

N-Heterocyclic compounds:-

- **Purines**: These are present in nucleotides such as ribonucleic acid (RNA) and deoxyribonucleic acid (DNA). Purines are present in living organisms and it is the essential unit of life. Purines are present in animals like silvery and golden fish.
- **Flavins**: In this type of compound two rings are present pteridin and benzene ring. The main compound of this group is Riboflavin and is present in all living microorganisms. It is also present in milk, vegetables (leafy), fish and meat.
- **Phenazines**: These are present in bacteria.
- **Phenoxazines**: These are present in insects and fungi.

Quinones:-

It is having a good number of coloring compounds and this is the biggest group in the variation of structure and number. Other than natural pigments these are widely distributed pigments except of carotenoids. Desaturated cyclic ketone is present in the structure of quinones which is a derived from compound like aromatic monocyclic or polycyclic. On the basis of structure quinones are divided as- benzoquinones, anthraquinones, naphthoquinones and quinones (miscellaneous)- dibenzoquinones, dinaphthoquinones and dianthraquinones. These are also found in higher plants, algae, bacteria, fungi, flowering plants, fungi, and in insects. Examples are-

menaquinones in bacteria,

naphthoquinones in animals and

anthraquinones in fungi, flowering plants, lichens and insects.

Some organisms produce quinones in large quantities such as fungi. Quinones produce different colors like red, brown or yellow colorations. (Delgado-Vargas *et al*., 2000)

Melanins:-

In these structures indole ring is present and they are polymeric compounds (nitrogenous). These are found in microorganisms, animals and plants. They produce different colors like black, brown and gray. Examples are-

Eumelanins are found in many invertebrate and vertebrate animals.

Allomelanins are found in spores, seeds and fungi.

Esclorotins are found in arthoppods. (Delgado-Vargas *et al*., 2000).

APPLICATIONS OF PIGMENTS

Many microorganisms are capable of producing pigments which have a wide range of applications in food industry, in pharmaceutical companies, in cosmetics, in textile industries etc. Microbes are capable of producing natural pigments in table1 which can replace artificial synthetic pigments. Fungi are the potential source of natural pigments. These pigments are very helpful in many ways.

Microbial pigments are having beneficial properties like –

- Anticancer
- Antibiotic
- Immunosuppressive
- Antiproliferative
- Biodegradability etc

Many microbes produce food grade natural pigments which are used in food industry like-

- Arpink Red
- β-carotene
- Monascus
- Riboflavin lycopene etc

Microbes produce pigments which are used in pharmaceutical industry which are used to treat diseases these are-

- Violacein
- Anthocyanin
- Prodigiosin etc (Kumar *et al*., 2015)

Table 1: List of microorganisms which are producing pigments.

Microorganisms	**Molecule or pigments**	**Color**
Algae		
Dunaliella salina	Beta-carotene	Red
Hematococcus	Canthaxanthin	
Chlorococcum	lutein	
Bacteria		
Agrobacterium aurantiacum	Astaxanthin	Pink-red
Paracoccus carotinifaciens	Astaxanthin	Pink-red
Flavobacterium spp.	Zeaxanthin	yellow
Staphylococcus aureus	Staphyloxanthin Zeaxanthin	Golden Yellow
Pseudomonas aeruginosa	Pyocyanin	Blue-green
Haloferax alexandrines	Canthaxanthin	Dark red
Fungi		
Aspergillus spp.		Orange-red
Blakeslea trispora	Beta-carotene	cream
Penicillium cyclopium		Orange
Fusarium sporotrichioides	Lycopene	Red
Monascus spp.	Monascorubramin Rubropunctatin	Red Orange
Monascus roseus	Canthaxanthin	Orange-Pink
Monascus purpureus	Monascin Ankaflavin	Red-yellow
Penicillium oxalicum	Anthraquinone	Red
Cordyceps unilateralis	Naphtoquinone	Deep blood red
Neurospora crassa	Beta-carotene	Yellow-orange
Pacilomyces farinosus	Anthraquinone	Red
Yeast		
Cryptococus spp.		Red
Phaffia rhodozyma	Astaxanthin	Pink-red
Saccharomyces neoformans		Melanin black

Chapter 2
Terminology

Terminology	Terms
PDA	Potato Dextrose Agar
PDB	Potato Dextrose Broth
RBA	Rose Bengal Agar
YMA	Yeast Malt Agar
CDA	Czapek Dox Agar
NA	Nutrient Agar

Chapter 3
Review of Literature

Atalla *et al*., 2010 tested eleven fungal strains by using a dye precursor H-acid (1-naphthol-8-amino-3, 6-disulfonic acid) in the medium of fermentation which produces different dyes like brown and reddish brown. The tested eleven fungal isolates are capable of producing dyes and it varying in both dye color which is (brown to reddish brown) and these dyes are having fastness properties to washing, and UV light. These dyes which are isolated from fungi strains were subjected to further analysis for inter-relations and their role in dye color and stability.

Bhat *et al*., 2013 isolated pigment producing bacteria from different food samples taken from different regions of local market of Kashmir. Isolated two bacteria those are producing orange and yellow pigmentation. These isolates showing morphological characteristics showing that these are Gram positive, cocci in shape and also showing non-motile. Isolates were identified as *Micrococcus nishinomiyaensis* and *Micrococcus luteus* on the basis of taxonomic characterization. At 35°C, pH 9 and at 4 % (W/V) NaCl concentration maximum production of pigment was observed.

Mendez *et al.*, 2011 used Czapek-Dox media with D-xylose as a carbon source to study the red pigment production by *Penicillium purpurogenum* GH2 with combined effects like pH and temperature. Three different pH values which is pH5, pH7, and pH9 and the two temperature conditions which is at 24˚C and at 34°C. The maximum production of red pigment (2.46 g/L) is at pH value of 5 and a temperature of 24 °C. This report demonstrated that *Penicillium purpurogenum* GH2 produces a pigment that can be used in food industry.

Stanly *et al*., 2013 isolated *Fusarium moniliforme* KUMBF1201 from paddy field soil for the production of natural pigments. Eight different solid and six different media were studied to obtain a suitable medium for enhanced pigment production. *Fusarium moniliforme* KUMBF1201 grew well on Potato Dextrose Agar (PDA) and Potato Dextrose Broth (PDB) media, which shows best growth, pigment production and sporulation at temperature (28±1°C) and pH (5.5). From results, the glucose (2 %) as the carbon source and yeast extract (2 %) as nitrogen source played a major role in enhanced cell growth and pigment.

Gunasekaran *et al*., 2008 isolated *Penicililum* species from western Ghat region capable of producing red color pigment and examined optimized culture condition, medium, effect of temperature and effect of pH on red pigment production and found that the maximum pigment product was found in soluble starch medium, pH 9 and at temperature 30°C. Those conditions were shown the best pigment production and growth also. The *Penicillium* sp. produces almost seven fold improvement in production of red pigments that was achieved.

Sasidharan *et al.*, 2013 isolated yellow pigment by bacterial strains. He used air and soil sampling method to isolate bacterial strains (13strains) that are producing yellow pigment. The pigments of four bacterial isolates like (RS7, RSS3, RS13 and RS1) were studied based on morphological characterization. The gene sequences of strains was done 16s rDNA and were compared with the sequence. Phylogenetic trees showed the isolate RS7 and RS13 were related to *Exigeobacterium aurantiacum*. Isolates like RSS3 and RS14 belong to the *Exiguobacterium* profundum.

Mawthols *et al.,* 2005 isolated fungi strains like *Aspergillus* Spp., *Alternaria* and *Fusarium* from tomato leaves, fruits of Dolichos and Amla and these were grown on GN medium. When the culture filtrate was done it shows color pink for *Fusariurm*, and for the *Aspergillus* and *Alternaria* showing yellow color. Development of pigments was measured in the culture filtrates at different pH. At pH 8.5 maximum pigment production was observed for the isolates like *Aspergillus niger* at 3.5 for *Fusarium* is at 5.5 and 6.5 for *Alternaria*. Culture filtrate of *Aspergillus* inhibited germination of seeds of Amla at all pH except pH 5.5. The 20% germination of seed was favoured by culture filtrate of *Alternaria* sp..

Robinson *et al*., 2011 investigated the use of red pigments for different use in applications (decorative). The two *Fusarium* species and two strains of *Arthrographis cuboidea* were inoculated onto sugar maple and after that incubated these plates for 6– 14 weeks, and observe on the basis of their ability to produce a high-saturation, penetrating stain. Both strains of *A.cuboidea* produced high amounts of surface and penetrating red stain. This report shows that under sterile conditions species *A.cuboidea* is suitable for of red pigmentation production on decorative wood applications.

Bissett., 1984 studied the variations in strains like *Trichoderma longibrachiatum* Rifai and the *T. pseudokoningii* Rifai species. He observed that *Trichoderma longibrachiatum* as compared to the other species showing conidiophores which is branched with a high phialides producing structure. It shows fast growth, produces bright yellow-green pigment which is observed in the back side of the colony. *Trichoderm acitrinoviride* Bissett has ellipsoidal conidia smaller compared to other species.

Sharma *et al.*, 2012 isolated three fungus, *Trichoderm avirens, Alternaria alternate, Curvularia lunata* which produces pigments and optimized their fermentation for the use of textile dyeing. Under static conditions the high optical density had obtained in Potato Dextrose Broth at 28℃ in 25 days. *Trichoderm avirens* showed the property of antifungal. Different techniques were used like paper and Thin Layer Chromatography which shows that the pigments which are isolated from different fungal species are multi-component in nature.

Musaalbakri *et al.*, 2006 was performed a technique which is Monospore isolation to obtain the strain which has high ability for production of red pigment. The ability of specie *Monascus purpureus* FTC 5391 which is wild strain and producing red pigment was isolated successfully by using technique which is performed starting of the experiment. Many carbon source like glucose, potato starch and rice starch where the three different monospore isolates of *M. purpureus* FTC 5391 (MP3, MP4 and MP5) which shows the best red pigment producers.

Sayyed *et al.*, 2015 studied that there is demand for non toxic dyes or eco-friendly which are used for food colors and child textiles. Fungi are capable of producing natural dyes and it is reported that source of natural pigments example anthraquinones. In this study they isolated different fungi from domestic fridges. They isolated four fungal strain which are capable of producing different natural pigments. The four different strains are IDP1, 2, 3 & 4. These strains are producing different pigments which is IDR, IDG, IDY, & IDBr. The highest pigment production was seen in mineral salt glucose medium. The colony characteristics and microscopic evaluation showed that all four strains were *Penicillium* Spp.. The pigment yields of four different fungal strains were 0.073mM, 0.133mM, 0.1056mM, and 0.159 mM. This study showed that pigments are multicomponent in nature.

Dhale *et al*., 2009 isolated *Penicillium* sp from the marine which is capable of producing red pigment. The pigment which is extracted from this strain scavenged 2, 2-diphenyl-1-pycrylhydrazyl (DPPH) radical. This species is grown in media which contains corn steep liquor 72-88% of DPPH radical. Fungus produces more pigment in solid-state fermentation (wheat) which shows the reading 9.232 OD units. These fungal strains secrete more amylase in medium which is about 246U/mg. The study of pigment production of *Penicillium* Spp. and its scavenging activity proved that it's having applications in food, pharmaceuticals, and nutraceutical industries.

Soumya *et al*., 2014 studied that fungi are the best source of natural pigments; they are having easy culturing and downstream production. In this study they isolated *Chaetomium cupreum* from the soil sample and *Chaetomium cupreum* is selected for the study of production of extracellular pigment. The aim of the study is based on the influence of different wavelengths on biomass and pigment production of *Chaetomium cupreum*. The maximum pigmentation was observed under green light incubation whereas white and yellow light incubation gives reduced pigment yield and low intensity. The growth of mycelia on solid media showed difference in the radial growth and the morphology of fungus showed less variation. Photoreceptors are active in fungi and play a major role in biomass and pigment production.

Joshi *et al*., 2010 isolated sixty-two *Trichoderma* sp. from different soil samples (rhizospheric) collected from different locations of western Himalayas regions. The growing colonies of *Trichoderma* Spp. bearing postulate or tufted and showing branched condiophores. Out of 62 isolates only 2 species were found which are *Trichoderma viride* and *Trichoderma hazianum.* The efficacy of these isolates against plant pathogens (soil borne) like *Rhizoctonia solani, Sclerotinia sclerotiorum and Sclerotium rolfsii* showed only 3 isolates amounting 5% of total collected three isolates of the region were found that they are highly antagonist. On the reverse side of colony showed pigment production and 70% of the isolates are not producing pigment in the medium. They measured the plant growth promotion of shoot and root length and observed it was higher than in control.

Saleem *et al*., 2012 isolated 14 dermatiaceous fungi (hypomycetes) from diseased leaves of bean plants and investigated their level of pathogenicity. Among all of them only 8 fungal isolates were positive and is able to infect bean leaves and showing symptoms of leaf spots.

In all of them one isolate is most virulent which is *Alternaria alternata* . 75% of infected leaves is due to *Alternaria alternate.* 6 fungal isolates showed negative result and does not show pathogenicity. The 4 photosynthetic pigments chlorophyll a, chlorophyll b and carotenoids decreased as a result of infection of phytopathogenic fungi. The pigment concentration was affected on the basis of degree of pathogenicity. *Alternaria citri*, *A. raphani* and A. tenuissima exhibit high pectinase activity and other 3 isolates *Alternaria alternata*, *Curvularia lunata* and *Ulocladium botrytis* showed moderate pectinase activity.

A. citri and *A. raphani* showed maximum pectinase production when it was observed after 8^{th} day at pH 6 and 30°C in the liquid medium which was supplemented with ammonium sulphate and citrus pectin.

Chitale *et al*. (2012) isolated *Trichoderma viride* which is producing brown colored pigment by using PDB (potato dextrose agar). The extracted pigment was purified by the technique silica gel column chromatography and after that it was analyzed by GC-MS and HPLC. The phytotoxicity study showed that pigment is non toxic in nature on the germination. The pigment which is produced from *Trichoderma viride* has applications in food industries and also it will replace the use of artificial synthetic pigments.

Chapter 4

Rationale and Scope of the Study

To achieve the goals fungal strains have been isolated from natural habitat such as soil serial dilution techniques. There after screening and identification of the isolated fungi has been done by Lacto phenol cotton blue method.

The best isolates have been used for detecting their pigment producing capacities. Extracellular and Intracellular pigment production has been done.

For enhancing the growth and production of pigments, optimization of the cultural on different medium has been performed parameters.

Chapter 5

Objective of the study

The various fields like Food industries, textile industries, Paper production etc use the natural pigments and synthetic dyes. Natural habitat and synthetic are the various sources for pigment production. Microorganisms from the natural habitat are preferred over chemical synthesis for higher yield of the pigment due to their greatest ability and cultivation technology. The Rationale of the study is to explore the different fungal species isolated from natural habitat, such as for the production and optimization of the pigment by using different cultivation parameters. The main objectives of our studies are:

- Isolation of fungal strains from natural habitat.
- Screening of pigment producing fungal strains.
- Identification of pigment producing fungal strains.
- Screening of suitable media for the enhanced growth and pigment production from isolates.
- Optimization of pigment producing fungal strain.

Chapter 6

Material and Research Methodology

6.1 Isolation of fungal strains from various soil sources (Aneja 2003).

Soil samples collected from different regions of Punjab, these regions include Phagwara, Jalandhar, and Lovely Professional University, from rice field and near to Industries. The soil sample spreaded on the potato dextrose agar (PDA) and incubate plates at 28°C for 48 hours.

6.2 Purification of Fungal strains (Aneja 2003).

The isolated colonies purified by sub cultured from master plates into PDA slants under aseptic conditions following standard protocol and incubated at 28° C for 48 hours.

6.3 Screening of pigment producing fungi strains:

6.3.1 Pigment production of fungal strains on solid medium (Stanly *et al*., 2013)

In this study five different media used for purified fungal isolates and observed their growth and pigment production. These five media are- Potato Dextrose Agar (PDA), Nutrient Agar (NA), Rose Bengal Agar (RBA), Yeast Malt Agar (YMA) and Czapex Dox Agar (CDA). Each petri plate were poured with 20ml sterilized medium and allowed each petri plate for solidification. After solidification precultured fungal isolates has been inoculated inside the medium incubated at 28°C for 7 days and observation recorded for their growth and pigment production.

6.4 Identification of fungal strain (Macroscopic and Microscopic characterization)

6.4.1 Macroscopic study of isolated fungal strains (Gilman, 2001).

The fungal isolated plates incubated at 28°C for 48-168 hours and macroscopic characteristics for color, pigment production and elevation were recorded.

6.4.2 Microscopic study of fungal strains (Alexopoulous and Mims, 1979,Gillman 1998 and Aneja and Mehrotra,1990)

The study of microscopic characteristic through Lactophenol Cotton Blue is done. A small drop of dye placed on clear slide and transferred a small amount of the fungal culture, preferably with upper part of the colony which is spores. Slowly teased the material by needles then mixed with dye. Placed a cover slip over the material and then

observed under microscope at different magnifications. Avoid bubble formation. Flame the needle while making slide.

6.5 Pigment Production

6.5.1 Pigment Extraction Method (Shivalkar *et al.*, 2014)

Extraction of Extracellular and Intracellular pigments from different fungal strains was carried out by the following steps:

Extraction of Extracellular Pigments:

- Inoculated fungal strain into 100ml of Potato Dextrose Broth (PDB)
- At 6000rpm centrifugation is done for 10 minutes
- Supernatant is separated by whatman filter paper for extracellular pigments
- Extracted pigment from supernatant by using 10ml of solvent (acetone) and absorbance was measured at 520nm.

Extraction of Intracellular Pigments:

- All the pellets were collected
- After collecting all pellets, 0.1N HCL(1:10) is added
- Placed the flasks in waterbath at 90°C for 10mins
- Cooled in ice water for 10mins
- Centrifugation is done at 6000rpm for 10mins
- After centrifugation supernatant is filtered by Whatsmann filter paper
- Extracted pigment from supernatant by using 10ml of solvent (acetone) and absorbance was measured at 520nm.

6.6 Characterization by Spectrophotometer at 500nm (Stanly *et al.*, 2013)

Both extracellular and intracellular material were taken for their absorbance by Spectrophotometer and their quantification done at 520 nm.

6.7 Optimization of pigment producing fungi strains (Stanly *et al.*, 2013)

6.7.1 Effect of pH

The effect of cultural conditions like different pH (3-9) on different pigment producing strains was studied separately by inoculating the previous old culture of each fungal strain into the medium and then kept at incubator at 28°C for 24 days. The growth and the pigment production was done by the extracellular and intracellular method and after that observed their absorbance at 520nm.

6.7.2 Effect of Carbon source

Various carbon sources such as glucose, fructose and sucrose were amended separately into the medium (PDB) at a concentration of 2%. Each isolates was inoculated to the media and incubated at 28°C for 24 days. After incubation the growth and pigment production were observed and quantified.

6.7.3 Effect of Nitrogen source

Various nitrogen sources such as sodium nitrate, urea and peptone were amended separately into the medium (PDB) at a concentration of 2%. Each isolates was inoculated to the media and incubated at 28°C for 24 days. After incubation the growth and pigment production were observed and quantified.

Chapter 7

Results and Discussion (Experimental Work)

9.1 Sample site:

A total of 5 sites, from in and around region of Jalandhar and Phagwara region were selected for the study. The sites have been shown in Table 9.1(a).

Table 9.1(a) Number of fungal forms isolated from different soil samples.

Serial No.	Sample Site	Site Code	No. of fungi
1	Agricultural field near the main gate, Lovely Professional University (Punjab)	S1	5
2	Agricultural field near Girls hostel 6B, Lovely Professional University (Punjab)	S2	6
3	Agricultural field near the Bus stand SABSBT, Jalandhar (Punjab)	S3	6
4	Agricultural Rice field, opposite the 25 block, Lovely Professional University (Punjab)	S4	4
5	Sugar mill Industry Phagwara,(Punjab)	S5	4

9.2 Isolation of fungal strains from samples:

Different soil samples from Jalandhar and Phagwara were taken for the isolation of different types of fungi. A total number of 25 fungal strains were isolated from 5 samples. Maximum fungi have been isolated from S2 and S3 followed by S1 and S4 and S5.

9.3 Purification of fungal strains (Aneja 2003)

The isolated fungal strains are purified by sub cultured from master plates into PDA slants under the aseptic conditions following standard protocol and incubated at 28°C for 48 hours. The figure has been shown in 9.3(a)

Figure 9.3(a) Purified fungal strains

9.4 Screening of pigment producing fungal strains:

9.4.1 Pigment production of fungal strains on solid medium (Stanly *et al.*, 2013)

In this study different five media used for different fungal isolates and observed their growth and pigment production. These five media are- Potato Dextrose Agar (PDA), Nutrient Agar (NA), Rose Bengal Agar (RBA), Yeast Malt Agar (YMA) and Czapex' Dox Agar (CDA). All fungal strains showing different characteristics. Some fungal strains showing best growth on PDA media like *Penicillium* sp. (JS-2), *Fusarium* sp. (RF-2), *Penicillium* sp. (IS-1), *Aspergillus* sp. (JS-1), Unidentified sp. (RF-1) and *Aspergillus* sp. (LPUS-1). *Penicillium* sp. (PS-1) fungal strain showing best growth on YMA as shown in table 9.4.1(a).

Table 9.4.1(a) Cultural characteristics of fungal strains on different 5 media.

Sample		PDA	NA	RBA	YMA	CDA
JS-2	**Growth**	++++	++	++	+++	+++
	Colony morphology and pigment production	Green colony with irregular margin and showing Yellow color pigment.	Green colony with irregular margin and showing no pigment.	Green colony with irregular margin and showing light yellow color pigment.	Green colony with irregular margin and showing yellow color pigment.	Green colony with irregular margin and showing light yellow color pigment.
RF-2	**Growth**	++++	+	++	+	+
	Colony morphology and pigment production	Pink cottony colony with irregular Margin and showing pink color pigment.	Pink cottony colony with irregular margin and showing pink color pigment.	Pink cottony colony with irregular margin and showing pink color pigment.	Pink cottony colony with irregular margin and showing pink color pigment.	Pink cottony colony with irregular margin and showing pink color pigment.
PS-1	**Growth**	+++	+	++	++++	+++
	Colony morphology and pigment production	Brown color colony with irregular margin and showing dark yellow color pigment.	Brown color colony with irregular margin and showing no pigment.	Brown color colony with irregular margin and showing light yellow color pigment.	Brown color colony with irregular margin and showing dark yellow color pigment.	Brown color colony with irregular margin and showing dark yellow color pigment.
IS-1	**Growth**	++++	+	++	+++	++
	Colony morphology and pigment production	Green colony with irregular margin and showing dark red color pigment.	Green colony with irregular margin and showing no pigment.	Green colony with irregular margin and showing light red color pigment.	Green colony with irregular margin and showing light red color pigment.	Green colony with irregular margin and showing light red color pigment.

Sample		PDA	NA	RBA	YMA	CDA
JS-1	**Growth**	++++	+	++	++++	+++
	Colony morphology and pigment production	Black spore forming colony with irregular margin and showing no pigment.	Black spore forming colony with irregular margin and showing no pigment.	Black spore forming colony with irregular margin and showing no pigment.	Black spore forming colony with irregular margin and showing no pigment.	Black spore forming colony with irregular margin and showing no pigment.
RF-1	**Growth**	++++	++	++	++	++
	Colony morphology and pigment production	Dark green colony with circular margin and showing no pigment.	Dark green colony with circular margin and showing no pigment.	Dark green colony with circular margin and showing no pigment.	Dark green colony with circular margin and showing no pigment.	Dark green colony with circular margin and showing no pigment.
LPUS-1	**Growth**	++++	+	++	+++	++
	Colony morphology and pigment production	Parrot green spore forming colony and showing no pigment.	Parrot green spore forming colony and showing no pigment.	Parrot green spore forming colony and showing no pigment.	Parrot green spore forming colony and showing no pigment.	Parrot green spore forming colony and showing no pigment.

9.5 Identification of fungal strains

9.5.1 Macroscopic characteristics

A total of seven pigment producing fungal forms were obtained from various soil samples. The macroscopic characteristics of seven pigment producing fungal strains has been shown in table 9.5.1(a) and in Figure 9.5.1 (b-h)

Table 9.5.1(a) Macroscopic characteristics of isolated Seven pigment producing fungal strains.

Isolates	Form	Colony color	Reverse side color	Elevation	Lower Pattern	Texture
JS-1	Irregular	Black	Creamish with light Black	Raised	Veined	Powdery
JS-2	Circular	Green	Yellow	Flat	Smooth	Powdery
PS-1	Irregular	Dark Brown	Brown	Flat	Smooth	Powdery
RF-1	Circular	Dark Green	Light Brown	Flat	Smooth	Powdery
RF-2	Circular	Pink	Dark Pink	Raised	Smooth	Cottony
LPUS-1	Circular	Parrot Green	Creamish with light Green	Flat	Veined	Powdery
IS-1	Irregular	Greenish with light Yellow	Red	Flat	Smooth	Powdery

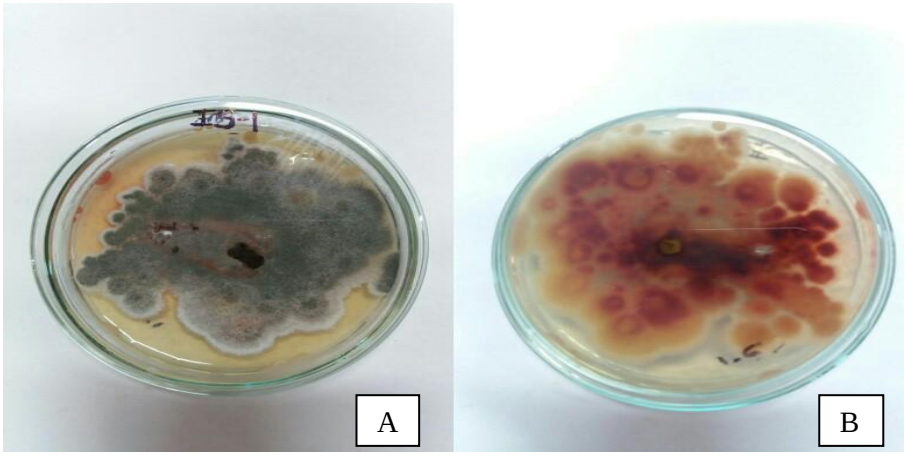


Figure 9.5.1(b) Fungal strain *Penicillium* sp. (IS-1)

A: Front View of Petriplate **& B**: Back View of Petriplate

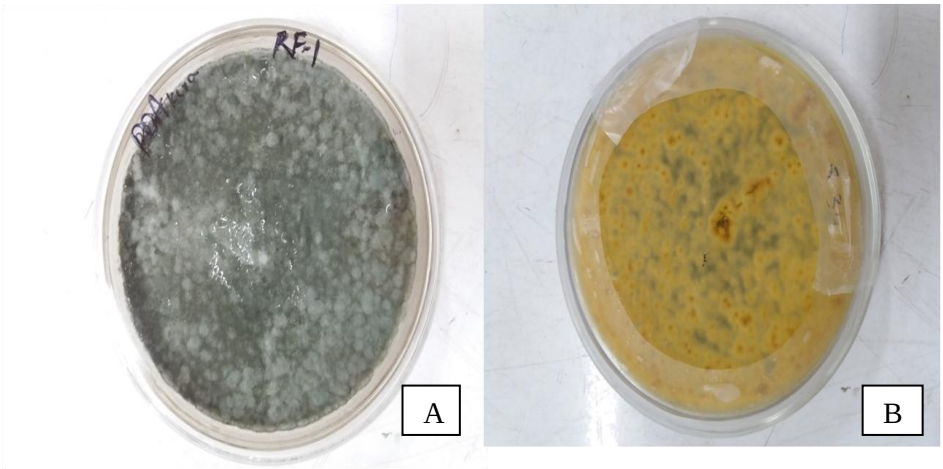


Figure 9.5.1(c) Fungal strain Unidentified sp. (RF-1)

A: Front View of Petriplate & **B**: Back View of Petriplate

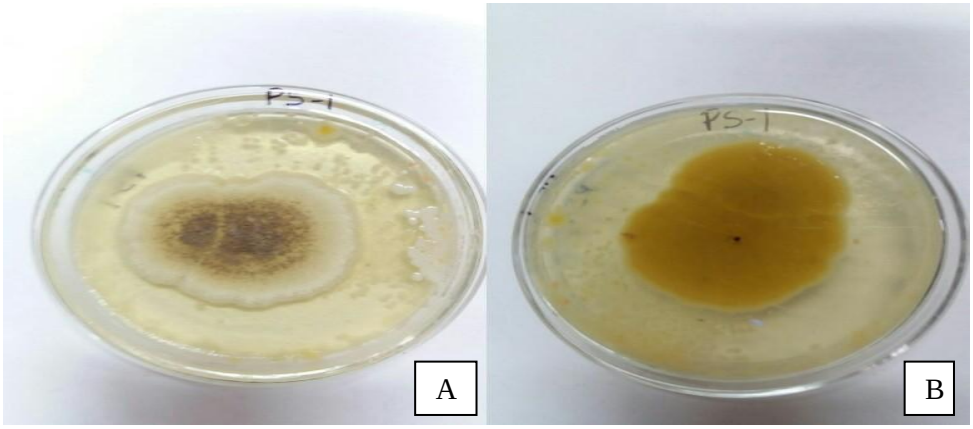


Figure 9.5.1(c) Fungal strain *Penicillium s*p. (PS-1)

A: Front View of Petriplate **& B**: Back View of Petriplate

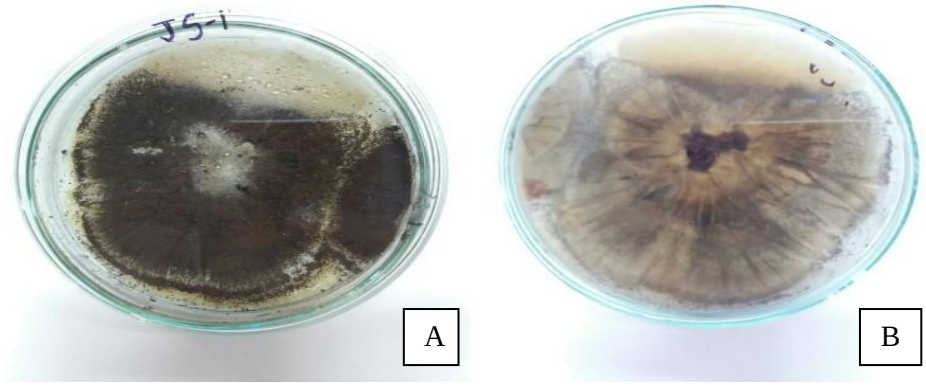


Figure 9.5.1(d) Fungal strain *Aspergillus* sp. (JS-1)

A: Front View of Petriplate **& B**: Back View of Petriplate

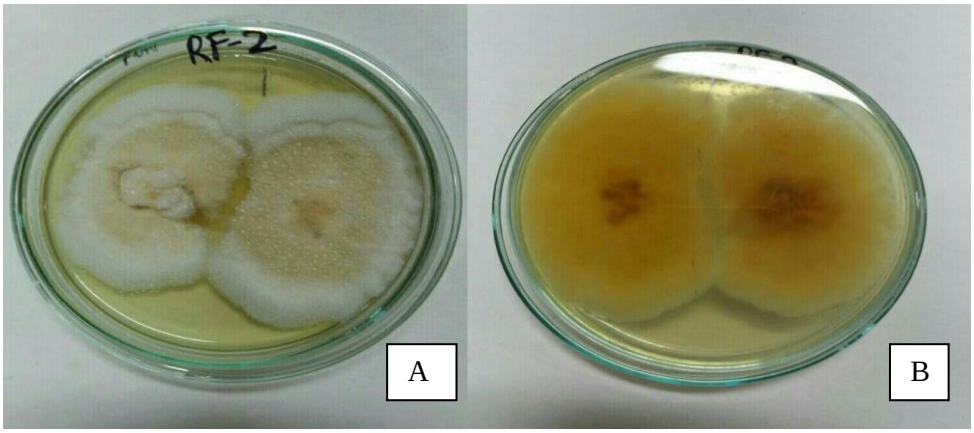


Figure 9.5.1(e) Fungal strain *Fusarium* sp. (RF-2)

A: Front View of Petriplate **& B**: Back View of Petriplate

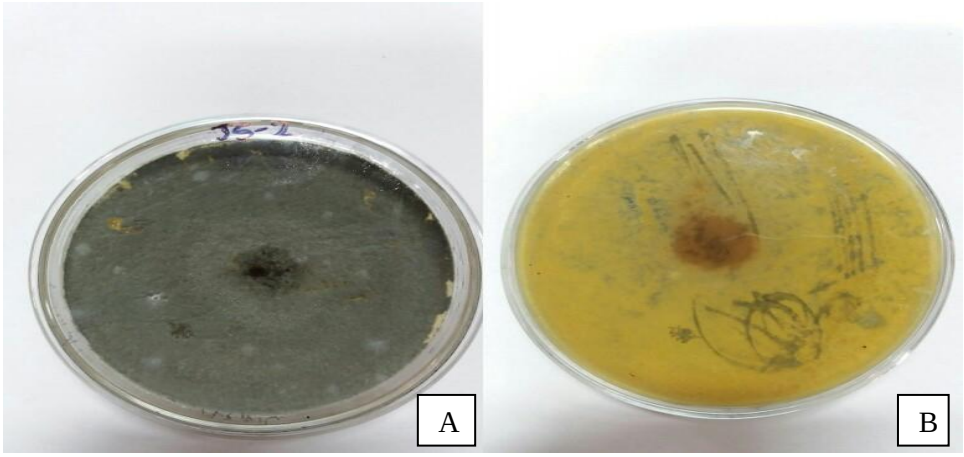


Figure 9.5.1(f) Fungal strain *Penicillium* sp. (JS-2)

A: Front View of Petriplate **& B**: Back View of Petriplate

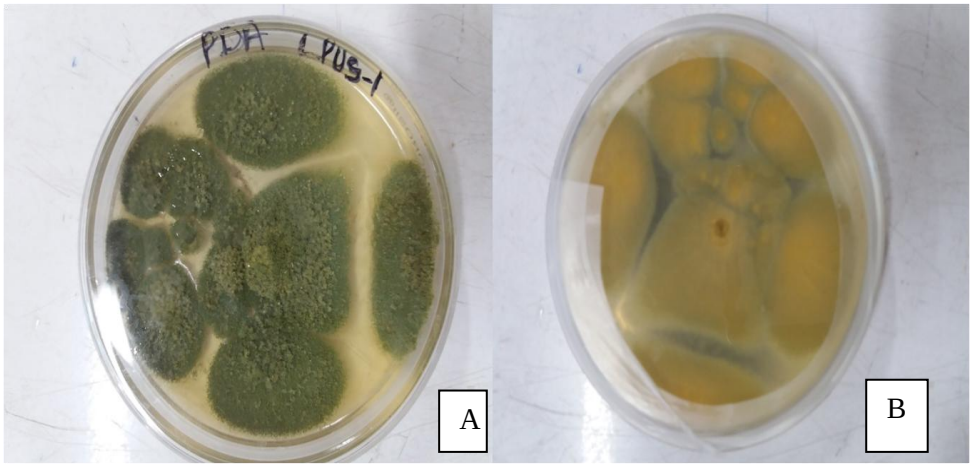


Figure 9.5.1(g) Fungal strain *Aspergillus* sp. (LPUS-1)

A: Front View of Petriplate **& B**: Back View of Petriplate

9.5.2 Microscopic characteristics observed by Lacto phenol Cotton Blue.

Microscopic characteristics of fungal strains were studied under light microscope at 40X and 100X magnification. According to the morphological characteristics (Alexopoulos and Mims 1979), (Gillman 1998 and Aneja and Mehrotra, 1990) we observed different characteristics and observed its microscopic view which are as under in figure 9.5.2(a-d)

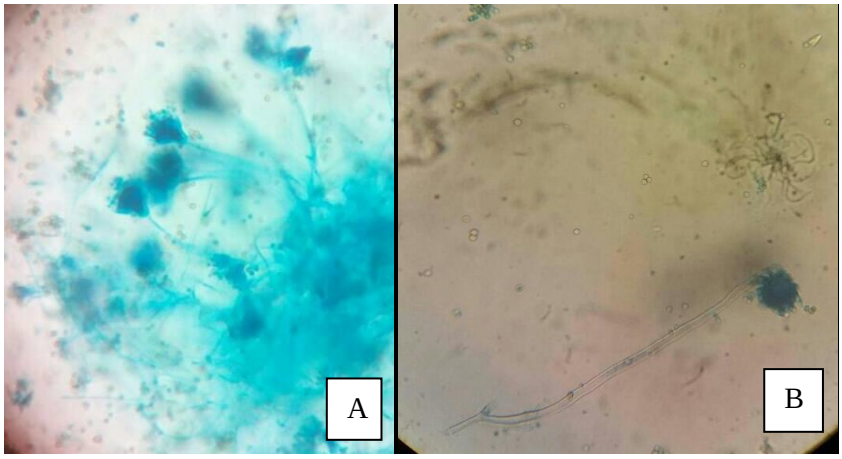


Figure 9.5.2(a) Microscopic View of fungal strain under 40X

A: *Penicillium* sp. (IS-1) & **B:** *Aspergillus* sp. **(**LPUS-1)

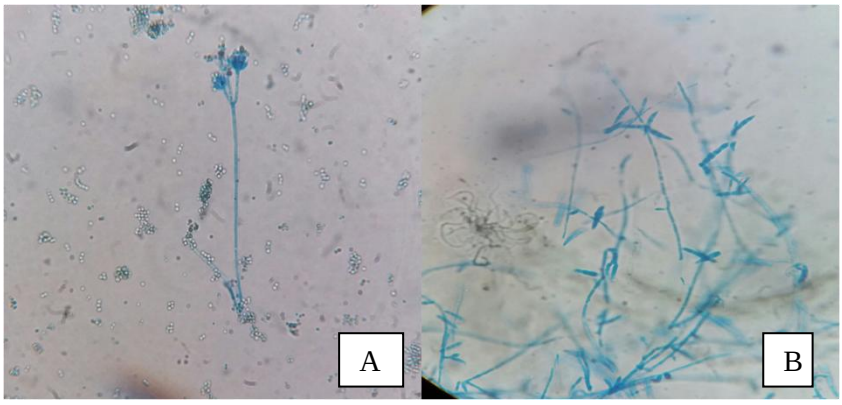


Figure 9.5.2(b) Microscopic View of fungal strain under 40X

A: *Penicillium* sp. (JS-2) & **B:** *Fusarium* sp. (RF-2)

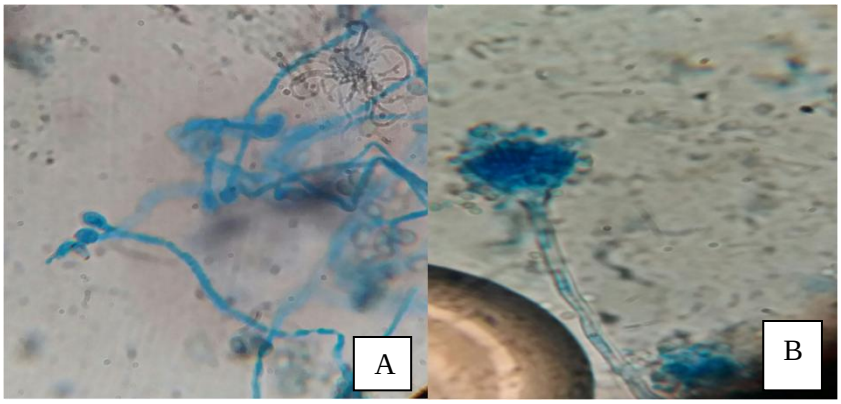


Figure 9.5.2(c) Microscopic View of fungal strain under 40X

A: Unidentified sp. **(**RF-1) & **B:** *Penicillium* sp. (PS-1)

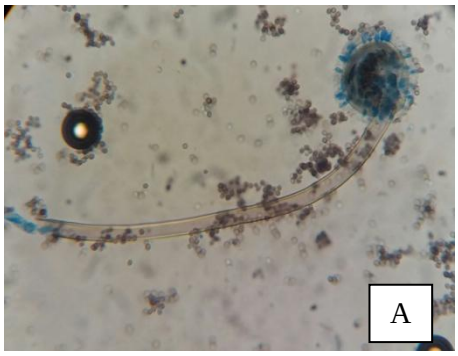


Figure 9.5.2(d) Microscopic View of fungal strain under 40X

A: *Aspergillus* sp. **(**JS-1)

9.6 Genus level characterization

All different seven fungal strains showing different characteristics under microscope. The two fungal strains showing characteristics like *Aspergillus* spp., three fungal strains showing characteristics like *Penicillium* spp., one strain showing characteristics like *Fusarium* sp. and one is unidentified. The data has been shown in table 9.6(a)

Table 9.6(a) Microscopic characteristics and genus level characterization

Isolates	Genus	Characteristics
PS-1	*Penicillium* sp.	Hyphae have produced conidiophores which has given rise to vesicle, brush like structure observed.
LPUS-1	*Aspergillus* sp.	Hyphae produces condiophore Abundantly from foot cells, spores were seen.
JS-1	*Aspergillus* sp.	Vesicle produced attached to condiophore, spores also observed.
JS-2	*Penicillium* sp.	Small brush like structures observed which are attached to condiophore.
RF-1	Unidentified sp.	Oval like structures attached to the condiophore
RF-2	*Fusarium* sp.	Fusiform to sickle shaped cells showing septate
IS-1	*Penicillium* sp.	Small compact brush Like Structures were Observed attached to the condiophore

9.7 Pigment Extraction Method (Shivalkar *et al.*, 2014)

Production of Extracellular and Intracellular pigments from isolated fungal were carried out and taken their absorbance at 520nm. The pigment production were observed at different days (8^{th}, 16^{th} and 24^{th} day), at pH-4. In Extracellular pigment production *Penicillium* sp. (IS-1) and unidentified sp. (RF-1) gives maximum absorbance at 24^{th} day. In Intracellular pigment production *Aspergillus* sp. (JS-1) gives maximum absorbance at 24^{th} day and these strains are taken for the optimization method. The data has been shown in table 9.7(a) and figures have been 9.7.1(b-n).

Table 9.7(a) Production of Extracellular and Intracellular pigments.

	Absorbance 8th day	**Absorbance 16th day**	**Absorbance 24th day**	
Sample	**Extracellular**	**Extracellular**	**Extracellular**	**Intracellular**
RF-1	0.115	0.161	0.433	0.427
RF-2	0.100	0.345	0.391	0.102
JS-1	0.117	0.206	0.211	1.208
JS-2	0.250	0.323	0.384	0.711
PS-1	0.194	0.211	0.264	0.294
IS-1	0.199	0.315	0.487	0.299
LPUS-1	0.112	0.172	0.096	0.676

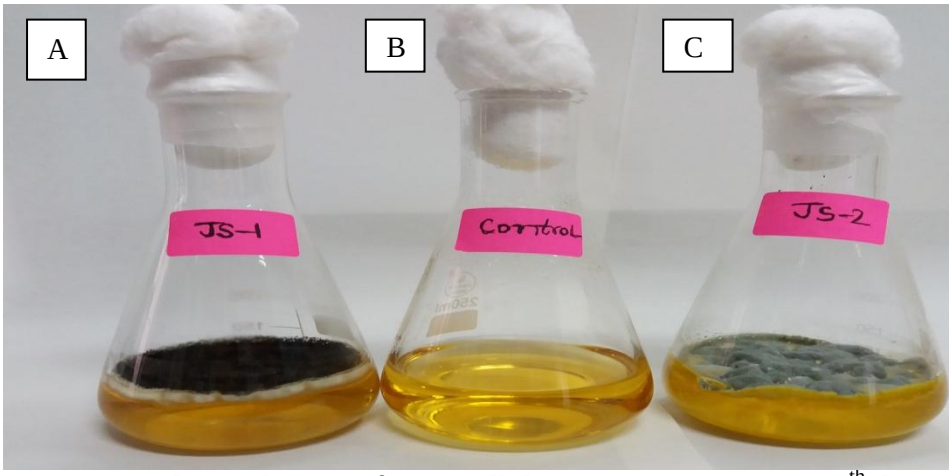


Figure 9.7.1(b) Production of Extracellular pigments on PDB at 8th day

A: *Aspergillus* sp. **(**JS-1), **B:** Control & **C:** *Penicillium* sp. (JS-2)

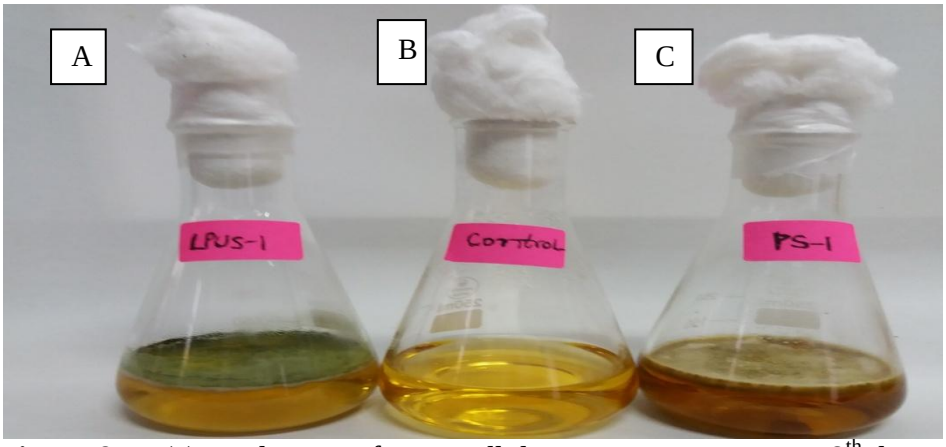


Figure 9.7.1(c) Production of Extracellular pigments on PDB at 8th day

A: *Aspergillus* sp. **(**LPUS-1), **B:** Control & **C:** *Penicillium* sp. (PS-1)

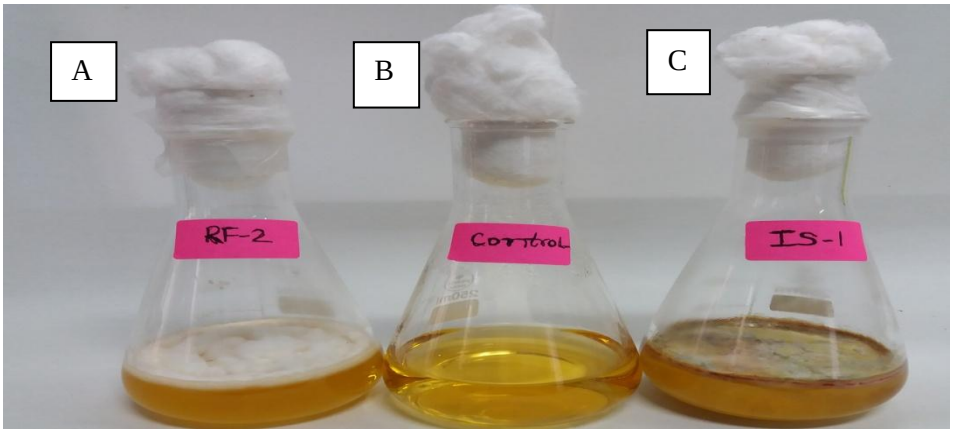


Figure 9.7.1(d) Production of Extracellular pigments on PDB at 8th day

A: *Fusarium* sp. (RF-2), **B:** Control & **C:** *Penicillium* sp. (IS-1)

Figure 9.7.1(e) Production of Extracellular pigments on PDB at 8th day

A: Control & **B:**Unidentified sp. (RF-1)

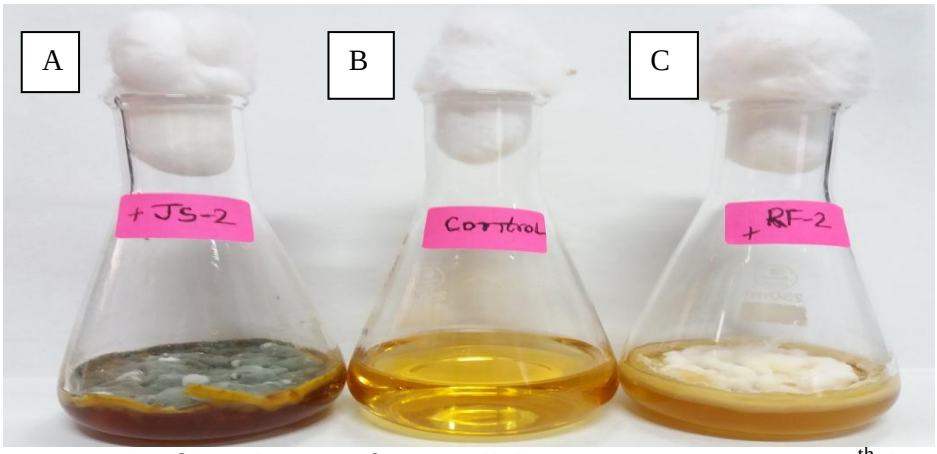


Figure 9.7.1(f) Production of Extracellular pigments on PDB at 16th day

A: *Penicillium* sp. (JS-2), **B:** Control & **C:** *Fusarium* sp. (RF-2)

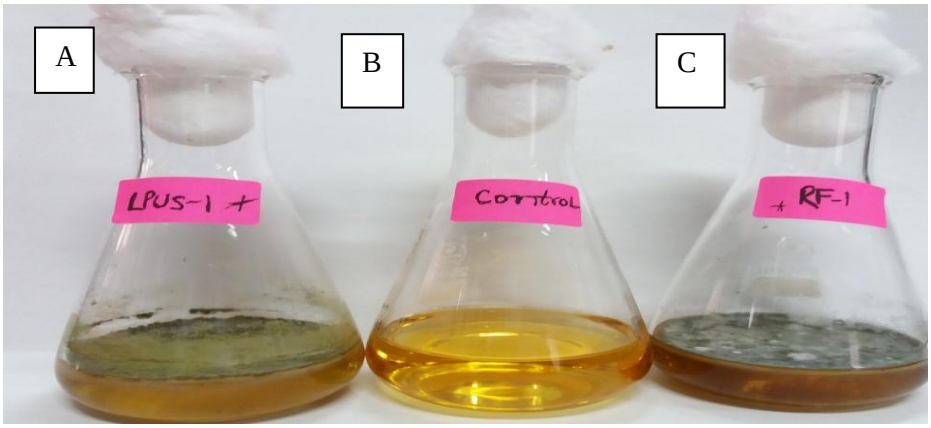


Figure 9.7.1(g) Production of Extracellular pigments on PDB at 16th day

A: *Aspergillus* sp. (LPUS-1), **B:** Control & **C:** Unidentified sp. (RF-1)

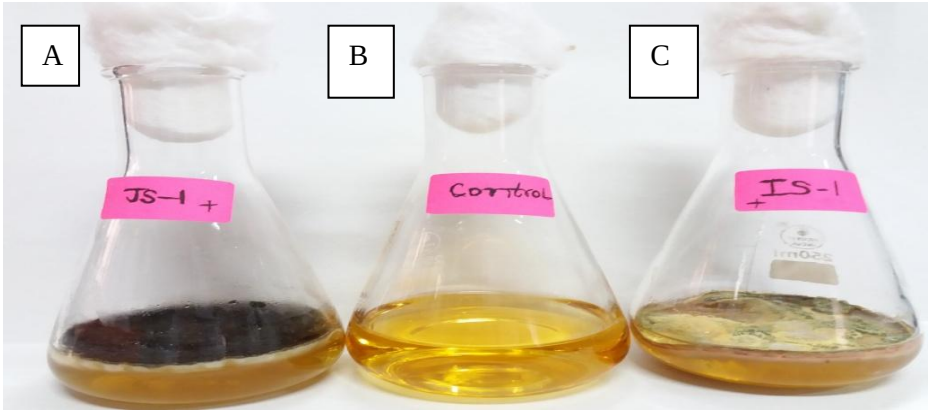


Figure 9.7.1(h) Production of Extracellular pigments on PDB at 16th day

A: *Aspergillus* sp. (JS-1), **B:** Control & **C:** *Penicillium* sp. (IS-1)

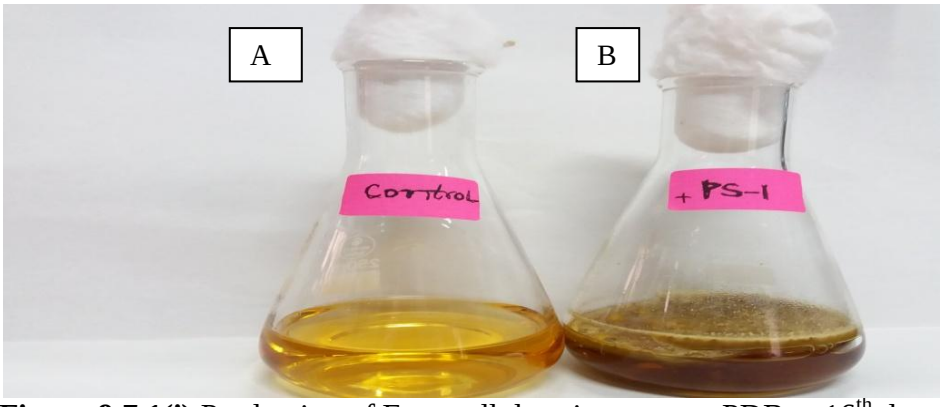


Figure 9.7.1(i) Production of Extracellular pigments on PDB at 16th day

A: Control & **B:** *Penicillium* sp. **(**PS-1)

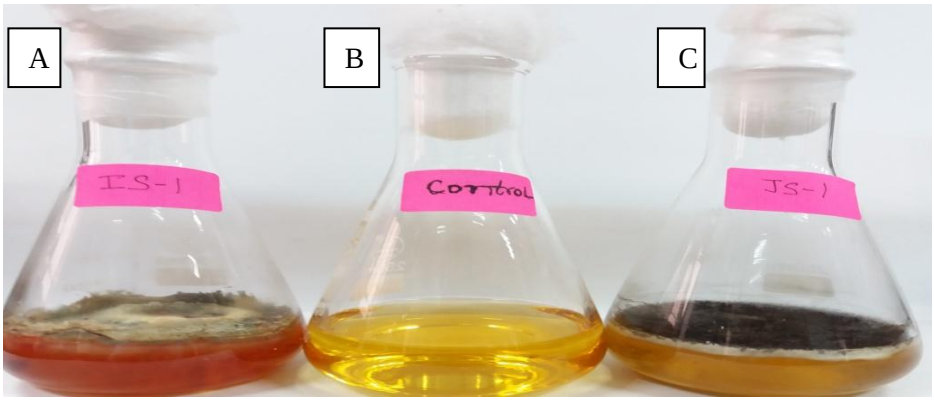


Figure 9.7.1(j) Production of Extracellular pigments on PDB at 24th day

A: *Penicillium* sp. (IS-1), **B:** Control & **C:** *Aspergillus* sp. (JS-1)

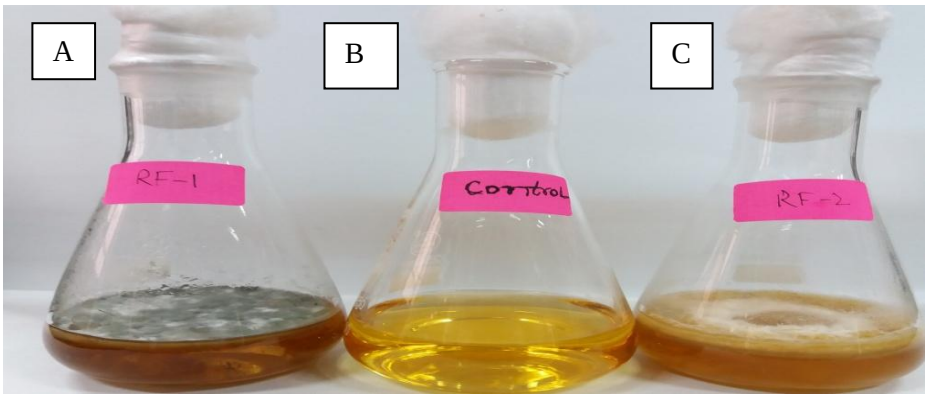


Figure 9.7.1(k) Production of Extracellular pigments on PDB at 24th day

A: Unidentified sp. (RF-1), **B:** Control & **C:** *Fusarium* sp. (RF-2)

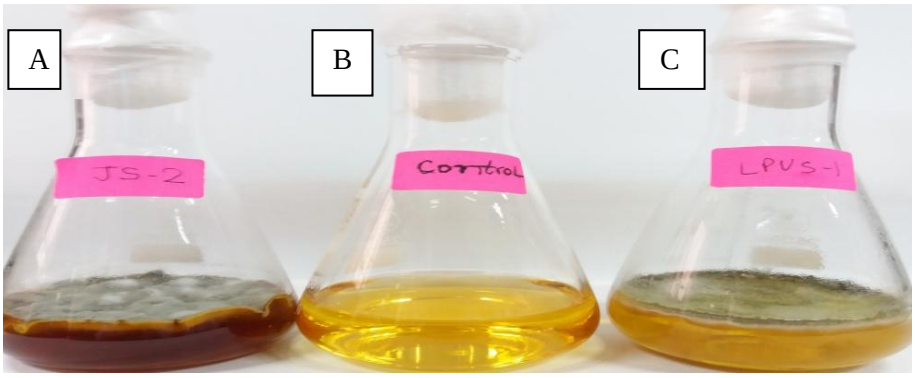


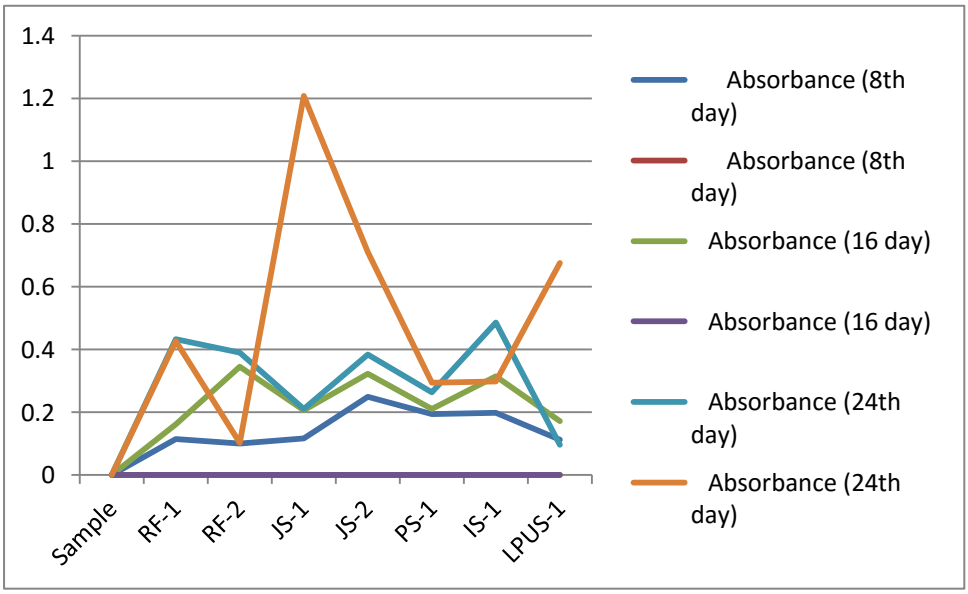
Figure 9.7.1(l) Production of Extracellular pigments on PDB at 24th day

A: *Penicillium* sp. (JS-2), **B:** Control & **C:** *Aspergillus* sp. (LPUS-1)

Figure 9.7.1(m) Production of Extracellular pigments on PDB at 24th day

A: *Penicillium* sp. (PS-1) & **B:** Control

Figure 9.7.1(n) Absorbance of production of Extracellular and Intracellular pigments of fungal strains at 520nm



9.8 Optimization of pigment producing fungi strains (Stanly *et al*., 2013)

9.8.1 Effect of pH

The effect of cultural conditions like different pH (3-9) on different pigment producing strains was studied separately. The growth and the pigment production was done by the extracellular and intracellular method and after that observed their absorbance at 520nm. The *Penicillium* sp. (IS-1) strain gives maximum absorbance at pH-9, the *Aspergillus* sp. (JS-1) strain gives maximum absorbance at pH-4 and unidentified sp. (RF-1) strain gives maximum absorbance at pH-4. The data has been shown in table 9.8.1(a) and the figures has been shown in figure 9.8.1(b-d)

Table 9.8.1(a) Production of Extracellular and Intracellular pigment on PDB at different pH

		Extracellular pigment			**Intracellular pigment**
Sample	**pH**	**Absorbance 8th day**	**Absorbance 16th day**	**Absorbance 24th day**	**Absorbance 24th day**
IS-1	pH-3	0.105	0.209	0.218	0.201
	pH-4	0.199	0.315	0.487	0.224
	pH-5	0.168	0.193	0.222	0.163
	pH-6	0.144	0.323	0.290	0.273
	pH-7	0.234	0.308	0.355	0.243
	pH-8	0.223	0.324	0.497	0.238
	pH-9	0.214	0.325	0.499	0.299
JS-1	pH-3	0.089	0.222	0.209	1.201
	pH-4	0.117	0.106	0.211	1.208
	pH-5	0.073	0.191	0.200	1.201
	pH-6	0.085	0.241	0.201	1.204
	pH-7	0.090	0.221	0.208	1.206
	pH-8	0.068	0.203	0.203	1.200
	pH-9	0.103	0.225	0.202	1.208

RF-1	pH-3	0.138	0.271	0.303	0.353
	pH-4	0.115	0.161	0.433	0.427
	pH-5	0.114	0.240	0.315	0.402
	pH-6	0.135	0.261	0.312	0.398
	pH-7	0.158	0.262	0.282	0.299
	pH-8	0.106	0.253	0.299	0.381
	pH-9	0.155	0.268	0.398	0.409

Figure 9.8.1(b) Absorbance of production Extracellular and Intracellular pigments of fungal strain *Penicillium* sp. (IS-1) at different pH (3-9)

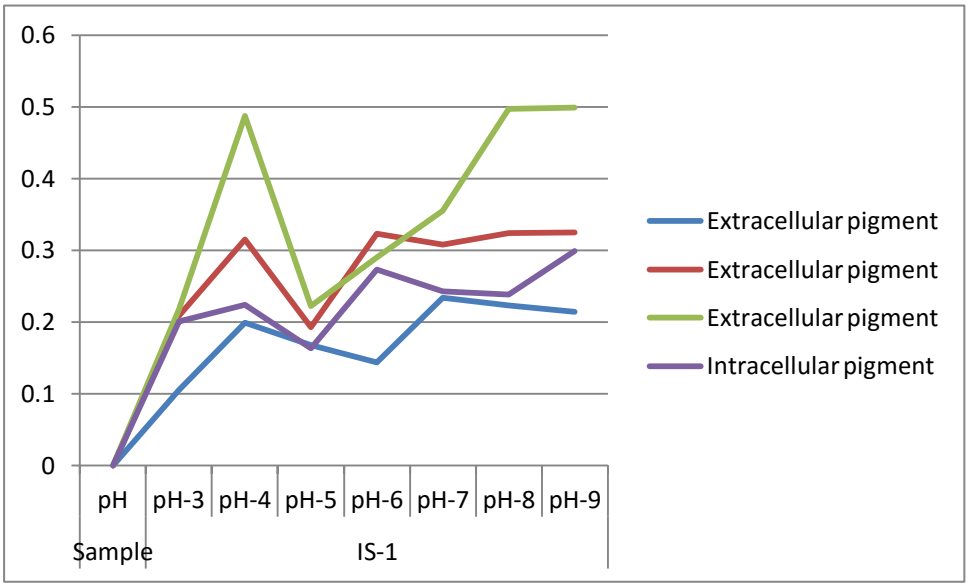


Figure 9.8.1(c) Absorbance of production Extracellular and Intracellular pigments of fungal strain *Aspergillus* sp. (JS-1) at different pH (3-9)

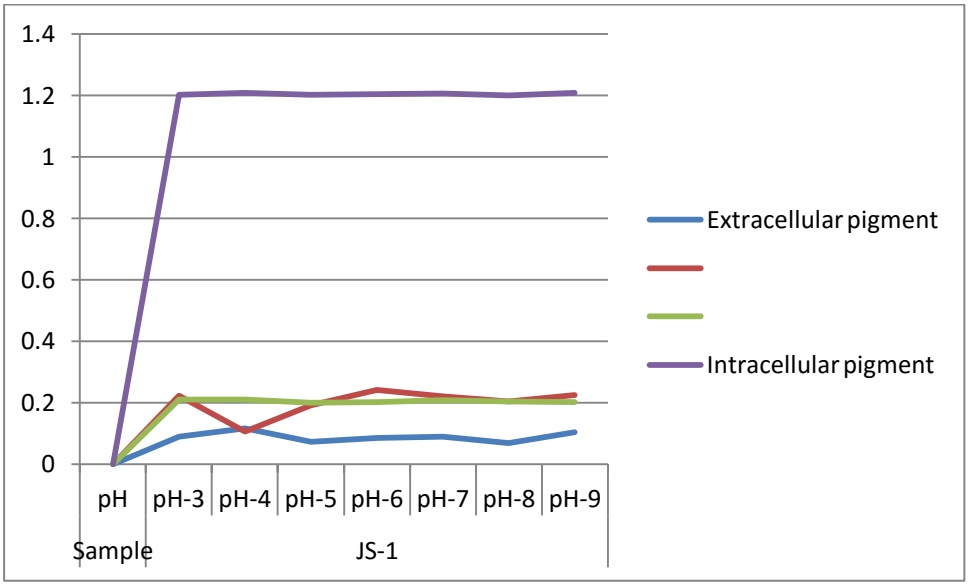
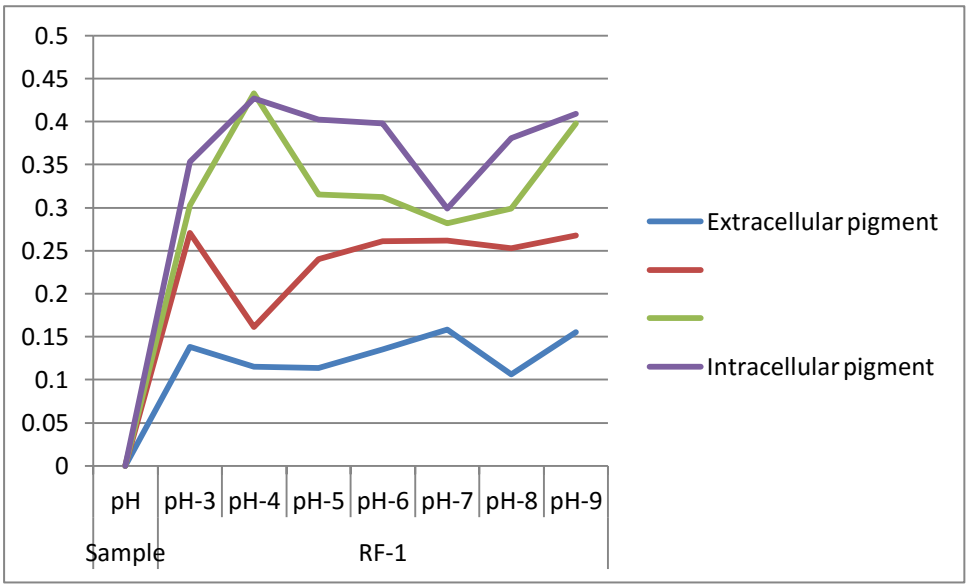


Figure 9.8.1(d) Absorbance of production of Extracellular and Intracellular pigments of fungal strain Unidentified sp. (RF-1) at different pH (3-9)



9.8.2 Effect of Carbon source and Nitrogen source

Various carbon sources such as glucose, fructose and sucrose were amended separately into the medium (PDB) at a concentration of 2%. Various nitrogen sources such as sodium nitrate, urea and peptone were amended separately into the medium (PDB) at a concentration of 2%. Growth of *Penicillium* sp. (IS-1) strain was done at pH-9, growth of *Aspergillus* sp. (JS-1) strain was done at pH-4 and growth of unidentified sp. (RF-1) strain was done at pH-4. After incubation the growth and pigment production were observed and quantified. The *Penicillium* sp. (IS-1) strain gives maximum absorbance in Glucose (Carbon source) and Peptone (Nitrogen source). The *Aspergillus* sp. (JS-1) strain gives maximum absorbance in Glucose (Carbon source) and Urea (Nitrogen source). The unidentified sp. (RF-1) strain gives maximum absorbance in Sucrose (Carbon source) and Peptone (Nitrogen source). The data has been shown in table 9.8.2(a) and in figures 9.8.2(b-d).

Table 9.8.2(a) Production of Extracellular and Intracellular pigment on PDB at different Carbon and Nitrogen source.

		Extracellular Pigment			**Intracellular Pigment**
Sample	**Carbon Source**	**Absorbance 8th day**	**Absorbance 16th day**	**Absorbance 24th day**	**Absorbance 24th day**
IS-1	Glucose	0.280	0.316	0.496	0.298
	Fructose	0.143	0.284	0.388	0.273
	Sucrose	0.243	0.309	0.422	0.226
	Nitrogen Source	**Absorbance 8th day**	**Absorbance 16th day**	**Absorbance 24th day**	**Absorbance 24th day**
	Sodium nitrate	0.245	0.311	0.408	0.296
	Urea	0.201	0.298	0.429	0.284
	Peptone	0.269	0.315	0.434	0.299
JS-1	**Carbon Source**	**Absorbance 8th day**	**Absorbance 16th day**	**Absorbance 24th day**	**Absorbance 24th day**
	Glucose	0.196	0.223	0.316	1.230
	Fructose	0.184	0.241	0.302	1.229

	Sucrose	0.156	0.220	0.284	1.209
	Nitrogen Source	**Absorbance 8th day**	**Absorbance 16th day**	**Absorbance 24th day**	**Absorbance 24th day**
	Sodium nitrate	0.114	0.273	0.296	1.200
	Urea	0.149	0.280	0.311	1.243
	Peptone	0.193	0.220	0.308	1.238
RF-1	**Carbon Source**	**Absorbance 8th day**	**Absorbance 16th day**	**Absorbance 24th day**	**Absorbance 24th day**
	Glucose	0.114	0.230	0.432	0.403
	Fructose	0.135	0.228	0.382	0.398
	Sucrose	0.155	0.243	0.444	0.421
	Nitrogen Source	**Absorbance 8th day**	**Absorbance 16th day**	**Absorbance 24th day**	**Absorbance 24th day**
	Sodium nitrate	0.101	0.193	0.402	0.388
	Urea	0.133	0.221	0.399	0.282
	Peptone	0.143	0.233	0.430	0.392

Figure 9.8.2(b) Absorbance of production of Extracellular and Intracellular pigments of fungal strain *Penicillium* sp. (IS-1) at different Carbon and Nitrogen sources at 520nm

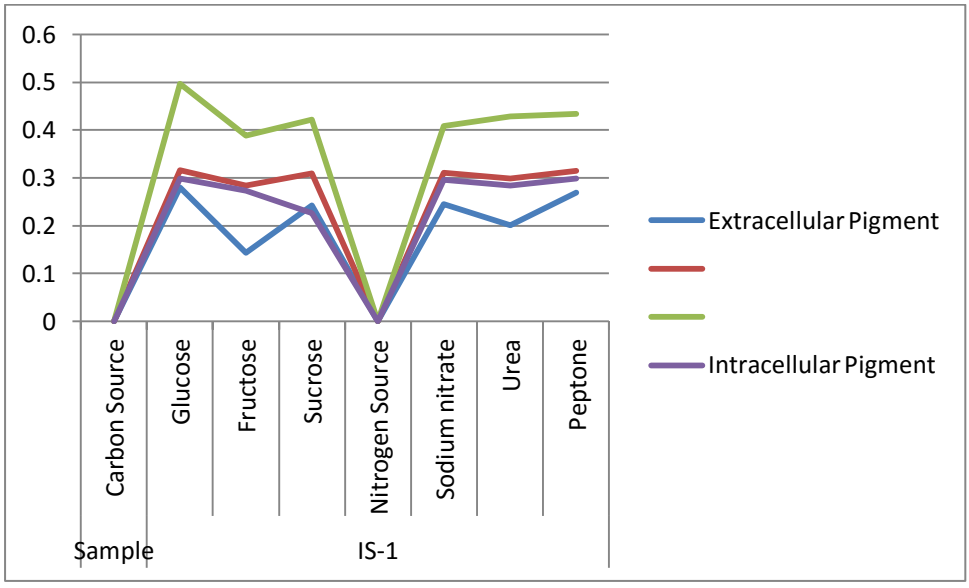


Figure 9.8.2(c) Absorbance of production of Extracellular and Intracellular pigments of fungal strain *Aspergillus* sp. (JS-1) at different Carbon and Nitrogen sources at 520nm

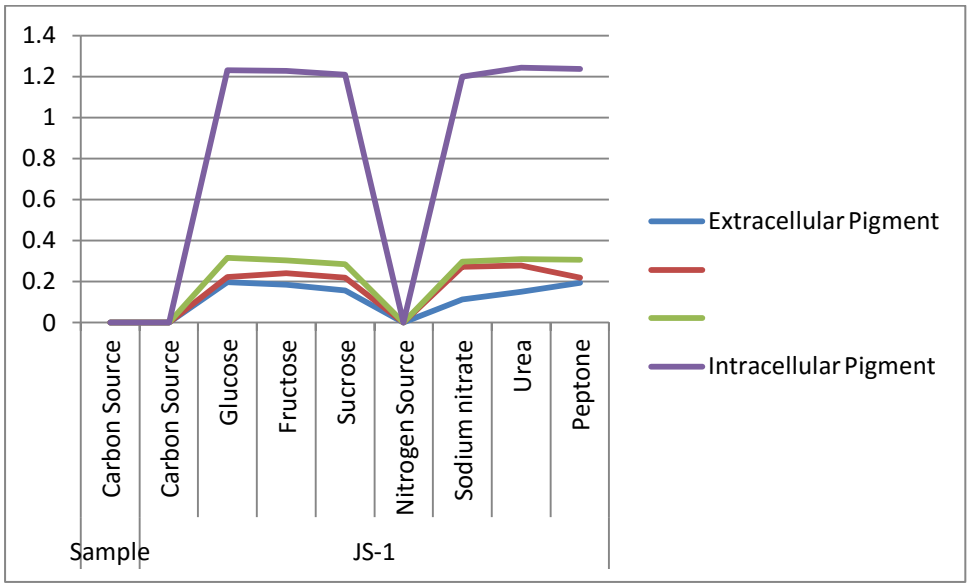
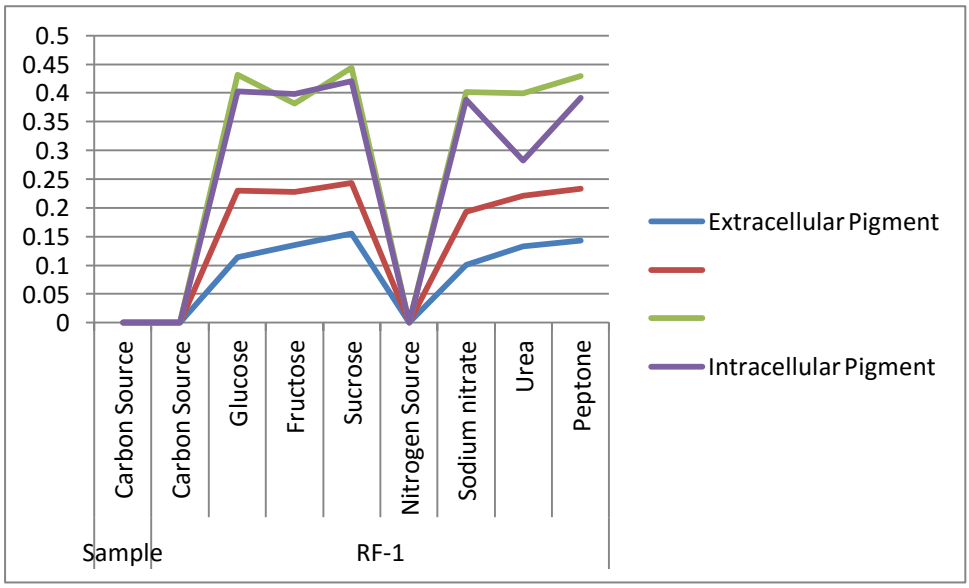


Figure 9.8.2(d) Absorbance of production of Extracellular and Intracellular pigments of fungal strain unidentified sp. (RF-1) at different Carbon and Nitrogen sources at 520nm



Chapter 8

Conclusion and Future Scope

Pigments are very useful in day to day life. These have great importance in various fields like in food industry where we can use these pigments as a food colorants, in cosmetics has great application etc. Different species of Microorganisms produce pigments like bacteria and fungi. Fungi are interesting source of pigment production microorganisms.

Different pigments like Melanin, carotenoids and lycopene have great importance in everywhere. Recent publications showed that around 47% of papers on fungi which are capable of producing pigments.

In this study, seven pigment producing strains have been isolated from soil sample. Identification has been done in which three are *Penicillium* spp., two are *Aspergillus* spp., one is *Fusarium* sp. and one is unidentified sp. Pigment extraction was done in which three fungal strains are giving maximum absorbance at 520nm. After selecting these three fungal strains, optimization has been done in which effect of pH and effect of different carbon and nitrogen sources has been done. The microbial pigments are having beneficial properties like –

Anticancer, Antibiotic, Immunosuppressive, Antiproliferative, Biodegradability etc

Many microbes produce food grade natural pigments which are used in food industry like-carotene, riboflavin etc.

Chapter 9

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Chapter 10
APPENDIX

Potato Dextrose Agar (PDA)

Components	Composition (g/l)
Potatoes (sliced washed unpeeled)	200.0gm
Dextrose	20.0gm
Agar powder	15.0gm

Rose Bengal Agar (RBA)

Components	Composition (g/l)
Dextrose	15.0gm
Papaic Digest of Soybean Meal	5.0gm
Monopotassium Sulfate	1.0gm
Magnesium Sulfate	0.5gm
Chloramphenicol	0.1gm
Rose Bengal	0.05 gm
Agar	15.0gm

Nutrient Agar (NA)

Components	Composition (g/l)
Beef Extract	3.0gm
Peptone	5.0gm
Agar	15gm

Yeast Malt Agar (YMA)

Components	Composition (g/l)
Peptic Digest of Animal Tissue	5.0gm
Yeast Extract	3.0gm
Malt Extract	3.0gm
Dextrose	10.0gm

Czapek Dox Agar (CDA)

Components	Composition (g/l)
Sucrose	30.0gm
Sodium Nitrate	2.0gm
Dipotassium Phosphate	1.0gm
Magnesium Sulfate	0.5gm
Potassium Chloride	0.5gm
Ferrous Sulfate	0.01gm
Agar	15.0gm

Potato Dextrose Broth (PDB)

Components	Composition (gm)
Potatoes, Infusion from	200.000
Dextrose	20.000