OPTIMIZATION OF NITROGEN SOURCE FOR PIGMENT PRODUCTION BY *Penicillium* sp.

SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE AWARD OF DEGREE OF

MASTER OF SCIENCE

IN

MICROBIOLOGY (HONORS)

SUBMITTED BY

NEETHU JAYAN

11307156

UNDER THE GUIDANCE OF

Dr. Loveleen Kaur

Assistant Professor

SCHOOL OF BIOSCIENCES AND BIOTECHNOLOGY

LOVELY PROFESSIONAL UNIVERSITY

PHAGWARA, PUNJAB-1444111

Academic year 2013-15

ABSTRACT

Pigments are the compounds with characteristic importance to many industries such as food, textile, cosmetic and paper. The awareness regarding adverse effects of synthetic pigments has led to an increase in the demand for natural colorants. Microbial pigments are an alternative to other natural pigments because of ease of production and independence of seasonal variations. In the present study, pigment production by *Penicillium* sp. was carried out by solid state fermentation using different substrates i.e. broken wheat supplemented with monosodium glutamate, ammonium sulfate and ammonium nitrate and organic sources: soybean meal, sunflower meal and mustard meal to determine the best nitrogen source that gives highest pigment yield. Monosodium glutamate and soybean meal showed highest pigment yield of 166 CV/gdfs and 75.75 CV/gdfs respectively on 18th day of incubation. Pigment stability was studied at different temperatures (30 to 70ºC), pH (4 to 9), exposure to visible light and organic solvents. Pigment was found to be stable upto 70ºC and at pH 5, 6 and 7. Pigment stability decreased with increase in light exposure while under dark conditions pigment retained its stability. Pigment was most stable in methanol (85.49%). Infrared spectrum (FTIR) of pigment was carried out for determining the functional groups present in the pigment.

Keywords: Pigment, Monosodium glutamate, Soybean meal, Stability, FTIR

ACKNOWLEDGEMENT

All praises and thanks to Almighty, who bestowed me, the potential, ability and opportunity to work on this dissertation for the award of degree of M.Sc. Microbiology. With great pleasure and respect, I express my heartfelt gratitude to my supervisor Dr Loveleen Kaur, Assistant Professor, Lovely Professional University, for her valuable guidance and constant encouragement in completing this research work successfully.

I express my gratitude to Dr Neeta Raj Sharma, Professor and Head of School of Biotechnology and Biosciences, Dr Ashish Vyas, Head of Department of Microbiology Domain.

I record my sincere gratitude to the lab technicians and other staff members of Biotechnology and Bioscience Department who have helped me directly or indirectly in the completion of my project work.

I would also like to express my deep thanks to my friends Kirandeep Kaur, Deeksha Agrawal, Shivangi Sharma and Sadam Hussain, who helped me in my research work.

Last but not least, with deep sense of love and respect, I express my heartfelt gratitude to my beloved parents for their support, love, and constant encouragement which motivate me to achieve success in every sphere of my life.

Neethu Jayan

DECLARATION

I hereby declare that this dissertation entitled **'Optimization of nitrogen source for pigment production by *Penicillium* sp.'** is an authentic record of my own work carried out at School of Biotechnology and Biosciences, Lovely Professional University, Phagwara, for the partial fulfillment of the award of Masters of Science in Microbiology (honors) under the guidance of Dr. Loveleen Kaur, School of Biotechnology and Biosciences, Lovely Professional University, Phagwara.

This work is my original and has not been submitted for any degree/ diploma in this or any other University. The information furnished in this dissertation is genuine to the best of my knowledge and belief.

Place: Phagwara Neethu Jayan

Date: 5/5/2015 11307156

CERTIFICATE

This is to certify that **Neethu Jayan (11307156)** has completed Dissertation project entitled **'Optimization of Nitrogen sources for the Pigment production by *Penicillium* sp.'** under my guidance and supervision. To the best of my knowledge, the present work is the result of their original investigation and study. No part of the report has ever been submitted for any other degree at any university.

This report is fit for submission and partial fulfillment of the conditions for the award of M.Sc. Microbiology (Honors).

Date:

Signature of Supervisor

Dr Loveleen Kaur
Assistant Professor
School of Biotechnology and Biosciences
Lovely Professional University
Phagwara, Punjab (India)

TABLE OF CONTENT

LIST OF TABLES

LIST OF FIGURES

CHAPTER 1

INTRODUCTION

Color is the most pleasing attribute of many articles. It provides attractive appearance to many commercial products such as food products, textiles and pharmaceutical products. Addition of color to the processed food is an old practice. Saffron, turmeric and vegetable dyes have been used to color food (Singhal and Kulkarni (1999). The term colorant refers to any chemical compound that imparts color, while pigment indicates normal constituents of cells or tissues that impart color. Artificial dyes like nitrodye, nitrosodye, azodye etc are toxic and non-biodegradable and cause environmental pollution. The awareness regarding the adverse impacts of synthetic food pigments by the consumer have given rise to a strong interest in natural colorants (Dufosse (2006). Recent increasing concern regarding the safe use of edible colorants has banned various synthetic coloring agents which may be potentially carcinogenic. As a result, there is a marked trend towards use of natural coloring additives in food and feed industry. Among the natural pigments of interest are various compounds such as carotenoids, anthocyanins, chlorophylls, melanins, betalains and quinones. Hence, natural colorants will not only be beneficial to the health of human beings, but it will be a boon for the preservation of biodiversity as harmful chemicals released into the environment while producing synthetic colorants could be stopped.

Pigments are the compounds with characteristic importance to many industries such as textile industries, paper production, food production, water science and technology and agricultural practices and researches (Tibor (2007). In food industry, pigments are used as color intensifiers, additives, antioxidants etc. (Abermound, (2011). Pigments come in different varieties of which some are water soluble. For these reasons many of these compounds have been isolated, produced and characterized (Duran *et al.,* (2002). Many companies have been utilizing plants and animals as a source of pigment. However, these pigments have numerous drawbacks such as instability and low water solubility, and are often not available throughout the year. Microbial pigments are of industrial interest owing to the

stability, solubility and availability of cultivation technology (Gunasekaran and Poorniammal (2008).

Microorganisms are associated with all the foods that we eat and are responsible for production of many food products by the process of fermentation. Industrial production of pigments by microbial fermentation process is a good approach as it has several advantages such as cheaper production, easy and fast growth, higher yield through strain improvement, colors of different shades, no lack of raw material, no seasonal variation ,easier production and extraction (Babitha (2009). Natural pigments possess anticancer activity, contain pro-vitamin and have some desirable properties such as stability to light, heat and pH. These colors are biodegradable and environment friendly. Some microbial pigments also have antioxidant, immunosuppressive and antiproliferative activity. That's why production of microbial pigments is one of the emerging fields of research to explore its potential for various industrial applications.

Many bacteria, mold, yeast and fungi are widely studied for their pigment producing capability. Among them are: filamentous fungi *Monascus* sp., *Penicillium* sp., *Paecylomices* sp. (Blanc *et al.*,(1994); Mendez-Zavala *et al.*,(2007); Mapari *et al.*, (2006). *Monascus* sp. has been studied in detail for its production of more than six red-colored, orange and yellow pigments. On the other hand, some pigments homologous to those of *Monascus* sp. by strains of *Penicillium* sp. had been reported (Mapari *et al.*, 2006; Jiang *et al.*, 2005). Bacterial strains like *Serratia marcescens*, *Pseudomonas magneslorubra*, *Vibrio psychroerythrous, Alteromonas rubra*, *Streptoverticillium rubrireticuli* and *Streptomyces longisporus* (Dufosse, (2006); Gupta *et al.*, (2011), yeast strains like *Rhodotorula glutinis*, *Xanthophyllomyces dendrorhous*, *Yarrowia lipolytica*, *Crytococcus sp.* and *Phaffia rhodozyma* (Dufosse (2006) have shown their potential in pigment production.

In the present study, pigment production by *Penicillium* sp is studied. *Penicillium* is a well known fungi occurring in a diverse range of habitats from soil to air to vegetation and various food products. Its main function in nature is to decompose organic materials as well as producing diverse range of mycotoxins (Frisvad *et al.*,

(2004). It is a genus of ascomycetous fungi. The mycelium consists of highly branched network of hyphae.

All the microbial processes which lead to the formation of commercially important product are called fermentation. There is high interest in pigment production by fermentation because of the intensive metabolite production that is possible in bioprocess (Babitha, (2009). Microbial fermentations are classified based on the process in which they are produced. These include submerged state and solid state fermentation. Solid state fermentation (SSF) has emerged as an effective alternative to submerged fermentation because recent studies report that SSF provides a more adequate habitat for fungi, resulting in high pigment production in a relatively low cost process. The substrates used in SSF supply the basic nutrients to the microorganisms and serve as an anchor for the cells. A variety of substrates used for SSF are obtained from agriculture byproducts and wastes such as rice bran, wheat bran, rice straw, sugarcane bagasse, hay, fruit and vegetable waste, paper pulp, coconut coir etc. An ideal pigment producing microorganism should be capable of using a wide range of carbon and nitrogen sources, have tolerance to pH, temperature and minerals concentration, possess moderate growth conditions, give reasonable color yield, should be non toxic and non pathogenic and easily separable from cell biomass.

The present study has been aimed at producing pigment from *Penicillium* sp. isolated from soil. *Penicillium* is a non- toxigenic fungal strain to serve as food colorants. The production of pigments from such potentially safe host is advantageous over traditional processes that involve *Monascus* sp., which risks the production of certain mycotoxins.

CHAPTER 2

TERMINOLOGY

2.1 Pigments: Pigment is defined as the coloring agent which can be produced either by living organisms or chemical reagents.

2.2 Bio pigments: The natural pigments that are derived from normal constituents of cells and tissues of plants, animals or microbes.

2.3 Centrifugation: It is the process which involves the use of centrifugal force for the sedimentation of heterogeneous mixtures or to separate two immiscible liquids

2.4 Fermentation: It is a metabolic process that converts sugars to acids, gases and alcohols.

2.5 Solid state fermentation: The fermentation process in which microorganisms grow on solid material without the need of free liquid.

2.6 Submerged fermentation: It utilizes the free flowing liquid materials such as nutrient broths, molasses, corn steep liquor etc. The enzymes or metabolic by products are secreted in to the broth.

2.7 UV Visible Spectroscopy: It refers to absorption spectroscopy or reflectance spectroscopy in the UV- Visible spectral region. It uses the light in the visible and adjacent ranges which directly affects the perceived color of the chemicals involved.

2.8 Fourier Transform Infrared Spectroscopy: It is a technique to identify the functional groups present in certain molecule. It can be used to identify a pure compound or to detect the presence of specific impurities.

CHAPTER 3

REVIEW OF LITERATURE

Pigment is defined as the coloring agent which can be produced either by living organisms or chemical reagents. The history of pigment application dates back to prehistoric cave painting, which gives evidence of the use of ocher, hematite, brown iron ore and other mineral-based pigments more than 30,000 years ago (Daniel, 1986).

Modern pigments serves more than just being appealing. They are used in various fields of everyday life such as food production (Pandey *et al.,* 2003), paper production, agricultural practices, textile industries, water science and technology (Tibor, 2007). Other industries where pigments have wide applications include cosmetics, paints, soaps, beverages, biomedicines, inks and tinting.

3.1 Types of pigments: Pigments can be classified as synthetic and natural pigments.

3.1.1 Synthetic pigments: Chemically synthesized colors were used extensively as they were easy to produce, less expensive and superior in quality, and can blend easily with food, giving no off-flavors. In pharmaceutical industry, the synthetic food dye is added in order to add color to many medicinal products, as well as to ensure the same color for all the batches of a given product. Although synthetic food dyes are long lasting and cheaper, some of them provoke allergic reactions in people. From the environmental point of view, a great variety of synthetic dyes used for textile dyeing and other industrial applications causes serious pollution when part of these dyes penetrates into waste water. Most of these compounds are toxic, carcinogenic and highly resistant to degradation (Chung *et al.,* 1992). The negative perceptions of synthetic pigments by the consumer have given rise to a strong interest in natural coloring alternatives (Dufosse, 2006).

Table 1: Pigments produced by different fungi

Fungi	Pigment	Color	Reference
Monascus sp	Monascorubramin Ankaflavin Rubropunctatin Monascin Ankaflavin *Monascus*ones	Red Yellow Orange Yellow Yellow Yellow	Blanc *et al.,* (1994)
Penicillium oxalicum var. armeniaca	Arpink Red	Dark red	Sardaryan, (2002)
Penicillium herquei	Atrovenetin	Yellow	Robinson *et al.,*(1992)
Penicillium oxalicum	Anthraquinone	Red	Sardaryan *et al.,(*2004)
Fusarium sporotrichioides	Lycopene	Red	Jones *et al.,* (2004)
Cordyceps unilateralis	Naphtoquinone	Deep blood red	Unagul *et al.,* (2005)
Neurospora crassa *Blakeslea trispora* *Fusarium sporotrichioides* *Phycomyces blakesleanus*	B- carotene	Yellow-orange	Hausmann & Sandmann, (2000) Jones *et al.,* (2004) Cerda-Olmedo,(2001)
Blakeslea trispora	Lycopene	Red	Malik *et al.,* (2012)

3.1.2 Natural pigments: Natural pigments are derived from plants, animals or microorganisms; hence also called bio pigments. Plant sources include roots, berries, flowers, barks and leaves. Red color (dyer's root from Madder plant, Brazilwood, beetroot, cranberry), orange color (stigmas of saffron flower), yellow color from (Camomile and Milkwort flowers and Weld), green color (ripe Buckthron berries,

ragweed) and blue color (Woad plant and *Spirulina*). The most important dyes extracted from animal sources are Natural Sepia (from the ink sac of the cuttlefish), Crimson (from the Kermes Louse) and Tyrian purple (from the Murex shellfish) (Soltan and Shehata, (2012). Although these natural colors are safe to use, but there are some limitations: their seasonal production, variation in quality and purity from source to source, availability in limited shades, low concentration in source material, difficulties in extraction from the source, instability during storage and use, high cost (Sharma,(2014). That's why microbes are now extensively studied and isolated for deriving pigments for commercial use.

3.2 Microbial production of pigments

Microorganisms like bacteria, mold, fungi and yeast produce a number of stable pigments like chlorophyll, carotenoids, anthoquionone, flavanoids, quinines etc. Among the microorganisms that produce pigments are: filamentous fungi *Monascus* sp., *Penicillium* sp., *Paecylomices* sp (Blanc *et al.,* (1994); Mendez- Zavala *et al.,* (2007); Mapari *et al.,* (2006), yeasts *Paffia rhodozyma* and microalgae *Porphyridium cruentum* and *Haematoccocus pluvialis* (Boussiba, (2000); Yoshimura *et al.,* (2006). Species of *Serratia, Cordyceps, Streptomyces Rhodotorula, Sarcina*, *Bacillus* sp., *Achromobacter*, *Yarrowia* also produce a large number of pigments.

There are various advantages of producing microbial pigments such as easy and fast growth which leads to high productivity of product (Jiang *et al.,* (2005), production independent of seasonal and geographical variations, color of different shades and growth on cheaper substrates such as agro- industrial waste residues which reduces water and environmental pollution (Joshi *et al.,* (2003). Also microorganisms can be genetically manipulated which helps in altering the metabolic pathways in the micro organism so that the color characteristics can be enhanced, resulting in high product yield. Apart from this, microbial pigments have numerous beneficial properties such as immunosuppressive, antibiotic, anticancer, antiproliferative and biodegradability. Microbial pigments have broad area of application in food, pharmaceutical and textile industries. Riboflavin, β-carotene, Lycopene, Arpink Red, and *Monascus* pigments are used in food industries. In pharmaceutical industry pigments like Anthocyanin,

Prodigiosin and Violacein are widely used to treat diseases. Several microbial pigments are also used in textile industry.

Amongst various microorganisms, many species of fungus have attracted special attention because they have the capability of producing different coloured pigments showing high chemical stability (Hajjaj *et al.,* (2000). *Monascus* sp., has been studied in detail for its production of more than six red colored, orange and yellow pigments.

The major pigment producing fungal species include *Monascus, Penicillium, Fusarium, Neurospora, Aspergillus.* Various pigment molecules produced by different fungi are given in table 1.

3.3 Pigment production

Pigment production can be carried out either by solid state or submerged fermentation.

Submerged fermentation (SmF) utilizes the free flowing liquid materials such as nutrient broths, molasses, corn steep liquor etc. This technique is best suited for the microorganisms which require high moisture for their growth such as bacteria. There are some disadvantages of SmF over SSF. High energy consumption, high water activity becomes the major cause of contamination, water makes downstream processing difficult and expensive, high amount of waste liquid is generated which makes the dumping difficult.

Solid state fermentation (SSF) is defined as the fermentation process in which microorganisms grow on solid material without the need of free liquid. In SSF, the moisture necessary for microbial growth exists in an absorbed state or in complex with solid matrix. Solid state fermentation offers greatest possibilities when fungi are used because fungi typically grow in nature on solid substrates such as pieces of wood, stems and roots (Bhargav *et al.,* 2008). Solid state not only provides nutrients but also act as an anchorage for the growth of fungi. Substrate itself is the source of energy and requires no medium for growth of microorganisms. There are various advantages of SSF over SmF for the pigment production. The low water availability reduces the possibilities of contamination by bacteria and yeast, SSF provides similar environment conditions to those of the natural habitats for fungi and inoculation with

spores facilitates its uniform dispersion through the medium. The use of various cheaply available substrates through solid state fermentation is feasible economically also for pigment production.

The pigment production by *Monascus* sp by SSF and SmF was compared and it was observed that maximum pigment was obtained in solid state (0.59AU/gds) than in submerged fermentation (0.34AU/gds) (Sugumaran *et al.,* 2014). The optimal culture conditions for pigment production by *Penicillium purpurogenum* IAM 15392 was investigated and it was found to be as follows: soluble starch 2%, peptone, pH 9.0, temperature 30^{o}C, agitation 200 rpm and inoculum age 4 days. The properties of pigment and their residual content were also studied (Arai *et al.,* (2013). The conditions required for the growth, sporulation and pigment production by *Fusarium moniliforme* KUMB1201 were optimized which was found to be: 2% glucose as carbon source, 2% yeast extract as nitrogen source, temperature 28^{o}C and pH 5.5 (Pradeep *et al.,* (2013). The feasibility of use of jackfruit seed powder as a substrate for pigment production by *Monascus purpureus* in solid state fermentation was investigated. A pigment yield of 25 OD Units/g dry fermented substrate was achieved. The optimized conditions are: 50% moisture content, incubation temperature of 30^{o}C for 7 days and 9x 10^{4} spores/g dry substrate inoculum (Babitha *et al.,* (2006).

Kamalam *et al.,* (2012) investigated the feasibility of jackfruit seed powder as a substrate for the production of pigments by *Monascus ruber* in solid-state fermentation (SSF).A pigment yield of 2.85 OD Units/gdfs was achieved by employing jackfruit seed powder. Pigment production was carried out with 50% initial moisture content, incubation temperature 30 °C, 1 × 106 spores/g dry substrate inoculum and an incubation period of 14 days.

3.4 Agro-industrial wastes as substrate

Agriculture sector generate a lot of waste in the form of seeds, whey, waste liquid, molasses, peels, bagasse, wheat and rice straw, oil cakes, coffee husk, coconut coir etc. These waste materials are rich in various nutrients such as carbohydrates, proteins, minerals fibres, and vitamins. Using these waste materials not only solves

the problem of disposal but also reduces environmental pollution. Also the use of these cheaply available waste residues makes the process cost effective and environment friendly. That's why researchers have shown a great interest in utilizing these waste materials for the production of value added products such as microbial pigments.

Velmurugan *et al.,* (2011) investigated the feasibility of corn cob powder as a substrate for production of pigment by *Monascus purpureus* KACC 42430.The use of corn cob powder as a substrate lead to the yield of 25.42 OD Units/gram of dry fermented substrate. Babitha *et al.,* (2006) and Subhasree *et al*., (2011) investigated the feasibility of jackfruit seed as a substrate supplemented with different carbon sources for the production of red pigment by *Monascus purpureus* in SSF. Durian seeds were found to be a promising substrate for angkak production (fermented red rice) using *Monascus* sp. yielding 50 mg/kg of Monacolin K (Srianta *et al.*, (2012).

The utilization of kinnow peel powder has been observed as a good substrate for the growth of *Monascus purpureus* MTCC 369, which resulted in considerable amount of pigment production (Panesar, (2014). Apple pomace, waste from apple juice processing industry has been utilized for microbial pigment production (Joshi & Attri, (2006). Corn and sugarcane bagasse have been used as starch source for microbial pigment production using *Monascus* sp. (Sugumaran *et al.,* (2014). Corn steep liquor was used as a source of nitrogen and salts for red pigment production by *Monascus ruber*, thereby substituting various salts and yeast extract (Hamano and Kilikian (2006).

3.5 Effect of nitrogen source on pigment production: The production of microbial pigments is influenced by the nitrogen source used depending upon the microorganisms.

In the present study, ionic and organic nitrogen sources are used to investigate the best source which gives highest pigment yield. Ionic nitrogen sources used include monosodium glutamate, ammonium sulfate and ammonium chloride. Different substrates that have been used as organic nitrogen source include soybean meal, sunflower meal, and mustard meal. These meals are the solid residue by-product

obtained after extraction of oil. These are an important source of proteins used to feed farm animals.

Dikshit and Tallapragada (2011) used peptone, yeast extract, monosodium glutamate at 1%, 5%, 10% concentration as nitrogen source for pigment production by *Monascus purpureus.* Maximum pigment yield was observed with rice supplemented with 5% yeast extract (29.9 CVU/gds) followed by 1% yeast extract (25 CVU/gds) and for 5% MSG (25.2 CVU/gds) followed by 1% MSG (18.1 CVU/gds) whereas 10% nitrogen content in all the substrates were found to be inhibitory in nature. This strain was not able to grow on peptone media. Silveira *et al.,* (2011) used peptone, soy protein isolate and ammonium chloride as nitrogen source for pigment production by *Monascus purpureus* and found that peptone and soy protein isolate had generated the best pigment yield whereas ammonium chloride had not supported high pigment production. Pradeep *et al.,* (2013) determined that peptone and yeast extract when used as nitrogen source supported the high pigment production by *Fusarium moniliforme* KUMBF1201 as compared to the medium without these nitrogen sources.

3.6 Stability of pigment

Any change in pigment properties can be studied by exposing it to various physico-chemical properties such as temperature, pH, sunlight, UV and preservatives like sodium bisulfate, ascorbic acid and citric acid.

Hailei *et al.,* (2011) co-cultured *Penicillium* sp HSD07B and *Candida tropicalis* that resulted in the production of a red pigment. Toxicity and mutagenic nature of pigment was determined. The pigment showed no acute toxicity in mice and was not mutagenic in Ames test. The pigment was stable between pH 2 and 10 and temperature 10-100^{o}C. Pigment also exhibited good photo stability and resistance to oxidation by hydrogen peroxide and reduction by Na_2SO_3.

Kaur *et al.,* (2009) checked the light, pH and heat stability of red pigment from *Monascus purpureus* MTCC 410. It was found that pigment extracted from SSF was less sensitive to light than from SmF. Pigment was thermolabile over 70^{o}C heating

and when exposed to 100^{o}C, color changed from red to blackish. Pigment was found to be relatively stable at pH from 6.0- 8.0.

The stability of *Monascus* pigments produced by SSF using corn cob substrate was investigated. The pigments were stable at acidic pH, high temperatures, and in salt solutions (Velmurugan *et al.,* (2011).

Silveira *et al.,* 2011 investigated the temperature and pH stability of red pigment produced by *Monascus purpureus* and found that pigment showed high stability at temperature range of 30-60ºC and at near neutral pH values 6.0-8.0.

3.7 Characterization of pigment: Pigment characterization can be done by various analytical tools such as HPLC, FTIR etc.

3.7.1 High Performance Liquid Chromatography (HPLC) is one of the most powerful tools in analysis of sample. It separates, identify and quantify the compounds present in any sample that can be dissolved in a liquid. The liquid solvent containing sample mixture is passed under high pressure through column filled with solid adsorbent material. Different compounds in sample interact differently with the solid adsorbent material, leading to the separation of the different compounds. Jiang *et al.,* (2005) analyzed the red pigment produced by *Penicillium* sp. by HPLC equipped with two 510 pumps and a spectrophotometer detector. The extract solution was analysed by using a Bondapak C18 column at 25ºC. Hailei *et al.,* (2011) analyzed the *Monascus*-derived pigment and the red pigment in *Penicillium* sp. co-cultured with *Monascus* and compared their retention time after HPLC analysis.

3.7.2 Nuclear Magnetic Resonance (NMR) Spectroscopy: NMR spectroscopy is a technique that exploits the magnetic properties of certain atomic nuclei. It determines the physical and chemical properties of atoms or the molecules in which they are contained. Chintapenta *et al.,* (2014) identified the compound present in the pigment produced by mangrove *Penicillium* using ^{1}H and ^{13}C NMR Spectroscopy and found the compound to be [2-(4-acetyl phenyl) acetic acid]. Chiba *et al.,* (2006) analysed the magenta pigment produced by fungus using ^{1}H NMR Spectroscopy.

3.7.3 Fourier Transform Infrared Spectroscopy (FTIR) uses an infrared radiation which is passed through the sample. Some of the infrared radiation is absorbed by the sample and some of it is passed through. The resulting spectrum gives the absorption and transmission, creating a molecular fingerprint of the sample. The spectrum represents absorption peaks which correspond to the frequencies of vibrations between the bonds of the atoms making up the material. FTIR analysis gives information about the unknown material, quality and consistency of sample and amount of component in a mixture. It has several advantages such as rapid results are obtained, highly sensitive, self caliberated and mechanical simplicity which makes the results obtained by FTIR accurate and reproducible.

Dhale and Vijay-Raj (2009) analyzed the red pigment produced by the *Penicillium* sp NIOM-02 grown on sugarcane bagasse. The FTIR spectrum of fraction was recorded on a Nicolet 5700 (Thermo Electron Corporation, Madison, WI, US.) spectrometer at room temperature. The fraction was mixed with KBr pellet and scanned in the range of 4000-400 cm-1. The KBr pellet was used as blank. Kamalam *et al.,* (2012) studied the infrared spectrum of red pigment produced by *Monascus ruber.* The main absorbance peak were obtained at 3424.95, 2915.12, 2361.62, 1654.35, 1458.06 and 1045.16cm-1. The peak at 3424.95cm-1 showed a broad peak so it might be OH group. The peak at 2915.12 is CH2 group. The peaks at 2361.62 and 1654.35 cm-1 indicated that there might be NH group present. The peaks at 1458.06 cm-1 and 1045.16cm-1 indicated the presence of C=O groups.

3.8 Applications of fungal pigments

Fungal pigments possess antioxidant and antibiotic properties. Lycopene produced by *Fusarium sporotrichioides* have anti-oxidative properties (Jones *et al.,* 2004). Beta-carotene produced by various fungi acts as antioxidant and has potential positive properties against certain diseases. *Monascus purpureus* produces pigments that contain compounds such as monocolin, responsible for inhibiting cholesterol synthesis and can decrease the level of total cholesterol and serum triglycerides (Mukherjee and Singh, 2011). *Monascus* pigments are used as food colorant such as in processed seafoods. Monascarubromine, a red pigment is widely used in meat, fish

and ketchup. Arpink red pigment produced by *Penicillium oxalicum* contain chromophore of anthraquinone type (Dufosse *et al.,* 2006). It is used as food colorant. The amount of Arpink red pigment in various food products was recommended by Codex Alimentarius Commission. *Monascus* derived pigments are used as a staining agent for endoscopy and are reported to be biologically safe in nature (Yamamoto *et al.*, 2006).

CHAPTER 4

RATIONALE AND SCOPE OF STUDY

Pigments are the compounds which find importance in many industries such as food, pharmaceutical, paper, textile and cosmetics. They are used as additives, color intensifiers, antioxidants etc. The demand for natural sources of pigments is increasing day by day because of the awareness of positive health benefits of natural compounds and the adverse effects caused by the synthetic pigments on environment as well as human health. Therefore, there has been an increased interest among the scientific community to explore various natural sources of pigments and their potentials in different areas.

The natural pigments derived from plants and animals have various drawbacks such as seasonal production, less stable and variation in color. Hence, microbes are being exploited as potential source for the pigment production.

Microbial fermentation has several advantages such as cheaper production, abundance of raw material and no seasonal variation because cheaply available agro-industrial residues can be utilized for the pigment production. Agro industrial residues which are otherwise burnt or dumped as such can be utilized and recycled which not only solves the problem of environmental pollution but also provide a good source for pigment production. The microbial pigments have also been found to have some health benefits. This area still needs to be explored more. Hence, there is wide range of scope in studying and identifying more pigment producing microbes having commercial importance.

In present study, the effect of different nitrogen sources on pigment production by *Penicillium* sp. was evaluated so as to determine the best nitrogen source which supports the high yield of pigment.

CHAPTER 5

OBJECTIVES OF THE STUDY

The broad objective of present work is to study the effect of ionic and organic nitrogen sources on pigment production by *Penicillium* sp. Keeping in mind the importance of pigment and effect of nitrogen sources on pigment production, the work was planned under following objectives:

1. To study the effect of ionic and organic nitrogen source on pigment production by *Penicillium* sp.
2. To study the stability parameters of pigment produced by *Pencillium* sp.
3. To partially characterize the pigment produced by *Penicillium* sp. by FTIR.

CHAPTER 6

MATERIALS AND RESEARCH METHODOLOGY

6.1 Materials

Equipments required in project work: Incubator, Laminar Air Flow Hood, Hot Air Oven, Centrifuge, Tray Drier, Rotary Shaker, Microscope were availed from the laboratories of School of Biotechnology and Biosciences, Lovely Professional University, Phagwara, Punjab.

The glasswares, chemicals and reagents were procured from CDH, Loba Chemie and Hi Media, School of Biotechnology and Biosciences, Lovely Professional University, Phagwara, Punjab.

6.2 Maintenance of microorganism:

The previously isolated *Penicillium* sp from soil was cultured on Potato Dextrose Agar (PDA) medium. Media was prepared and autoclaved at 121^{o}C at 15 psi pressure for 15min. PDA was then poured in petriplates under sterile conditions, allowed to solidify, inoculated with the culture and incubated at 28±2^{o}C for 5-7 days. Stock cultures were maintained on PDA slants, preserved in refrigerator at 4^{o}C and sub-cultured once every 4 weeks.

Composition of PDA (HiMedia):

Materials	Grams/Litre
Potatoes, infusion form	200.0
Dextrose	20.0
Agar	15.0
Final pH (at 25^{o}C)	5.6±0.2

6.3 Morphology study

6.3.1 Colony morphology:

Penicillium culture was grown on Potato Dextrose Agar (PDA) plates and their macroscopic characteristics: colony appearance, texture and color were analyzed.

6.3.2 Microscopic examination:

Direct microscopic examination of the colonies was performed with lactophenol cotton blue stain. A drop of lactophenol cotton blue stain was placed in the center of a clean, grease free slide. A fragment of fungal colony from the colony edge was removed with a sterile inoculating needle, placed in the drop of stain and teased gently so that it mixes with the stain. The cover slip was then placed with the help of a forcep in such a way that no air bubbles were trapped. Then the slide was observed under microscope at 10, 40 and 100X. The microscopic characteristics were recorded.

6.4 Radial growth measurement:

The experiment was carried out in replica by method of Pradeep *et al.,* (2013). Each petriplate were poured with 20 ml of sterilized PDA media and allowed for solidification. 8mm bits of 7-day old precultured *Penicillium* grown on PDA were taken out with the help of cork borer and inoculated at the centre of each Petri plate. After inoculation, petriplates were incubated at 28±2°C. The diameter of colony was recorded in two directions at right angles to each other with a scale after the interval of 24 h, till the full expansion of growth.

6.5 Pigment production:

6.5.1 Substrate preparation: Broken wheat, soybean meal, mustard meal and sunflower meal were obtained from local markets of Jalandhar, Punjab. 10 gm of broken wheat was weighed in flasks and soaked in water for 4-5 hours. After soaking, excess water was drained so as to maintain moisture content of 50- 60 % and 0.1 gm of nitrogen sources i.e. monosodium glutamate, ammonium sulfate and ammonium chloride were added in the different flasks and mixed well. In meals, water was added to 10 gm of substrate to maintain the moisture content of 50-60 %. The flasks were then autoclaved at 121^{o}C at 15psi for 15 min.

6.5.2 Inoculum preparation: Inoculum preparation for solid-state fermentation was performed as described by Babitha *et al.,* (2007). A solution of

100ml distilled water containing 0.5ml Tween 80 was prepared. 10ml of this solution was added to fully sporulated agar slant culture and the spores were scraped from the agar slants under aseptic conditions to produce a spore suspension. This spore suspension was used as inoculum.

6.5.3 Solid State Fermentation

The experimental set ups were performed in replica. The flasks containing the sterile substrates were inoculated under aseptic condition with 1ml of spore suspension and mixed well. The flasks were incubated at 28±2^{o}C for 6, 12 and 18 days to study the effect of incubation time on pigment yield.

6.5.4 Pigment extraction

When the biosynthesis of pigment was completed after 6 days of incubation, first the flasks were autoclaved at 121^{o}C at 15psi for 15min. The content of flasks were transferred to aluminium foil and dried in air drier at 50^{o}C till it is dried completely. The dried contents were then crushed using mortar and pestle and the powder obtained is stored in small air tight polythene bags.

For extracting pigment, 0.1gm of powder obtained above was dissolved in 10 ml of 80% methanol. Flasks were kept on rotary shaker at 200 rpm for 1hour and then centrifuged at 5000 rpm for 30min. The liquid was collected which contain the pigment which was analyzed spectrophotometrically. The same procedure was repeated after 12 and 18 days of incubation.

6.5.5 Quantification of pigment:

Fungal pigments were quantified by taking O.D of collected supernatant in double beam spectrophotometer at 410 nm which corresponds to maximum absorption of yellow-orange color. Dilutions were prepared wherever required. Color Value (CV) was calculated by formula:

$$CV = \frac{\text{Optical density} \times \text{Dilution} \times \text{Volume of extract (ml)}}{\text{Amount of sample (gm)}}$$

6.6 Pigment stability:

The stability of pigment obtained was studied under different temperature and pH by the method of Srivastava *et al*.,(1999) and Perumal *et al*.,(2009) The solvent stability and light stability were also studied.

6.6.1 Temperature stability:

10 ml of pigment extract was taken in different test tubes and the initial absorbance was taken at 410nm and recorded. Then test tubes are subjected to the temperature of 30, 40, 50, 60, 70, 80 and 90^{o}C in water bath for 1 hour. Test tubes were cooled to room temperature and absorbance was determined again at 410nm and percent stability was calculated by the formula:

$$\text{Percent stability} = \frac{\text{Initial absorbance} - \text{Final absorbance}}{\text{Initial absorbance}} \times 100$$

6.6.2 pH stability:

10ml of pigment extract was taken in a set of test tubes. Initial absorbance was recorded and then pH was adjusted to 4, 5, 6, 7, 8, and 9 and mixed and kept for 1 hour. After that absorbance was taken at 410nm and percent stability was calculated.

6.6.3 Light stability:

10ml of pigment extract taken in a set of test tubes was kept in light for 6 days and the controls were kept in dark. Initial absorbance was recorded and the absorbance was taken after every 24 hours and percent stability of pigment when exposed to normal light conditions and dark was determined.

6.6.4 Solvent stability:

Pigment was dissolved in 10ml of organic solvents; chloroform, acetone, ethanol, and methanol. Initial absorbance was measured. Then the test tubes were kept in dark at room temperature for 7 days. After 7 days absorbance was measured again and percent stability was calculated.

6.7 Characterization of pigment:

Pigment was characterized by Fourier Transform Infrared (FTIR) spectrophotometer**.** The FTIR spectrum of the pigment was recorded on a FT-IR 8300, Shimadzu spectrometer at room temperature. The sample was ground with KBr (spectroscopic grade) before pressed into 10 mm diameter disks under 6 tons of pressure. Under pressure, pellet disc obtained was analyzed between 4000 and 400 cm^{-1}.

CHAPTER 7

RESULT AND DISCUSSION

7.1 Morphology study:

The culture was maintained on PDA medium at 28±2^{o}C by frequent subculturing and colony morphology and microscopic examination was carried out.

7.1.1 Colony morphology:

As observed from plate 1, the colonies were found to be flat and velvety in texture. The colonies were initially white and became greenish in color with time because of sporulation. Green colored spores were observed. The pigment diffuses out and can be observed from the reverse of the plate, turning the medium surrounding the colony to yellow. The *Penicillium* sp. was found to be slow growing.

Plate 1: Growth of *Penicillium* sp. on Potato Dextrose Agar medium showing white growth with greenish center.

7.1.2 Microscopic features:

Fungal hyphae was stained with lactophenol cotton blue and observed under microscope at 10, 40 and 100X (Plate 2). Long branched septate hyphae terminating in branched conidiophores were observed. Metulae, phialides and conidia were observed at 100X. Metulae are secondary branches that form on conidiophores. Conidia were held to hyphae by metulae and metulae carry the flask- shaped

phialides. Phialides form brush- like clusters at the tip of conidiophores known as penicillus. Conidia were round or globular in shape.

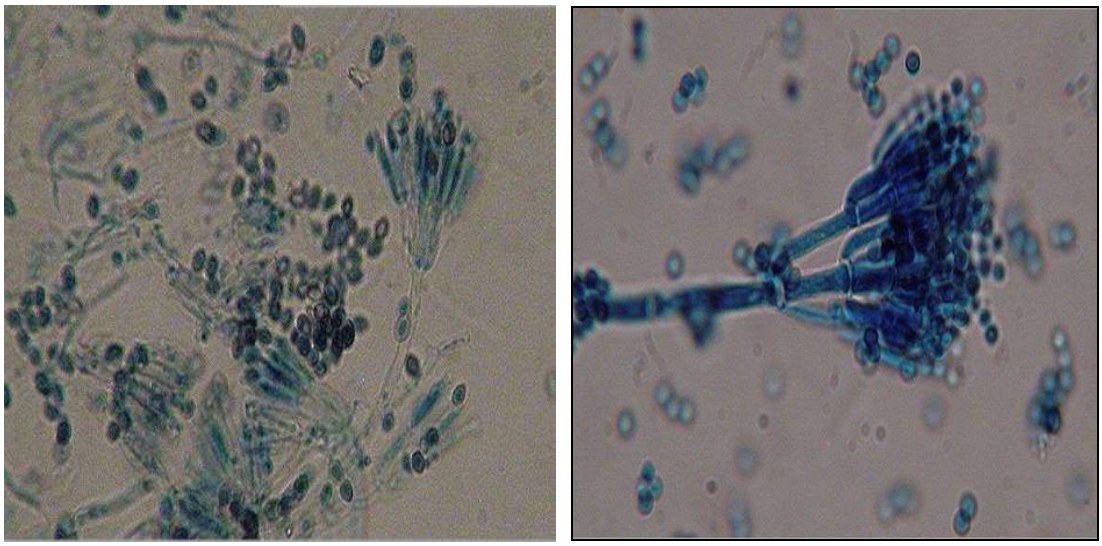


Plate 2: Microscopic view of *Penicillium* at 100X after staining with lactophenol cotton blue stain

7.2 Radial growth measurement:

Penicillium sp. was grown on PDA medium at 28±2^{o}C and the colony diameter was recorded after 24 hrs daily for 10 days to evaluate the growth rate of *Penicillium* sp. For initial 4 days (A, B, C and D) white colored mycelia were observed. From 5th day pigment was found to start diffusing out and yellow pigment was observed at the center (E, F and G). With the increase in incubation time more pigment diffused out and the medium surrounding the colony became orangish-yellow (H, I and J).

The growth rate of *Penicillium* sp. was studied by determining its colony diameter. Growth rate of 33.21mm/week was observed. Growth was rapid for initial 5 days after that marginal growth was observed for next 5 days. This may be because the *Penicillium* sp. was slow growing. Therefore, for initial 5 days the cells utilized the nutrients from the PDA medium and multiplied rapidly, hence rapid growth rate was observed. For next 5 days, because of nutrient depletion or cells being in stationary phase marginal or slow growth rate was observed.

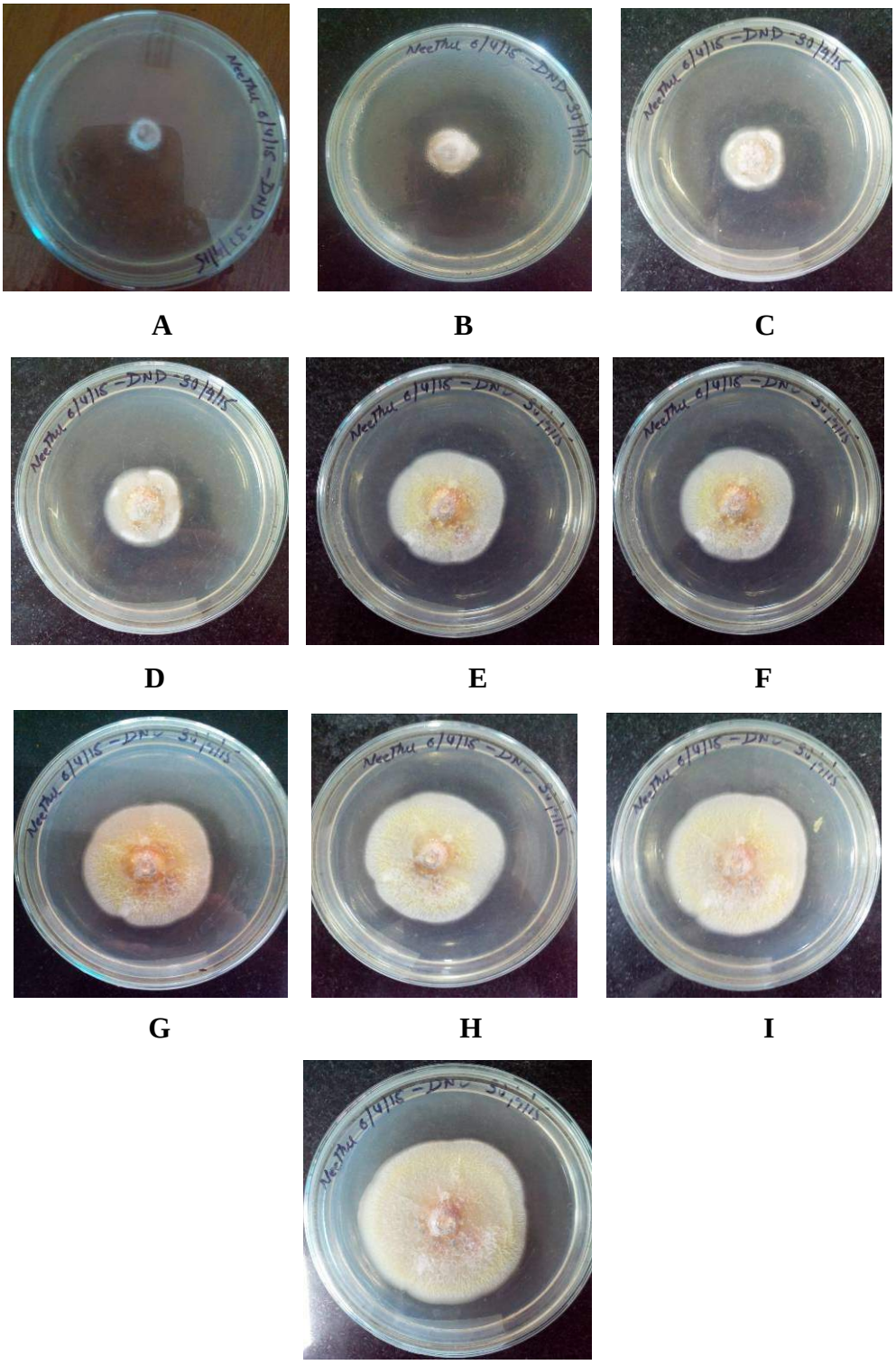


Plate 3: The measurement of radial growth of *Penicillium* sp. grown on Potato dextrose Agar medium w.r.t. number of days. A (Day 1), B (Day 2), C (Day 3), D (Day 4), E (Day 5), F (Day 6), G (Day 7), H (Day 8), I (Day 9), and J (Day 10).

Table 2: Measurement of growth of *Pencillium* sp. by determining colony diameter with time

Time (hours)	Diameter(mm)*	% increase in growth/ day
24	11.5	0
48	19	65.2
72	24	26.3
96	33.5	39.58
120	41.5	23.88
144	49	18
168	54	10.2
192	56.5	4.62
216	60	6.19
240	63.5	5.83
CD@ 5%	0.60	

*****Average of three replicatesTemperature of incubation- 28±2^{o}C, Medium used- Potato Dextrose Agar Medium

CD@ 5% Substrate: 0.83

No. of Days: 0.96

Substarte X No. of days 1.66

7.3 Pigment production:

7.3.1 Pigment production using ionic nitrogen source:

The *Penicillium* sp. when grown on broken wheat supplemented with ionic nitrogen sources showed the growth patterns as shown in Plate 4. White to grayish green colored mycelia was observed on the petriplates. With the increase in the incubation period, the growth of *Penicillium* also increased. After 18 days of incubation, the petriplate become fully covered with the mycelia. Substrate also started becoming dry after 18 Days.

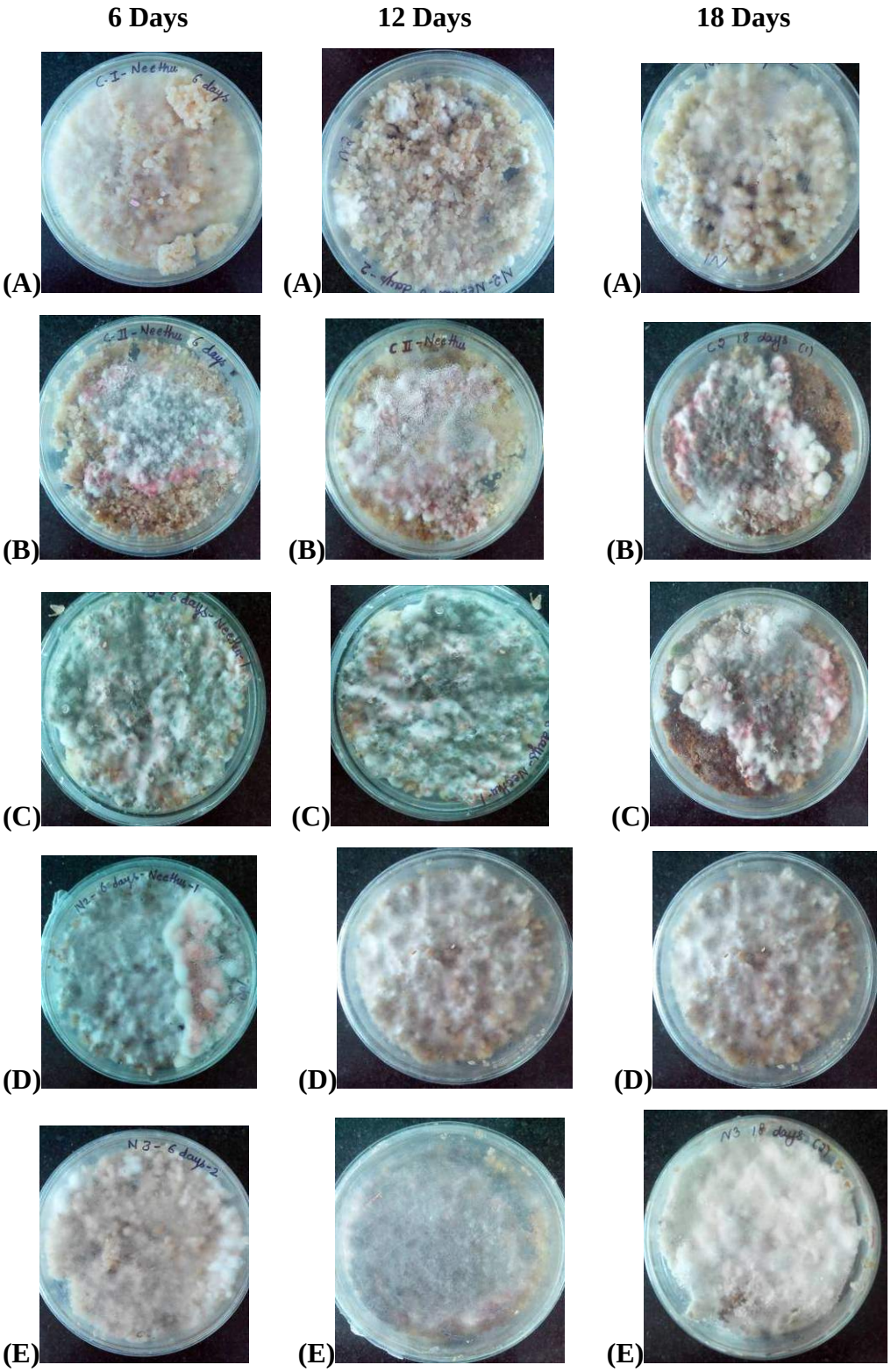


Plate 4: Growth of *Penicillium* sp. on broken wheat supplemented with ionic nitrogen sources. A (Uninoculated control), B (Inoculated control), C (Monosodium glutamate), D(Ammonium sulfate) and E (Ammonium chloride) w.r.t incubation time

7.3.2 Pigment production using organic meals as nitrogen source

The growth pattern of *Penicillium* sp. on different organic sources are as shown in plate 6. Sunflower meal showed the highest biomass production. Grayish black colored growth was observed on meals.

6 Days

A **B** **C**

12 days

A **B** **C**

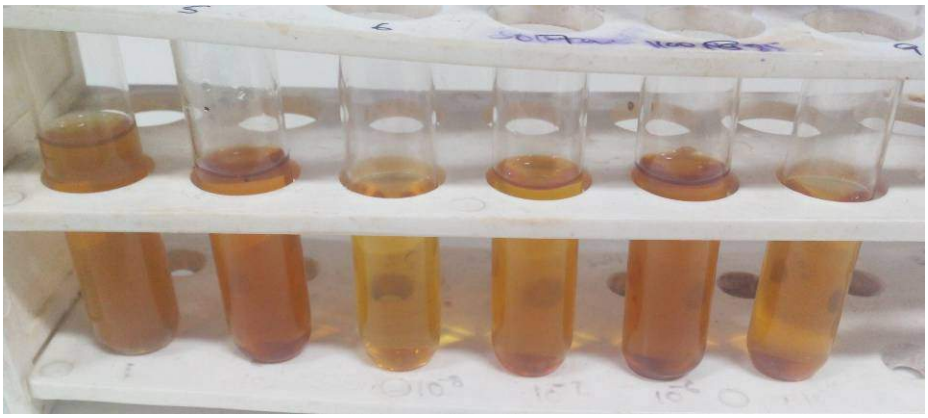
18 Days

A **B** **C**

Plate 5: Growth of *Penicillium* sp on A) Soybean meal B) Sunflower meal C) Mustard meal after incubation period of 6, 12 and 18 Days

7.4 Pigment extraction and quantification:

Plate 6 shows the pigment extracted from the different dried fermented substrates using 80% methanol as solvent. As observed by visible examination, after extraction with methanol the color of methanol became yellow. On spectrophotometric analysis, the maximum absorbance was observed at 410 nm.



1 2 3 4 5 6

Plate 6: The pigment extracted from *Penicillium* sp. grown on different substrates: 1 (Broken Wheat + Monosodium glutamate), 2 (Broken wheat + Ammonium Chloride), 3 (Broken wheat + Ammonium sulfate) 4 (Mustard meal), 5 (Soybean meal) and 6 (Sunflower meal)

7.4.1 Pigment produced when ionic nitrogen sources are used:

Pigment yield in case of broken wheat supplemented with ionic nitrogen source was higher as compared to broken wheat alone used as control, which shows that the pigment production was affected by the nitrogen sources. The results obtained are given in Table 3. With the increase in incubation time, pigment yield also increased. The highest pigment yield was obtained with monosodium glutamate followed by ammonium chloride and ammonium sulfate. Monosodium glutamate gave 166 CVU/gdfs after 18 days of incubation whereas ammonium sulfate showed least pigment production of 140.85 CVU/gdfs after 18 days of incubation.

Table 3: Yield of pigment (CV/gdfs) produced by *Penicillium* sp. on various ionic nitrogen sources w.r.t time.

Substrate	6 Days	12 Days	18 Days
Broken wheat (control)	11.85±0.20	31.4±0.40	51.75±0.60
Broken wheat +MSG	48.3±0.63	75.8±0.34	166±0.98
Broken wheat + NH_4SO_4	27.65±0.49	44.10±0.46	108.15±0.14
Broken wheat + NH_4Cl	30.55±0.49	56±0.59	140.85±0.89
CD@ 5%	1.56	1.50	2.39

CD@5% Substrate: 0.83

No. of Days: 0.96

SubstrateX No. of Days: 1.66

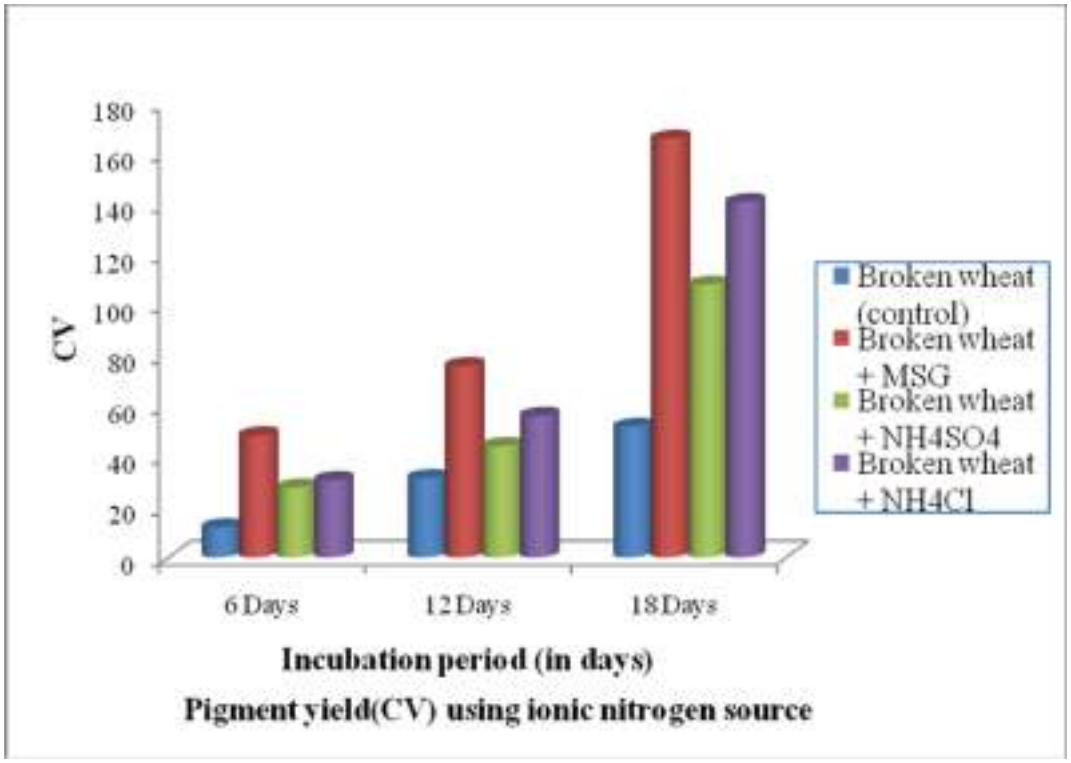


Figure 1: Yield of pigment (CV/gdfs) produced by *Penicillium* sp. on various ionic nitrogen sources w.r.t time.

The highest yield was obtained when MSG was used. This might be because MSG is inducing the metabolic pathway of Penicillium in a way which is favoring the pigment production. The results obtained are similar to Dikshit and Tallapragada (2011) on *Monascus purpureus* who observed highest pigment yield when MSG was used as a nitrogen supplement. Lee *et al.,* (2001) also reported that MSG gave the highest specific productivity of red pigments by *Monascus purpureus* out of all nitrogen sources tested. Miyake *et al.,* (2008) reported enhanced yellow pigment production upon addition of 0.5% MSG for pigment production by *Monascus* strain. Therefore, MSG was found to be the most favorable ionic nitrogen source and our results also showed the same.

7.4.2 Pigment produced when organic nitrogen sources are used:

The feasibility of organic sources i.e. soybean meal, mustard meal and sunflower meal as a nitrogen source was also determined. The results obtained are shown in Table 3. Out of the organic meals used as nitrogen source, soybean meal showed the highest pigment yield of 75.75±0.43CVU/gdfs followed by mustard meal (46.20±0.46 CVU/gdfs) and sunflower meal (29.40±0.23 CVU/gdfs). With the increase in the incubation period pigment production also increased (Fig 2).

Table 4: Yield of pigment (CV/gdfs) produced by *Penicillium* sp. on various organic nitrogen sources w.r.t time.

Substrate	6 Days	12 Days	18 Days
Soybean meal	26.75±0.14	43.20±0.51	75.75±0.43
Mustard meal	17.75±0.14	35.20±0.40	46.20±0.46
Sunflower meal	9.90±0.28	14.60±0.69	29.40±0.23
CD@5%	0.70	1.9	1.35

CD@5% substrate: 0.69

No.of days: 0.69

SubstrateX No. of days: 1.20

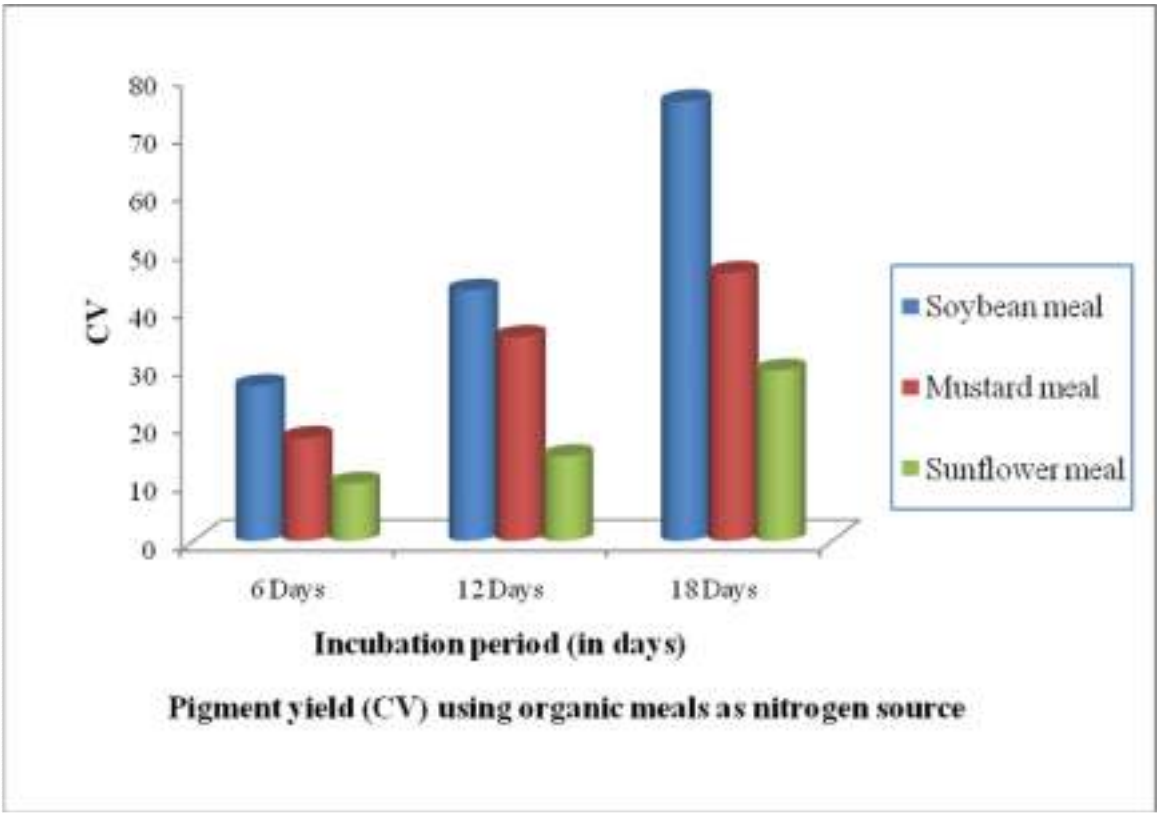


Figure 2: Yield of pigment (CV/gdfs) produced by *Penicillium* sp. on various organic nitrogen sources w.r.t time.

7.5 Stability of Pigment:

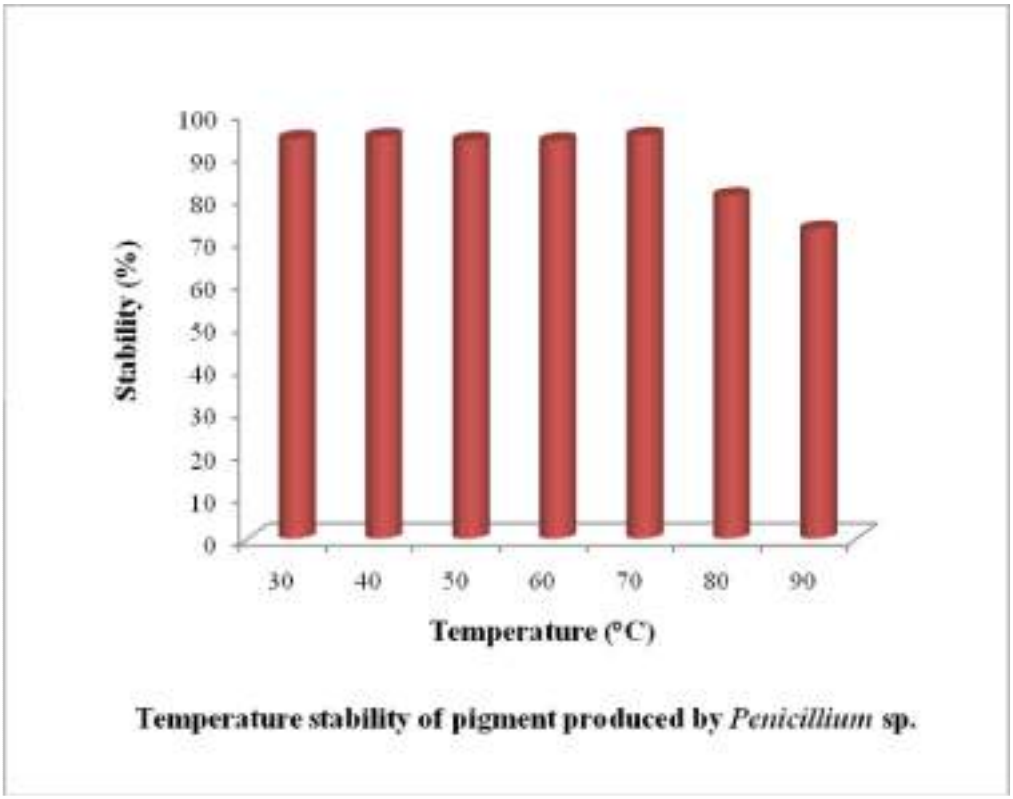
7.5.1 Temperature stability: Pigment extract was subjected to different temperature treatment to determine the thermostablity at 30 to 90ºC and the results are presented in Table 5. It was observed that pigment was stable from 30-70ºC and its stability decreased to 80.6% at 80ºC and on further increasing the temperature to 90ºC stability decreased to 72.8ºC.

Table 5: Effect of temperature on stability of pigment produced by *Penicillium* sp.

Temperature (^{o}C)	Stability (%)*
30	94
40	94.5
50	93.6
60	93.46
70	94.8
80	80.6
90	72.8
	2.10

* Average of two replicates

Solvent used- 80% Methanol



Temperature stability of pigment produced by *Penicillium* sp.

Figure 3: Effect of temperature on stability of pigment produced by *Penicillium* sp.

The pigment produced by *Penicillium* isolate was found to be stable upto 70^{o}C. The decrease in stability of pigment might be because of the degradation of certain compounds present in the pigment or bond breakage of the functional groups which resulted in the decrease in the optical density, and hence the stability.

7.5.2 pH stability: Pigment extract was adjusted to a pH range from 4 to 9 to check the stability and results obtained are presented in Table 5. It was found that pigment was stable at pH 5, 6 and 7. 100% stability was retained at pH 6. Under more acidic (pH 4) and alkaline (pH 8) conditions pigment stability decreased to 86% and at pH 9 stability further decreased to 83.75%. Hence, it was concluded that the pigment showed maximum stability at neutral or near neutral conditions.

Table 6: pH stability of pigment produced by *Penicillium* sp.

pH	Stability (%)*
4	86
5	97.28
6	100
7	98.9
8	86
9	83.75
CD@5%	0.38

* Average of two replicates

Solvent used: 80% methanol

Temperature: 30±2^{o}C

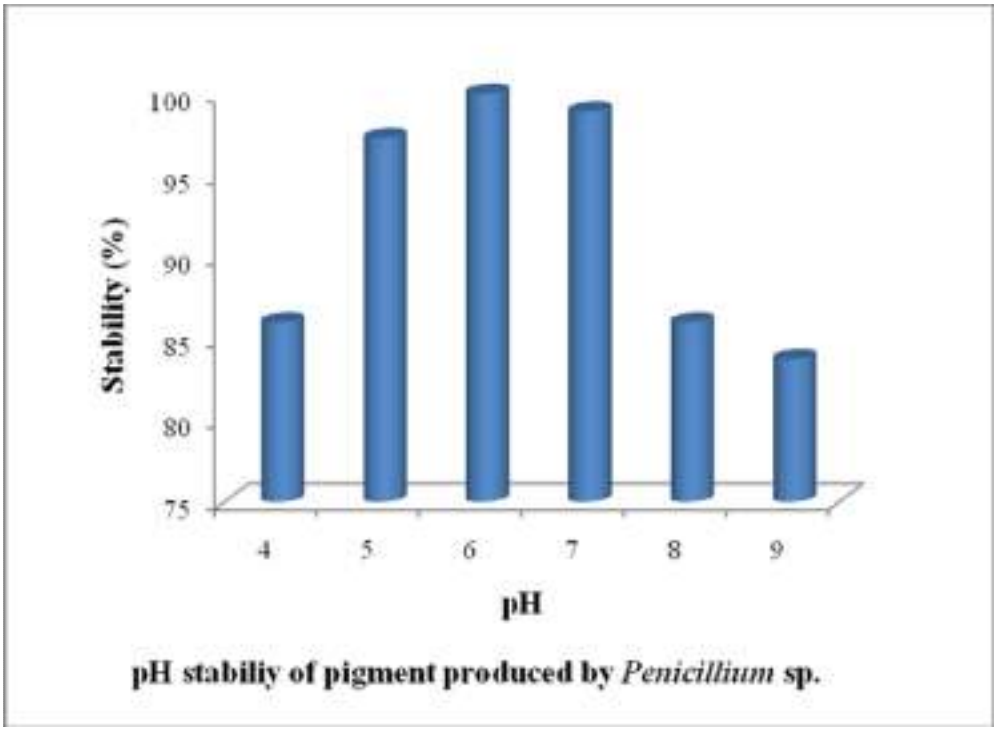


Figure 4: Effect of pH on stability of pigment produced by *Penicillium* sp.

The decrease in stability can be attributed to protonation or dissociation below or above the molecular dissociation constant of the pigment molecules. The studies of Velmurugan *et al.,* 2011 on *Monascus* also observed that original color retained at pH 5, 6 and 7. The studies of Kaur *et al.,* 2008 on *Monascus purpureus* MTCC showed the stability at pH 6, 7 and 8. Wongjewboot and Kongruang (2011) studied the pH stability of *Monascus purpureus* and found the pigment to be most stable at pH 8. The pigment is more than 80% stable of at pH 4, 8 and 9 which shows the importance of the pigment even under acidic and alkaline conditions.

7.5.3 Light stability: To study the effect of light on pigment stabilty, the pigment was exposed to light for 5 days and the results obtained (Table 7) was compared with the stability of pigment kept under dark conditions for same time period. It was observed that under dark conditions, pigment retained its stability but when exposed to light the pigment stability decreased with the increase in exposure time. Pigment was 84.26 % stable on first day of exposure of light and stability decreased to 63.34%

after 5 days whereas under dark conditions pigment was 96.5 % stable on first day and 91.4 % stable after 5 days.

Table 7: Light stability of Pigment produce by *Penicillium* sp.

	Stability (%)*	
Days	**Control (dark)**	**Light**
1	96.5	84.26
2	94.6	82
3	93.3	77.88
4	92.8	72.06
5	91.4	63.34
CD@5%		

* Average of two replicates

Solvent used- 80% methanol,

Temperature- 30±2^{o}C

The results obtained in the present work, resembles with the work of Hailei *et al.*,(2011) on *Penicillum* sp. co-cultured with *Candida tropicalis*. Pigment color did not change in dark even after 30 days but under light conditions decrease in color value was observed. This might be because some compounds in the pigment were not photostable. The visible light might denatured certain constituents present in the pigment which resulted in decrease in stability

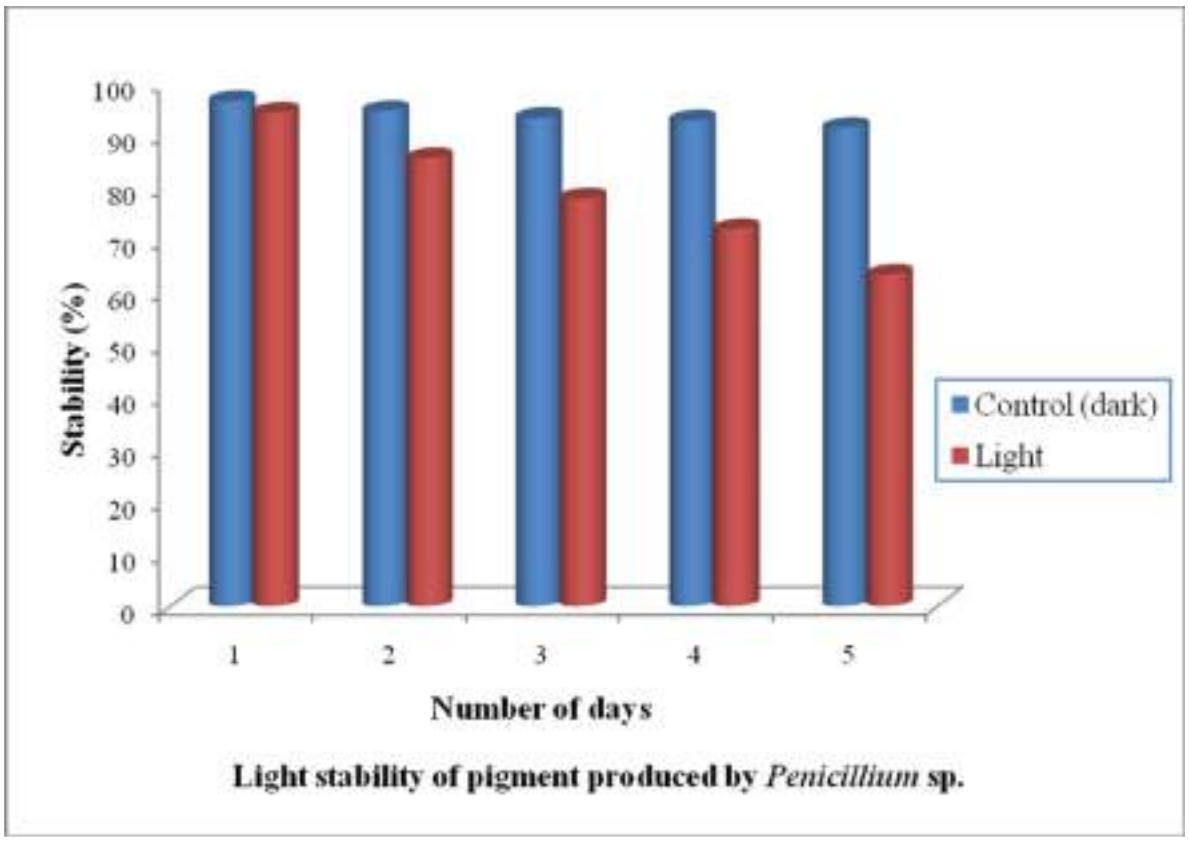


Figure 5: Effect of light on stability of pigment produced by *Penicillium* sp.

7.5.4 Solvent stabiliy: The solvent stability of pigment was studied by measuring the pigment concentration initially and after 6 days of incubation of the pigment dissolved in different solvents kept under dark conditions at 30^{o}C. The pigment stability was calculated and results were expressed in percentage (Table 7). The results showed that pigment degraded with time. It was observed that pigment was most stable in methanol (85.49%) followed by ethanol (80.25%). Pigment stability decreased when chloroform and acetone are used. Pigment was least stable in acetone (44.35%).

Table 8: Solvent stability of pigment produced by *Penicillium* sp.

Solvent (10 ml)	Stability (%)*
Ethanol	80.25
Methanol	85.49
Chloroform	49.1
Acetone	44.35 0.29

* Average of two replicates

Temperature: 30±2ºC

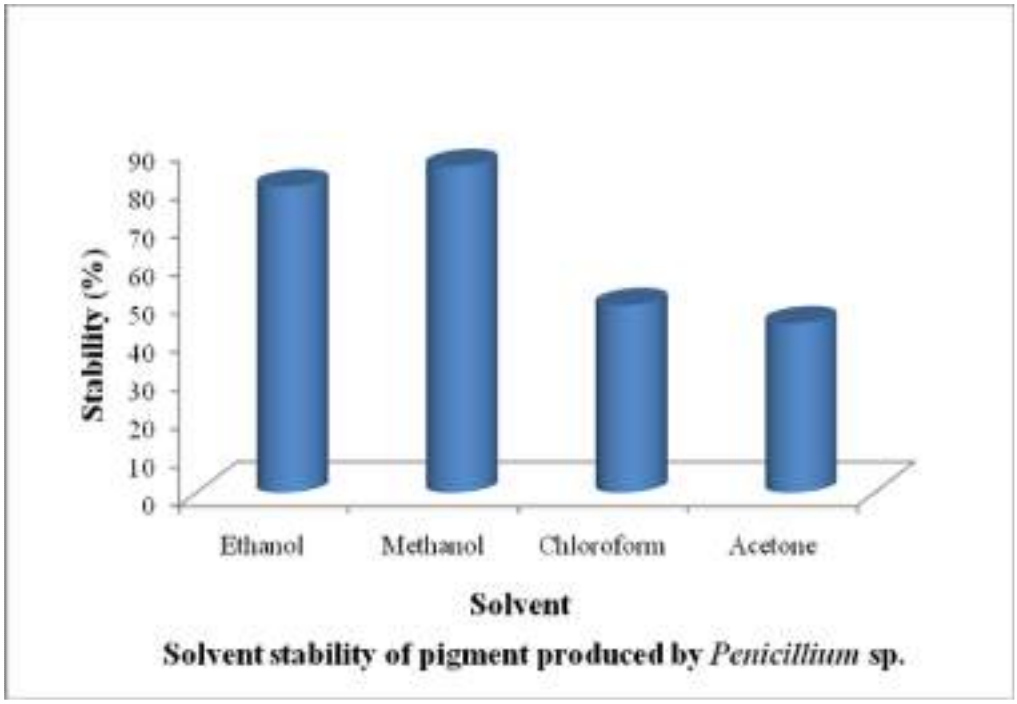


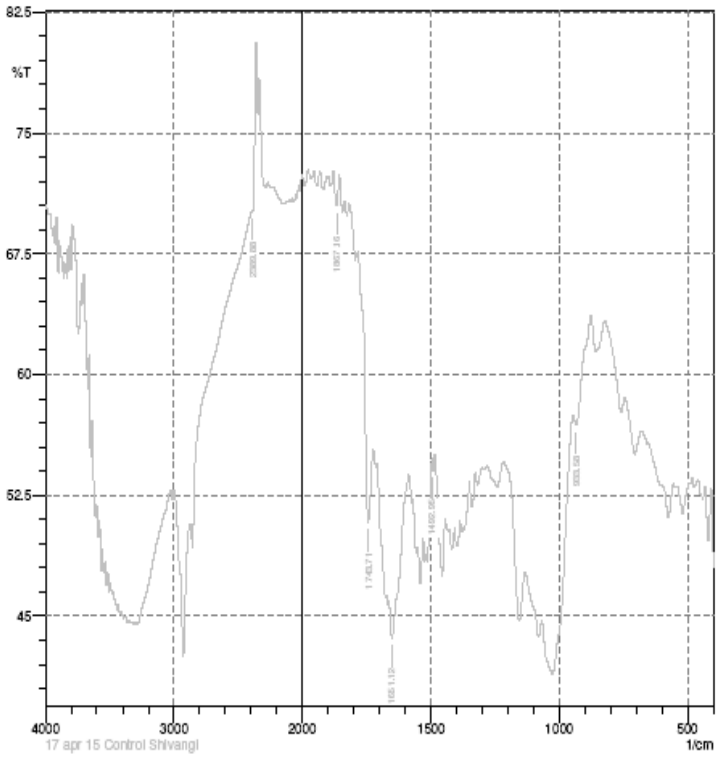
Figure 6: Effect of solvent on stability of pigment produced by *Penicillium* sp.

From the results obtained, we concluded that chloroform and acetone are not favorable for the pigments in terms of stability. The possible reason might be that the organic solvents have different polarity indices. Chloroform and acetone being less polar than methanol and ethanol might have resulted in less stability of pigment in these solvents. The results obtained are similar to those of Chiba *et al.,* (2006). They

found methanol to be most suitable solvent followed by ethanol. However, Wongsorn *et al.,* (2011) showed that *Monascus purpureus* pigment was not stable in methanol.

7.6 Pigment characterization by FTIR

Figure 7 shows the infrared spectra of the broken wheat used as substrate to determine the functional groups present. The main peaks were obtained at 2389.88, 1867.16, 1743.71, 1651.12, 1492.95 and 933.58 cm^{-1} which corresponds to –C≡C, –C=O stretch,-C=O- stretch, -C=C- stretch, N-O asymmetric stretch and O-H bend respectively. Therefore, the functional groups present in broken wheat are alkyne, anhydride, aldehydes, alkenes, nitro compounds and carboxylic acids. The peaks in these ranges were eliminated when analyzing the IR spectrum of pigment as these peaks were present in the control.

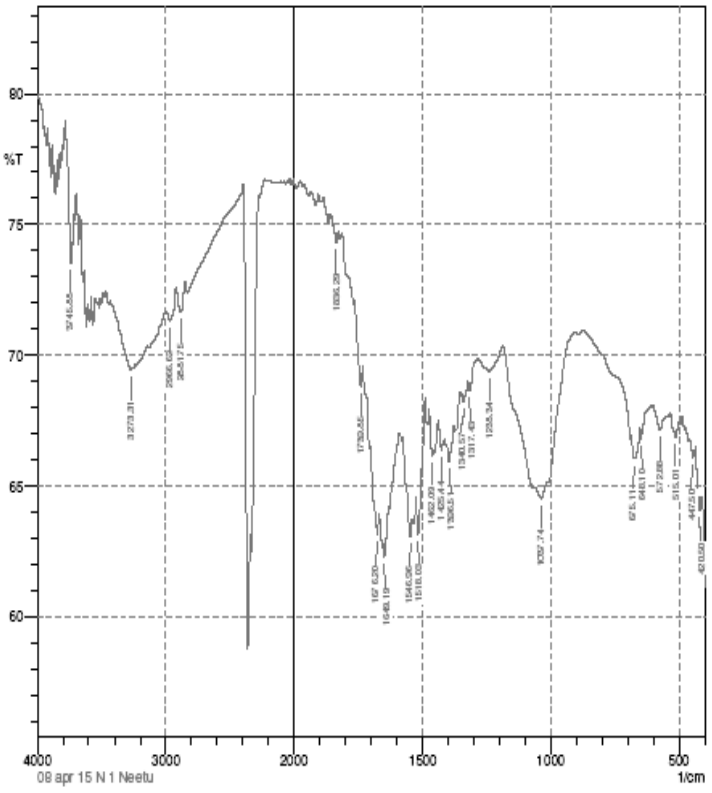


	Peak	Intensity	Corr. Inte	Base (H)	Base (L)	Area	Corr. Are
1	933.58	56.855	1.822	947.08	877.64	15.507	0.348
2	1492.95	54.073	0.789	1496.81	1485.24	3.055	0.041
3	1651.12	43.591	2.445	1664.62	1635.69	10.094	0.347
4	1743.71	50.811	8.242	1784.21	1726.35	13.57	1.234
5	1867.16	70.478	1.995	1878.73	1857.51	3.127	0.162
6	2389.88	70.137	1.246	2393.74	2360.95	4.383	0.325

Figure 7: Infrared spectrum of broken wheat used as control

The FTIR analysis of pigment (Figure 8) showed a broad band at 3273.31 cm^{-1} which corresponds to O–H stretching. So alcohol or phenol group might be present. A broad medium band at 1037.74cm^{-1} corresponds to C-N stretching which indicates presence of aliphatic amines. A number of small peaks were obtained at 648.10, 675.11, 1238.34, 1317.43, 1340.57, 1396.51, 1425.44, 1462.09, 1676.20, 2881.75 and 2966.62 cm^{-1} These peaks corresponds to –C=C-H or C-H bend, C-Br, C-N, C-N, N-O, C-C, C-C or C-H, C=O stretch, C-H stretch, C-H stretch respectively. Small and sharp peaks were obtained at 1518.03, 1546.96 and 1739.85 cm^{-1} which were not considered as these peaks were also present in control. A sharp medium peak was also obtained at 3745.88 cm^{-1}.

From the results it was suggested that the main functional groups present in the pigment produced by *Penicillium* sp. are alkynes, alkyl halides, aliphatic amines, alcohols, carboxylic acids, esters or ethers, nitro compounds, aromatic compounds, alkanes, carbonyls.



No.	Peak	Intensity	Corr. Inte	Base (H)	Base (L)	Area	Corr. Are
1	420.5	64.057	1.132	435.93	412.78	4.359	0.106
2	447.5	66.08	0.529	461	435.93	4.46	0.037
3	515.01	66.885	0.706	534.3	501.51	5.661	0.089
4	572.88	67.148	0.696	601.81	551.66	8.549	0.109
5	648.1	66.796	0.501	651.96	617.24	5.922	0.021
6	675.11	66.041	1.001	686.68	651.96	6.172	0.152
7	1037.74	64.536	1.369	1062.81	906.57	26.869	0.551
8	1238.34	69.403	0.124	1244.13	1188.19	8.752	0.048
9	1317.43	68.663	0.5	1323.21	1296.21	4.331	0.044
10	1340.57	68.232	0.392	1346.36	1323.21	3.803	0.036
11	1396.51	65.937	0.843	1404.22	1379.15	4.453	0.08
12	1425.44	66.365	0.521	1440.87	1419.66	3.716	0.036
13	1462.09	66.177	0.728	1473.66	1456.3	3.06	0.058
14	1518.03	63.171	2.258	1525.74	1491.02	6.493	0.33
15	1546.96	63.096	0.939	1560.46	1541.18	3.78	0.075
16	1649.19	62.29	0.723	1653.05	1629.9	4.622	0.054
17	1676.2	63.122	1.294	1701.27	1670.41	5.928	0.196
18	1739.85	68.831	1.212	1766.85	1734.06	5.042	0.134
19	1836.29	74.321	0.605	1853.65	1828.58	3.175	0.048
20	2881.75	71.655	1.072	2918.4	2848.96	9.875	0.269
21	2966.62	71.318	0.72	2997.48	2918.4	11.442	0.227
22	3273.31	69.45	0.008	3275.24	3259.81	2.441	0.001
23	3745.88	73.568	0.68	3753.6	3742.03	1.498	0.022

Figure 8: Infrared spectrum of crude pigment produced by *Penicillium* sp

CHAPTER 8

CONCLUSION AND FUTURE SCOPE

In the present study, the effect of nitrogen source on pigment production by *Penicillium* sp. was studied. The substrate broken wheat was supplemented with ionic nitrogen sources (monosodium glutamate, ammonium sulfate and ammonium chloride) and agro-industrial waste residues (soybean, sunflower and mustard meal**)** as organic sources are used to study the effect of nitrogen source on pigment production by *Penicillium* sp. We observed that with the increase in incubation period, pigment production also increased. On 6^{th} day, monosodium glutamate showed highest pigment yield of 48.3±0.63, followed by ammonium chloride (30.55±0.49) and ammonium sulfate (27.65±0.49). Similarly on 12^{th} and 18^{th} day, pigment yield was in the order Monosodium glutamate> Ammonium chloride > Ammonium sulfate. Out of the organic sources, Soybean meal showed highest pigment yield of 26.75 CVU/gdfs on 6^{th} day of incubation followed by mustard meal (17.75CVU/gdfs) and sunflower meal (9.9CVU/gdfs). Similarly on 12^{th} and 18^{th} day, pigment yield was in the order Soybean meal> Mustard meal> Sunflower meal. The stability of pigment was also determined at different temperature, pH, light and solvents. The pigment was stable upto 70^{o}C and pH 4-6. Stability decreased with increase in exposure to light while it retained its stability under dark conditions. Also the pigment was found to be most stable in methanol and least stable in acetone. The FTIR analysis of pigment was done and main functional groups were found to be alkynes, alkyl halides, aliphatic amines, alcohols, carboxylic acids, esters or ethers, nitro compounds, aromatic compounds, alkanes and carbonyls.

CHAPTER 9

REFERENCES

Aberoumand A (2011) A review article on edible pigment properties and sources as natural biocolorants in foodstuff and food industry. *World Journal of Dairy and Food Sciences* 6(1): 71-78.

Arai T, Koganei K, Umemura S, Kojima R, Kato J, Kasumi T and Ogihara J (2013) Importance of the ammonia assimilation by *Penicillium purpurogenum* in amino derivative *Monascus* pigment, PP-V, production. *AMB Express* 3:19.

Babitha S, Soccol CR and Pandey A (2007) Solid-state fermentation for the production of *Monascus* pigments from jackfruit seed. *Bioresource Technology* 98: 1554–1560.

Bhargav S, Panda BP, Ali M and Javed S (2008) Solid State Fermentation: An Overview. *Chemical and Biochemical Engineering Quarterly* 22(1) 49-70.

Blanc PJ, Loret MO, Santerre AL, Pareilleux A, Prome D, Prome JC, Laussac JC and Goma G (1994) Pigments of *Monascus*. *Journal of Food Science* 59:862–865.

Boussiba S (2000) Carotenogenesis in the green alga *Haematococcus pluvialis*: Cellular physiology and stress response. *Physiologia Plantarum* 108:111-117.

Cerdá-Olmedo E (2001) *Phycomyces* and the biology of light and color. *FEMS Microbiology Reviews* 25: 503–512.

Chiba S, Tsuyoshi N, Fudou R, Ojika M, Murakami Y, Ogoma Y, Oguchi M and Yamanaka S (2006) Magenta pigment produced by Fungus. *Journal of General and Applied Microbiology* 52: 201–207.

Chung KT, Stevens SV and Cerniglia CE (1992) The reduction of azo dyes by the intestinal microflora. *Critical Review of Microbiology* 18: 175-190.

Daniel MM (1986) Handbook of U.S. colorant for food, drugs and cosmetics. Wiley Interscience, U.S.A 2: 167-180

Dhale AM and Vijay-Raj AS (2009) Pigment and amylase production in *Penicllium* sp. NIOM-02 and its radical scavenging activity. *International Journal of Food Science and Technology* 44(12): 2424-2430.

Dikshit R, Tallapragada P (2011) *Monascus purpureus*: a potential source for natural pigment production. *Journal of Microbiology and Biotechnology Research* 1:164-174.

Dufosse L (2006) Food Grade Pigments. *Food Technology and Biotechnology* 44(3): 313-321.

Duran N, Teixeira M.F.S, De Conti R and Esposito E (2002) Ecological-friendly pigments from fungi. *Critical Review in Food Scence and .Nutrition* 42:53–66.

Frisvad JC, Smedsgaard J, Larsen TO, Samson RA (2004) Mycotoxins, drugs and other extrolites produced by species in *Penicillium* subgenus *Penicillium*. *Studies in Mycology (Utrecht)* 49:201-242.

Gunasekaran S and Poorniammal R (2008) Optimization of fermentation conditions for red pigment production from *Penicilliumj* sp. under submerged cultivation. *African Journal of Biotechnology* 7(12): 1894-1898.

Gupta C, Garg AP, Prakash D, Goyal S and Gupta S (2011) Microbes as potential source of biocolors. *Pharmacology online* 2:1309-1318.

Hailei W, Zhifang R, Ping L, Yanchang G, Guosheng L and Jianming Y (2011) Improvement of the production of a red pigment in *Penicillium* sp. HSD07B synthesized during co-culture with *Candida tropicalis*. *Bioresource Technology* 102:6082–6087.

Hajjaj H, Blanc P, Groussac E, Uribelarrea JL, Goma G and Loubiere P (2000) Kinetic analysis of red pigment and citrinin by *Monascus ruber* as a function of organic acid accumulation. *Enzyme and Microbial Technology* 27: 619-625.

Hamano PS, Kilikian BB (2006) Production of red pigments by *Monascus ruber* in culture media containing corn steep liquor. *Brazilian Journal of Chemical Engineering* 23:443-449.

Hausmann A and Sandmann G (2000) A single five-step desaturase is involved in the carotenoid biosynthesis pathway to b-carotene and torulene in *Neurospora crassa*, *Fungal Genetics and Biology* 30: 147–153.

Jiang Y, Li HB, Chen F, Hyde KD (2005) Production potential of water-soluble *Monascus* red pigment by a newly isolated *Penicillium* sp. *Journal of Agriculture and Technology* 1(1):113-126.

Jones JD, Hohn TM and Leathers TD (2004) Genetically Modified Strains of *Fusarium sporotrichioides* for Production of Lycopene and b-Carotene. *Society of Industrial Microbiology* 4: 91-95.

Joshi VK, Attri D (2006) Solid state fermentation of apple pomace for value added products. *Natural Product Radiance* 5:289-296.

Joshi VK, Attri D, Bala A and Bhushan S (2003) Microbial Pigments. *Indian Journal of Biotechnology* 2:362-369.

Jung H, Kim C, Kim K, Shin CS (2003) Color characteristics of *Monascus* pigments derived by fermentation with various amino acids. *Journal of Agriculture and Food Chemistry* 51:1302-1306.

Juzlova P, Martinkova L, Kren V (1996) Secondary metabolites of the fungus *Monascus*: A review. *Journal of Industrial Microbiology* 16:163-170.

Kamalam JN, Anburaj J, Kuberan T, Sundaravadivelan C, Kumar P, Starlin T, Selvi AT, Devi MV (2012) DNA amplification and characterization of pigment producing gene from *Monascus ruber*. *European Journal of Experimental Biology* 2:427-435.

Lee BM, Park NH, Piao HY and Chung WJ (2001) Production of Red Pigments by *Monascus purpureus* in Submerged Culture. *Biotechnology and Bioprocess Engineering* 6(4):341-346.

Malik K, Tokkas J and Goyal S (2012) Microbial Pigments: A review. *International Journal of Microbial Resource and Technology* 1(4): 106-110.

Mapari SA, Meyer AS and Thrane U (2006) Colorimetric characterization for comparative analysis of fungal pigments and natural food colorants. *Journal of Agriculture and Food Chemistry* 54(19): 7027-7035.

Méndez-Zavala A, Contreras-Esquivel JC, Lara-Victoriano F, Rodríguez-Herrera R, Aguilar CN (2007) Fungal production of a red pigment using a xerophilic strain of *Penicillium purpurogenum* GH2. *Revista Mexicana de Ingenieria Química* **6**:267-273.

Miyake T, Isato K, Nobuyuki N and Sammoto H (2008) Analysis of Pigment Compositions in Various *Monascus* Cultures. *Food Science and Technology Research* 14: 194–197.

Mukherjee G and Singh SK (2011) Purification and characterization of a new red pigment from *Monascus purpureus* in submerged fermentation. *Process Biochemistry* 46:188-192.

Pandey A (2003) Solid-state fermentation. *Biochemical Engineering Journal* 13: 81–84.

Panesar R (2014) Bioutilization of kinnow waste for the production of biopigments using submerged fermentation. *International Journal of Food Sciences and Nutrition* 3:9-13.

Perumal K, Stalin V, Chandrasekarenthiran S, Sumathi E and Saravanakumar A (2009) Extraction and characterization of pigment from *Sclerotinia* sp. and its use in dyeing cotton. *Textile Research Journal* 79: 1178–1187.

Pradeep FS, Begam MS, Palaniswamy M and Pradeep BV (2013) Influence of Culture Media on Growth and Pigment Production by *Fusarium moniliforme* KUMBF1201 Isolated from Paddy Field Soil. *World Applied Sciences Journal* 22 (1): 70-77.

Rajeswari TR, Ponnusami V, Sugumaran KR (2014) Production of *Monascus* pigment in low cost fermentation. *International Journal of ChemTech Research* 6:2929-2932.

Robinson N, Wood K, Hylands PJ Gibson TM (1992) Blue pigments of *Penicillium herquei*. *Journal of Natural Products* 55:814-817.

Sardaryan E (2002) Strain of the microorganism *Penicillium oxalicum var. Armeniaca* and its application. US Patent 6,340,586 B1.

Sharma D (2014) Understanding biocolour- A review. *International Journal of Scientific and Technology Research* 3(1)

Silveira ST, Daroit DJ, Brandelli A (2011) Pigment production by *Monascus purpureus* in grape waste using factorial design. *Food Science and Technology* 41:170-174

Singhal RS and Kulkarni PR (1999) Production of food additives by fermentation in biotechnology: Food Fermentation. Educational publishers, New Delhi 2:1145-1200

Soltan S and Shehata M (2012) The effects of using color foods of children on immunity properties and liver, kidney on Rats. *Food and Nutrition Sciences* 3: 897-904.

Srianta I, Novita Y, Kusumawati N (2012) Production of *Monascus* pigments on durian seed: effect of supplementation of carbon source. *Journal of Pure and Applied Microbiology* 6:59-63.

Srivastava RC, Goel M and Gulrajani ML (1999) Color gamut of natural dyes on cotton yarns in: Convention on natural dyes, department of textile technology. IIT Delhi, New Delhi. pp 288-296.

Subhasree RS, Babu P, Vidyalakshmi R, Chandra V (2011) Effect of carbon and nitrogen sources on stimulation of pigment production by *Monascus purpureus* on Jackfruit seeds. *International Journal of Microbiology Research* 2:184-187.

Sugumaran KR, Rajeswari TR, Ponnusami V (2014) Production of *Monascus* pigment in low cost fermentation. *International Journal of ChemTech Research* 6:2929-2932.

Tibor C (2007) Liquid Chromatography of Natural pigments and synthetic dyes. *Journal of Chromatography Library* 71:11-19.

Unagul P, Wongsa P, Kittakoop P, Intamas S and Srikitikulchai P (2005) Production of red pigments by the insect pathogenic fungus *Cordyceps unilateralis* BCC 1869. *Journal of Industrial Microbiology and Biotechnology* 32: 135-140.

Wongjewboot I and Kongruang S (2011) pH Stability of Ultrasonic Thai isolated *Monascus purpureus* pigments. *International Journal of Bioscience, Biochemistry and Bioinformatics* 1(1): 250-274.

Wongsorn H, Wongjewboot I and Kongruang S (2011) Solvent stability of ultrasonic mutants of *Monascus purpureus* pigments. *International Journal of Bioscience, Biochemistry and Bioinformatics* 1:206-211.

Yamamoto A, Limori Y, Koyama M, Ishiguro M and Saze M (2006) Historian composition for endoscope. US Patent 20070077202.

Yoshimura S, Ranjbar R, Inoue R, Katsuda T and Katoh S (2006) Effective utilization of transmitted light for astaxanthin production by *Haematococcus pluvialis. Journal of Bioscience and Bioengineering* 102:97-101.