**EFFECT OF CARBON SOURCES ON PIGMENTS PRODUCTION BY *PENICILLIUM SP.***

**SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENT**

**FOR THE AWARD OF THE DEGREE OF**

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## Abstract

There is a wide range of significant health hazards associated with the use of synthetic colorants. Synthetic dyes are not only involved in inducing various cancers and other major dreadful health hazards but they are also one of the major causes of the environmental pollution. These disadvantages have decreased the use of synthetic dyes, and hence the scientific community is more concerned about finding alternative colorants from the natural sources. In the present study the effect of carbon sources on the pigment production by *Penicillium* sp was evaluated. Broken wheat, wheat straw and wheat bran were used as a carbon sources and the effect of purified carbon sources dextrose, soluble starch, lactose as inducers was also investigated. Out of these, the maximum yield of pigments was obtained on wheat bran induced with starch (122.4± 4.07 CVU/ml). Wheat straw failed to support the growth as well as pigment production (17.6±0.06) by *Penicillium* sp. The pigment produced by the *Penicillium sp.* was found to be stable up to 70ºC and at an pH range of 5-8.The pigment was found to be photo stable having maximum stability in methanol (85.49%)

**Acknowledgement**

I take this opportunity to present my votes of thanks to all those guidepost that really acted as lightening pillars to enlighten my way throughout this project that has led to successful and satisfactory completion of this study.

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# DECLARATION

I hereby declare that this thesis entitled **"Effect of Carbon sources on 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 Master of Technology in Biotechnology 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:

Date:

Shivangi Sharma

Reg.No: 11304967

# CERTIFICATE

This is to certify that Shivangi Sharma **(11304967)** has completed Dissertation project report (BTY 731), entitled "**Effect of Carbon Sources on Pigment Production by *Penicillium* Isolate"** 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 the partial fulfillment of the conditions for the award of M. Sc. in Microbiology**.**

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# CHAPTER -1

# INTRODUCTION

Pigments refer to those chemical compounds that can absorb light under visible spectra at a specific wavelength range. Natural pigments have been in use for pre historical times when they were widely used in the cosmetics as well as in textile dyeing. These colors were earlier derived from that of the roots of the plants, vegetables, fruits and seeds etc. Since these pigments are of bio origin hence they are called as bio colors. Natural pigments have a very important role in nature such as photosynthesis (chlorophylls, carotenoids, porphyrins, anthocyanins, betalins).In animals pigments helps to transport oxygen and carbon dioxide (hemoglobin or myoglobin). Vargas *et al*., (2000) Under stress conditions plants synthesized certain molecules that are very important in conversion of light energy into chemical energy (flavonoids and quinones) stated by Jaiswal. *et al*.,(2002)

The scientific community has been increasingly focusing on the development of technology for the pigment production from natural sources due to major health issues with various artificial synthetic colorants. These synthetic dyes had been widely used in foodstuff, cosmetics and pharmaceutical manufacturing industries stated by Kim *et al.,* (1995). The dyestuff industry is also facing a lot of problem in the manufacturing process of synthetic dyes since they are owing to use of expensive feed stock as well as high amount of energy consumption for dye synthesis. In addition industries are also under pressure to minimize the damage to the environment (Nelson *et al.,* 2002). Various synthetic dyes that had been banned by many countries due to their toxic, carcinogenic and polluting nature Singh by *et al.,* (2000) .It has been observed that the synthetic colors can also induce adverse behavarioul effect in children which was first reported in 1975 by a scientist named Feingold stated by Weiss *et al*; (1980). These drawbacks have led to an increased attention towards natural sources of pigments.

There are few elements use in the synthesis of artificial colouring that have tremendous health risk such as dioxins, toxic heavy metals (chrome, copper) and some formaldehyde are found to be carcinogen and hormone disrupter stated by Brit *et al*.,(2008).

The natural food colorant which are being abundantly used are of either animals or plant origin , however they have been associated with many disadvantages which includes instability against light, heat ,adverse ph, low water solubility and limited available sources.

Microorganism can also serve as the source of pigments (Gunasekaran *et al.,* 2008).Bacteria and fungi are potent sources among microorganisms for pigment production. These organisms produces pigments either for photosynthesis (phycobilins) or it may be produce during sporulation (mainly in fungi) to protect the spores from extreme environmental conditions. Sometimes the pigments also play a major important role in the pathogenesis as well as for the survival of those particular organisms. (Calvo *et al.,* 2002).

Efforts have been concentrated on investigation of a potential microbial isolate that can produce pigments of wide range specially brown and reddish brown natural dyes as well as their isolation, purification and structural detection. Microorganisms are capable of growing rapidly, hence high productivity can be obtained, and product can be produced throughout year (Jiang, 2005). More attention has been given to the strains that belong to the *Meniscus*genus of filamentous fungi. Some authors refer these fungi as potent producers of natural pigments (Blanc, 1994); Tseng *et al.,* 2000); (Carvalho, *et al.,*2003).It is so because they possess the ability to produce various range of pigments that include several chemical classes such as melanins, carotenoids, flavins, phenazines, and more specifically monascins, violacein or indigo (Duffose *et al.*, 2014).

The natural pigments besides having no side effects property also has certain health benefits such as they act as antioxidant, anticancer, anti-inflammatory anti angiogenic anti-obesity and neuroprotective activities (Pangestuti *et al.*, 2011).

### 1.1: Distribution of natural pigments

#### 1.1.1: Tetrapyrrole Derivatives

These compounds consist of the pyrrole rings in a cyclic or linear array. For an example the heme group contains porphyrin ring which is bounded to an iron atom (haemoglobin, myoglobin, cytochromes).chlorophyll possess the most essential subgroup of the pigment within the tetrapyrrole derivatives

#### 1.1.2: Isoprenoids Derivatives

It is also known as terpenoids. Isoprenoids derivatives are almost found in all kingdom were they are involve in multiple function such as hormone, phytoalexins, pigments etc. The isoprenoids are further classified into three major groups which include quinones, carotenoids, iridoids. (present in approximately 70 different families).

### 1.1.3: Benzopyran Derivatives

Benzopyran derivatives are the phenolic compounds consist of two aromatic rings that are held by C3 unit that is a central pyran unit this derivative is further divided into various subgroups on the basis of oxidation state of the pyran rings as well on the specific characteristic color such as antho cyanins, aurons, chalcones, yellow, flavanones, di hydro flavanols, dihydro chalcones, isoflavonoids and flavans. (Vargas *et al* .,2000**)**

### 1.1.4: Melanins

They are polymers of the nitrogenous compound having indole ring as a monomer. It is present in a mixture of macromolecules and are not considered as homo polymers. The compound is responsible for black, brown grey coloration in animal, plants and microorganisms. Allo melaninns has been reported to be present in plant seed or spores of the fungi.

Various diseases can be treated by incorporating the various beneficiary substances in the food items recently the scientists are more concern about the microbial production of zeaxanthin. There are only few microbial sources reported to produce zeaxanthin one of them is flavo bacterium the pigment produced by this bacteria consists of 95to 99% zeaxanthin which is very much similar to that of thee pigment of zea mays. The particular pigments play a vital role in the prevention of age related macular degeneration (AMD), the leading cause of blindness (Sajilata 2008)

The productions of pigments from filamentous fungal strains have a potent use in various industries because they are well known for the production of wide range of pigments that are highly stable at extreme environmental condition. The only negative side of these fungal species is that it is associated with the mycotoxins production. Various fungal species such as *Aspergillus terreus Aspergillus niveus, Monascus purpeureus* including the *Penicillium* species (*camemberti, citrinun*) are known to produce citrinin. The citrinin is mycotoxin that has been reported to cause the yellow rice disease (first reported in japan). It is a nephrotoxins and has significant health risks (Bennetj *et al.,* 2003) hence before commercializing any pigment its purity must be assessed properly. These pigments are homologues pigments which have similar chromophore polyketides (Mapari *et al.,* 2008a) and are of various strains of the species *Epicoccumnigrum* that produces yellow pigments. (Mapari *et al.,*2008).

Many fungi have been reported to produce non-carotenoid pigments but only few of these has to be found as possible food colorants (Sameer *et al.*, 2006). There are number of fungus which have the capability to produce high yield of Pigments, including species of Paecilomyces, Monascus, Cordyceps, Serratia, Streptomyces and yellow-red and blue compounds produced by *Penicillium jerquer* and *Penicillium atrovenetum.* Amongst them, various species of fungus have attracted special attention because they have the capability to produce different coloured pigments showing high chemical stability (Hajajj *et al.*, 2007).

The success of any pigment produced by fermentation depends upon its acceptability on market, capital investment required to bring the product to market as well as its stability at adverse conditions and regulatory approval. A few years ago, there were confusions regarding the successful commercialization of fermentation-derived food grade pigments because of the high capital investment for fermentation process and the intensive studies requirement about the toxicity. Nowadays some fermentative food grade pigments are on the market such as *Monascus* pigments, astaxanthin (*Xanthophyllomyces dendrorhous),* a pinkish Red pigment from *Penicillium oxalicum,* (Dufosse, 2006*).* One recent development has been with the B-carotene from the fungus *Bkesleatrisporala.* This is currently being marketed as a natural food colorant by Gist. (Rai *et al; 2009*)

Solid state fermentation (SSF) has been widely used for the production of various bioactive compound such as to produce feedstock fuel, food including the pigment production .A large number of microorganism including fungi is used for pigment production in solid state fermentation were agro industrial residues are generally consider the best substrate for pigment production.(Pandey *et al.,*2001).Solid state fermentation is the recent trend for the production of various compound and hence it would have very much importance in the future

At present the *Penicillium* species has been investigated which has potent capability of the pigment production since they can grow at a faster rate at a wide range of substrates which are readily available at a very less expenses such as agro industrial waste, as well as they are well known for producing various biotechnological valuable metabolites. The genera of *Penicillium* are more frequently found in soil than any other fungus. The genera can be identified by distinct characteristics which includes, the *Penicillium* species usually produces greenish colonies and sometimes white. They are fast grower comparative to other fungus.

Microscopically Conidiophores rise from surface hyphae, long stipes comprising a cluster of suppressed metulae. (Semra *et al.*,2005).

The growth of *Penicillium* species is effected by various parameters such as medium ingredients, volume of media, size and shapes of petri dishes as well as method of inoculation and incubation period and conditions (temperature, pH, exposure of light and aeration).(Robert *et al.,* 2000). For example *Penicillium purpurogenum* GH2 were found to produce red pigment in a submerged fermentation the fungus produces the maximum pigment at temperature 24ºC around pH5 .the pigment was found to be very much soluble in water (Mendez *et. al.,* 2011).

The Monascus pigment produces yellow, purple, red like pigments and these colorants are widely used in industries either in the cosmetics or as a food colorants but as it is known that the Monascus species are also potent producers of mycotoxin their used has been limited. Therefore the scientific community is searching for another genus of filamentous fungi that has the capability to produce the monascus like pigments and on the other hand they should be free from mycotoxins. The two species had been identified of the genus *Penicilliuim* (*Penicillium pinophilum, Penicillium aculeatum*) that produces the pigments similar to that of the Monascuss sp. (Sameer A.S *et al.,* 2008).

Quercetin glycoside is an orange color pigment produced by *Penicillium* species. The *Penicillium* purpurogenum SX01is known to produce a water soluble red pigment which was first identified from Gingko Biloba , this pigments is widely used as food colorant in various European countries.(Mishra *et al.*,2015). Another strain of the *P. purpurogenum* has an effective activity against some pathogenic microbes and might have a potential application in pharmaceutical drug industry. (Geweely*et al.*,2011).

Pigments are the secondary metabolites produced by the microorganisms which are very well exploited by the human population to fulfil our needs. It has its very potent role in food industry as a food colorant not only for making food more palatable, but also to preserve food items, the bio colors are also used in various cosmetics as well as in textile dyes since the synthetic colorants are associated with large no. of health hazards as discussed earlier. There is a need of continuous production of these bio colors so that the use of synthetic colorants can be avoided. The present study is associated with the screening of carbosources that can potentially enhance the pigment production by *Penicillium* species and to further check the stability of the pigment produced.

# CHAPTER-2

# TERMINOLOGY

**2.1: Spectrophotometer:**

It is an instrument which works on the principles of Beer Lamberts law which states that the amount of light is proportional to the concentration of absorbing particles; it is done by measuring the intensity of light at a specified wavelength which passes through the tested samples.

**2.2: Solid state fermentations:**

It is a fermentation process in which the microorganisms are grown on a solid support used as a source of nutrition for the production of bioactive compounds. This type of fermentation is widely used in pharmaceuticals, foods, textiles, fuels

**2.3: Bio colours:**

The bio colours are the colorants or the pigments that are derived from natural sources like plants, animals, microorganisms.

**2.4: Submerged fermentation:**

It is a type of fermentation which the microorganisms are grown on a liquid medium to produce bioactive compounds. Widely used for the production of antibiotics.

**2.5: FTIR analysis (FOURIER TRANSFORM INFRARED SPECTROSCOPY):**

It is a technique used to measure the photoconductivity or Raman scattering activity of solids, liquid, gas. It is also used to measure the absorption and emission activity on infrared spectra.

**2.6: Pigments:**

These are the substances produced by microorganisms or any other living organisms due to the absorption of light at a specific wavelength, such as melanin and carotenoids**.**

**2.7: Colour value:**

It is a unit used to express the yield of pigments in CVU/ml.

**2.8: Centrifugation:**

It is a process used for the separation of heterogeneous mixture process based on the sedimentation rates of particles. Widely used in the industrial process as a part of down streaming procedures also used in laboratories.

**2.9: Agro industrial substrates:**

Wastes of the agricultural industries that are used to culture microorganisms to obtain the biotechnological valuable compounds are called as agro industrial substrates.

# CHAPTER-3
# RATIONALE AND SCOPE OF STUDY

There is a significant role of pigments in various industries such as textile industries, food industries, and pharmaceutical industries and also in various cosmetics industries. The earlier existing chemical dyes are toxic, carcinogenic as well as polluting in nature and in addition to this the production of this dye process are very expensive and energy consuming.

Colorants from plant and animal origin are no longer considered as efficient food colorants due to drawbacks such as instability towards light, heat, adverse pH, low water solubility associated with them.

The biological way of extraction of these pigments from the microbial flora is an alternative efficient method which resolves all these problems. Fungal pigments can be easily grown on the cheaper substrates such as wheat bran, wheat straw, rice straw, and bagasse as compared to bacteria. A large quantity of agro industrial residues are generated that can be utilized in a positive way for pigment production

In the present study *Penicillium sp.* was found to be a potent pigment producer. In addition, the yield of the pigments at the industrial level can be enhanced by using wheat bran as a substrates induced with starch and other sugars. Agro industrial residues are readily available, cost effective, are of good source of nutrition and can be successfully recycled. The present study was designed so as to evaluate the effect of agro-industrial residues as well as pure sugars as inducers on the production of pigments from *Penicillium* sp. that can be used in food industry.

# CHAPTER-4
# OBJECTIVE OF STUDY

The topic of the present work is screening of the carbon sources for the pigment production by *Penicillium* species. Keeping in mind the importance of pigment and effect of carbon sources on pigment production. The work was planned under following objectives

1. To screen the agro industrial wastes as a potent carbon source for pigment production by *Penicillium sp.*
2. To evaluate the effect of inducers on pigment production by *Penicillium sp*.
3. To partially characterize the pigment produced by *Penicillium sp.*
4. To evaluate the stability of the pigment produced by *Penicillium sp.*

# CHAPTER-5

# REVIEW OF LITERATURE

Pigments like melanin are produced by certain filamentous fungi to survive in the harsh environmental conditions such as UV radiation, high concentration of heavy metals and salts, drying. Gessler *et al.,* (2013) stated that these pigments has also proven their efficiency in protecting the pathogenic fungi by the mechanisms of reactive oxygen species.it has been noticed that in unfavorable environmental condition such as at high altitude (mountains), plants surfaces or any dessert area there is an increase in the melaninzed fungus.in comparisons with the other micro biota of that particular region.

## 5.1: PONTENT PIGMENT PRODUCERS

Wolf *et al.,* (1960) stated that *Penicillium chrysogenum* which is well known to produce penicillin is also a potent yellow color pigment producer. This characteristic also helps to distinguish the *Penicillium chrysogenum* with that of the *Penicillium notatum,* since notatum doesnot produces pigments. The study of this yellow color pigments was done by clutterbuck, lovell and raistrick and they named it chrysogenin .in the earlier days these chrysogennin was considered as the undesirable by product in the penicillin production and hence it was separated to obtained a high quality of penicillin the crude pigment can be extracted by the acidification of the cultures of *Penicillium chrysogenum* at pH 3.5 to obtain a flocculate precipitate. The precipitate is further extracted by using ether solutions, the pigments was very much soluble in organic solvents but least soluble in petroleum ether and in water.

Espinoza *et al.,* (2004) had isolated and characterized three *Penicillium* strains from the Mexican semi-desert (found to be xerophilic in nature) that are able to produce red pigments, at an optimum temperature of 24 °C, at an initial pH10 in potato dextrose agar. The maximum yield was obtained in the submerged fermentation using malt extract as nutrient sources.

Many studies had been conducted which reveal that there are various fungal strains which are capable of producing pigments. Several filamentous fungi produce non carotenoid pigments such as anthraquinones, naphthaquinones, dihydroxy naphthalene, melanin (a complex aggregate of polyketides) and flavin compounds such as riboflavin. Anthraquinone (octaketide) pigments like catenarin, chrysophanol, cynodontin, helminthosporin, tritisporinand erythroglaucin generally produced by *Eurotium* spp., *Fusarium* spp., *Curvularia lunata*and *Drechslera* spp. (Mapari *et al.*, 2005).

Certain ascomycetous filamentous fungi belonging to the genera *Epicoccum, Penicillium, Monascus* have the ability to produce pigments exogenously Mapri *et al.,* (2006) isolated these organisms from natural environment and had quantify their pigments by colorimetric analysis which revealed that these pigments are water soluble. The fungal extracts showed additional color hues in the red spectrum and some similar hues in the yellow spectrum in comparison with the references (natural colorants). It was very much similar or brighter in terms of chroma to some of the reference used in that study. The diversity of color was not only present among different fungal genera or species but also within the same species, when media for their growth was changed.

In the trend of searching the new natural colorants sources many other microbial sources were investigated. A study investigated by Mendez *et al.,* (2007) isolated three fungal strains *Quercus sp. Larreatridantata* they were morphologically, molecularly physiologically, characterized. Various temperature (8, 16, 20,24 and 32ºC) and pH (4, 6, 7, 8and 10) were evaluated in order to determine the optimum conditions for pigment production in submerged as well as solid state fermentation these strains were identified as *Penicillium purpurogenum*(GH2) and *Penicillium pinophilum (EH2* and *EH3).*The maximum pigment was produced by *Penicillium purpurogenum* (GH2) at 24ºC and at ph6 in solid state fermentation.

Attla *et al.,* (2010) investigated eleven different fungal strains including *Acrostalagmus* sp. (NRC 90), *Alternaria alternata*(NRC117), *Alternaria* sp. (NRC 97), *Aspergillus niger*(NRC 95), *Bisporomyces*sp. (NRC 63), *Cunning hamella*(NRC 188), *Penicilliumchrysogenum*(NRC 74), *Penicilliumitalicum*(NRC E11), *Penicilliumoxalicum*(NRC M25), *Penicillium regulosum*(NRC 50), *Phymatotrichum* sp. (NRC 151) in order to check the ability of these isolates for production of brown and reddish textile dyes. Dye precursor were used in the medium during fermentation. In this particular study H- acids (1-naphthol-8-amino-3, 6-disulfonic acid) were used as a dye precursor,. There was an significant increase in the yield of pigments was observed. The organisms were initially maintained on czapaks dox agar.

In the same year Wanghailei *et.al.*(2011)had done the co-culture of *Penicillium sp. HSD07B* and *Candida tropicalis*. It produces a red pigment which includes six different components identified by TLC and HPLC. The pigments gave negative results of Ames test and were stable between pH 2-10 and in temperatures range of 10–100ºC. It was observed

that the pigments produced were much more photo stable and was stable between pH 2-10 and temperature 10ºC - 100ºC.

Isarra *et al.,* (2011) stated that *Monascus* species are able to produce various types of pigments as well as secondary metabolites such as citrinin and monalicin K which makes them undesirable as a food colorants .This problem was solved by inducing mutation by ultrasonic waves continuously for the four generation on the above mentioned fungal species. The mutant *Monascus* species does not show the presence of secondary metabolites and the stability of the pigment was found to be much more at pH8.

One such study includes the pigment production by a new strain of *Fusarium moniliforme KUMBF1201*, which was isolated from the paddy field near Coimbatore area in Tamil Nadu, India. The aim of work was to check the suitable medium for the growth of the fungus. Out of six different liquid media and eight different solid media; it was found that the fungus could efficiently grow on potato dextrose agar (PDA) and potato dextrose broth (PDB). The optimum temperature of growth was found to be about 28ºC. An enhancement in growth was observed when 2% yeast extract and 2% glucose was incorporated in the media. (Stanly *et al.,* 2013)

Tudor *et al.,* (2013) certain lignocellulose fungi produce pigments under stressful environmental conditions to protect their mycelia. Due to variation in stressful conditions there can be minor changes in the color intensity of the pigments. Melanin pigments were produced by decaying wood at a maximum pH4.5.

Rashmi *et al.,* (2013) had isolated *Monascuss sp.* from that of the pomegranate and the cultural condition for the particular fungus were optimized in an submerged fermentation maximum pigment was found to be produced on $16^{th}$ day of incubation at pH6.5 and at a temperature of 30ºC.

Recently Chintapenta *et al.,* (2014) had isolated the mangrove *Penicillium* that produces red pigments. In order to check the effect of bio elements, the pigments were subjected to various salts such as sodium chloride, sodium nitrate, ammonium sulphates, and calcium, zincs iron. The study revealed that the effect of bio elements was not much effective in enhancing the pigment production but as the concentration of the salts increases the pigment production also increases.

## 5.2: POTENT SUBSTRATES FOR PIGMENT PRODUCTION

Johns *et al.*,(1991)reported that the rice with that of the synthetic medium can be used to produce pigments by *Monascus purpureus* .The composition can be solidified by using carrageenan. The pigments were much more produced at ph5 and at moisture content of 56%. It was observed as the moisture content decreased the yield of pigments also decreased the pigments were highly sensitive to physical parameters.

Pandey *et al.,* (2000) stated that due to the advancement in biotechnology the waste generated by the agro industries can be now utilized to produce various biotechnological value able compounds such as Sugarcane bagasse, it is a complex material obtained from the sugar cane industry. It contains about 25% hemicellulose and 25% lignin 50% cellulose, .as they are rich in nutrient content, it can be used as an ideal substrate for culturing microorganisms. Various trials has been made to produce bioactive compounds from that of the potent agricultural wastes such as protein-rich animal feed, amino acids organic acids and other compounds of pharmaceutical importance, etc. Generally, pre-treatments required to enhance the substrate utilization process by the microbes. The solid-state fermentation technology can be one of the efficient methods to use this substrate and to produce bioactive compounds.

The researches have also used ethanol as a substrate for the production. Julova *et al.,* (2002) stated that ethanol at 2%v/v can be used as a sole source of carbon by *Monascus purpeurus.* The yield obtained was relatively higher than the yield obtained by using maltose for the pigment production. Ammonium chloride and ammonium hydroxide were found to support the production of orange colour pigment and the amino acids were found to enhance the production of yellow and red color pigments.

Carvahlo *et al.,* (2007) stated that agro industrial wastes such as corn, wheat, cassava and rice are the potent substrates for natural colorants production. However cassava does not much support the pigment production but its low price can compensate for it for increase in the yields of pigments this substrate can also be enriched with nutrient medium.

Palanivel *et al.,* (2011) stated that the corn cob act as a potential substrate for the pigment production in solid state fermentation by *Monascus purpeureus KACC 42430*.Relatively high pigment yield production was observed than any other agro industrial wastes used as a substrate. The pigments were stable at a point of view of industrial aspects.

Nadzari *et al.,* (2012) had optimized the solid state fermentative conditions by using the agricultural products as a substrate such as corn, papaya, banana, guava, meat, white rice for the pigment production by *Monascus* species. The maximum pigment production occur on seven days inoculum on banana having moisture content of about50% at temperature 30ºC and pH6.

Wood can also be used as a substrate for pigment production Tudor *et al.,* (2012) had cultured eight different fungus using wood as a substrate, the pigments produced were tested for the reactivity in various moisture content. The intensity of the pigments can be stimulated by controlling the moisture content of the wood substrates.

The quantity and the quality of the pigment produced is also based on the type of nutrition provided to the pigment producers Silveria *et al.,* (2013) investigated the effect of various inorganic nitrogen sources on the *Monascus purpuerus* producing red pigments in a submerged fermentation using sugarcane bagasse as a carbon sources and it was observed that the peptone and soy protein isolate enhances the pigment production whereas ammonium chloride did not much support the pigment production.

Since the colorants have a wide range of application in the food industries, the pigments of *Monascus*s has been used as a food colorants in many Asian countries Rajeswari *et al.,*(2014) investigated both solid state fermentation and submerged fermentation in order to check the more potential mode of fermentation that enhance the pigment production. The relatively more yield was obtained in case of solid state fermentation as compared to submerged fermentation using corn (pH3.6) and sugarcane bagasse (pH5.6) as a substrate.

Panesar *et al.,*(2015) stated that industrial processing of agricultural products generates a large quantity of agro-industrial wastes throughout the year such as straw, stem, stalk, leaves, husk, peel, legumes, bagasse, spent grains, and many more. Because of the large availability and being rich in nutritive compounds it could have been use in various other processes, the reuse of the wastes has been the great interest either for environmental as well as well as economic aspects.

## 5.3: PIGMENTS PRODUCTION

Different researchers use different protocols in their studies for the pigment production. Generally the pigments are produced by two fermentative process either by submerged fermentation or by solid state fermentation Lee *et al.*,(2001) conducted a study on a red pigment producer (*Monascus purpurus*) with an objective to optimize the submerged fermentative conditions as well as the media composition. It was observed that the Mn2+ and Fe2+ with proper agitation at the speed of 700rpm induces the pigment production and when glucose and monosodium glutamate is incorporated in the growth medium they also enhances the pigment production.

Cho *et al,*(2002) in order to check the effect of carbon sources, rheology, morphological characteristics of a red pigment producer (*Pacilomyces sinclairii).* The fungus was grown on the medium containing sucrose and starch. The organism's specific growth rates were much higher in sucrose medium and the yield of pigment production was relatively much higher in starch as compared to sucrose.

Gunasekaran *et al.,* (2008) had isolated ascomyceteous filamentous fungi producing extracellular pigments (*Penicillium sp.*) from the soil near Coimbatore region of Tamil Nadu, India. Their cultural conditions were optimized at minimum inoculation of 4 days at pH 0.9, and at temperature 30ºC it showed maximum pigment production on the presence of peptone and soluble starch.

Alejandro *et al.*,(2011) has conducted a study in which the combined effect of temperature, pH and fungal morphology of *Penicillium purpurogenum* producing red pigments was evaluated. Czapek-Dox media was used incorporated with D- xylose as a source of carbon. Out of three different pH range (5, 7, 9) and two different temperature (24ºC and 34ºC) the maximum yield of pigment was obtained on 24ºC at pH5.

Dikshit *et al.*,(2011) stated that the temperature and pH plays an important role in intensity of pigment production as well as the growth pattern of the organisms in an submerged fermentation. The maximum pigment was obtained on the $16^{th}$ day of incubation at 30ºC at pH5.5. The yield of pigments was significantly increased when rice along with 5% yeast extract and 1% MSG was supplemented along with a substrate used.

Padmavathi *et al.,* (2013) has investigated that the two *Monascus* species *M. sanguiens* and *M. purpuereus MTCC410*. These organisms were found to be producing more pigments on potato peels coconut residues, respectively.

Bio pigments are not only used to provide good health or to coat standard preparation of the pharmaceutical but they are also used in the food industries as a preservative. They are also beneficial's to reduce the environmental pollutions by limiting the use of synthetic colorants since; synthetic colorants are not easily degraded by the microorganisms. Mugesh *et al.,* (2014) had checked the effect of various chemical constituents of various media in the bio pigment production by *Clerodendrum viscosum.* The maximum pigments were observed on MGYP and CYA media.

## 5. EXTRACTION AND CHARACTERIZATION OF PIGMENTS

After the production of pigments the next step is the extraction and characterization of pigments .This the crucial step in pigment production because the pigments which are obtained must be free from any other secondary metabolites and the mycotoxin and should be stable under various environmental stress conditions. The pigments produced can be extracted and characterized in numerous ways such as HPLC, TLC etc.

A study conducted by Yihzhong *et al.,* (2005) has described the use of high performance liquid chromatography in the separation and identification of the natural pigments like betalins (plant origin). From the 37 species of 8 genera 16 beta cyanins were extracted and among that three beta xanthin has been characterized by using RP-HPLC and mass spectroscopy. The study revealed that betacyanins includes 6 gomphrenin types, 8amaranthine and 2 betanin.

Mapari *et al.,*(2006) conducted a study in which the colorimetric characterization of the pigments produced by ascomyceteos filamentous fungi (*Penicillium, Epicoccum, Monascus*) was done. The extracts of the color were compared with that of the natural colorants which are widely used .The colorimetric analysis of the fungal pigments revealed that their angle of hues ranges from 40 to 110 indicating it possess orange yellow, red colors in them. Hence the study indicates that there are other filamentous fungi other than *Monascus* that can produce orange, yellow, red shades color.

Centano *et al.,* (2007) had done the surface enhanced Raman scattering (SERS). It was found to be the appropriate method to characterise the complex pigments produced by the microorganisms. In their work with that of the melanin pigments from that of the cuttle fish (*Sepia offinalis*). A significant increment in the spectra was observed in the sample of *Sepia* then compared to pure sample that are available commercially.

A comparison between the micro FTIR and micro Raman had been done by Duran *et al.,* (2009) by using the sample that are of cultural heritage of southern Spain such as *Priss*

blue (ultramarine, Prussian blue) and red (vermillion, red ochre), yellow (malachite copper), yellow (realgar), white (gypsum, calcite) .The characterization was associated with certain difficulties in some pigments and this was solved by using SEM EDX technique. Thus the combination of SDX EDX with that of the micro FTIR and micro Raman was found to be an efficient method for characterization of pigments.

Robinson *et al.,* (2014) conducted a study which involves testing the twelve different solvent for their efficiency to extract the pigment from *Chlorociboria aeruginosa* and *Scytalidium cuboideum* the basis of the assessment was the saturation of pigment produced in different solvents ,their ability to diffuse through filter paper and to check whether the pigments can react with solvents or not. The study revealed that the dichloromethane was a best solvent for extracting pigments from the above mentioned microorganisms.

## 5.5: PIGMENTS STABILITY

The stability of the pigments are of much concern the pigments which has been extracted from the isolated microorganisms must be stable in extreme environmental conditions. And this factor plays a major role during the commercialization of any pigments onto the market.

Natural pigments stability varies in the various environmental conditions one of the important stability is the photo stability. Natural colorants which are in used (plant or animal origin) are not much photo stable. Mapari *et al.,* (2009) had conducted a study in which he had study the photo stability of the pigments produced by *Penicillium* and *Eppinoccum*species in a soft drink models. He observed that these pigments were highly photo stable. The chemistry of photo degradation was assessed by using High performance liquid chromatography – diode array detection mass spectroscopy it was observed that a structural analogue of sequoiamonascin compound foramation occurred which is similar to pigments produced by *Monascus* sp. and also beneficial in maintain the stability of the pigments.

Among various stability factors one of them is solvent stability. Wongsorn *et al.,* (2011) has check the stability of the ultrasonic mutants of *Monascus purpureus* pigment on different solvents. The yield of pigments was much more produced in mutants as compare to that of the wild type and was found to have higher residual stability in wide range of solvents.

## 5.6: INDUSTRIAL APPLICATION

Various fungal species produces the pigments like Prodigiosins (red pigment) they have been used as antiprotozoal cytotoxic, anti-inflammatory and antibacterial properties. A pigment produced by *Monascus purpureus* consists of a compound that has the ability to inhibit the synthesis of bad cholesterol. Chengaiah *et al.*,(2010) stated that due to the toxic effect and the costly raw materials required for the synthesis of the artificial colorants the natural dyes are nowadays more in trend .these natural colorants are not only able to overcome the disadvantages of the artificial colorants but also possess certain properties which can be used by mankind as a therapeutic agents.

The pigments having a wide range of application in pharmaceutical industries mainly in coating the tablets, liquid orals, toothpastes, ointments etc. Allam *et al.*,(2011)the colorants are not only used to maintain the stability of the standard for a long duration of time but these pigments are also known to increase the efficacy of the standard products.

Ideal properties of the natural colorants to be used in pharmaceutical

1. It should be nontoxic and must not have any physiological activities and free from any harmful components such as mycotoxins in fungal pigments.
2. Must be readily available and inexpensive.
3. The tectorial efficiency power should be high so that large quantity of standard preparations can be colored using a small quantity.

According to Panesar *et al.*,(2015) certain pigments have been patented to be used for endoscopy as a staining agent .Various pigments are also being used in research purpose with fluorescent molecules to label antigens and antibodies Pigments also play a major role in providing good health to human's such as certain pigments like melanin used in various cosmetics to avoid the harmful effect of UV radiation on skin. The pigments are also very much important as pharmaceutical ingredients as well as they also act as a SCP (single cell protein) for aqua cultured animals. Currently the carotenoids are being commercialised as the potent source of vitamin A.

# CHAPTER-6
# RESEARCH METHODOLOGY

## 6.1: Equipment Required:

Autoclave, Hot air oven, spectrophotometer, Laminar air flow, Centrifuge machine, weighing machine, rotatory shaker, incubator all these equipment were availed from the School of Biotechnology and Biosciences, Lovely Professional University, Phagwara, Punjab.

## 6.2: Materials Required:

Glass wares, chemicals and reagents used in this project work were supplied by School of Biotechnology and Biosciences, Lovely Professional University, Phagwara, Punjab.

## 6.3: MAINTAINANCE OF THE FUNGAL CULTURE:

The *Penicillium* culture was isolated from the soil and the fungal culture was sub cultured by transferring the spores from the preserved culture into the fresh medium potato dextrose agar slants which were prepared earlier in an aseptic condition .The inoculated slants were incubated for 4-5 days and this days old culture is further sub cultured. to maintain the culture for further use.

**Table:1: Media composition of potato Dextrose agar**

| Ingredients | Gms/ltrs |
|---|---|
| Potatoes infusion form | 200 |
| Dextrose | 20 |
| Agar | 15 |
| Final pH(at25ºC) | 5.6±0.2 |

Downes F.P. *et al.,* (2001)

## 6.4: MACROSCOPICAL STUDY:

The *Penicillium sp.* culture was grown on potato dextrose agar plates by incubating it for 4 days at 28±2ºC and their macroscopical characteristics were analysed.

### 6.5 : MICROSCOPIC EXAMINATION:

The selected interested fungal isolate was microscopically identified after staining it with LCB (lacto phenol cotton blue) which is done by placing a drop of LCB in a clean grease free slide and then adding a loop full culture, the culture was mixed with LCB.A clean coverslip was placed over the suspension and care was taken that there should not be any bubble formation while placing coverslip onto the slide. The slides are next observed microscopically under 10X 40X and 100X (oil immersion).

### 6.6: RADIAL GROWTH:

From the plates a equal sized (8mm) bits of agar covered with fungal mycelium was cut by using bore well. The agar bit containing fungal culture was transferred to the centre of freshly prepared potato dextrose agar plates. The cultures were incubated at 28º±2C and their radial growth pattern was observed at an interval of 24 hours for 10 days.

### 6.7: INOCULUM PREPARATION:

0.5% tween 80 was prepared by adding 0.5 tween 80 to 100ml of distilled water and autoclaving it at 121ºC for 15min. Tween 80 (polysorbate80) is a hydrophilic non-ionic surfactant. 3ml of tween 80 was added to pre cultured potato dextrose agar slants and the fungal growth was scrubbed with the help of sterile inoculation needle, so that the spores can be suspended into tween 80. This was used as a mother inoculum for inoculating the experimental setup.

### 6.8: EXPERIMENTAL SETUP:

Wheat bran, broken wheat and wheat straw were procured from the local market of Phagwara. Ten grams of each agro waste was weighed and small quantity of water about 10ml was added to provide 65-70% moisture content. Wheat straw was soaked overnight and the moisture content was adjusted to about 62-65% .The substrate were autoclaved at 121ºC for 15min and then inoculated from with mother inoculum by transferring 1ml of spore suspension into each substrate aseptically. The setup was performed in replica. The inoculated substrates were incubated at 28±2ºC and examined for pigment production after 6, 12, 18 days

Ten grams of the substrate that was found to enhance the pigment production from the first setup (wheat bran) was weighed and the moisture content was adjusted to 65-70%.To evaluate the effect of carbon sources (as inducers) in the pigment production by *Penicillium sp.* 0.1g of each purified carbon sources was added to the substrate. Wheat bran inoculated with *Penicillium sp.*. In the present study three different carbon sources were used, *viz*., lactose, soluble starch and glucose, in order to study their effects as inducers on pigment production by *Penicillium sp.*. These sources were added to the substrate and mixed well with the glass rod. The substrates were autoclaved at 121 ºC for 15 minutes. From the prepared inoculum 1ml of the tween 80 along with the spores was inoculated to each one of the flasks and mixed well with the help of sterile glass rod in aseptic conditions. Then the inoculated flasks were observed for various incubation period for the efficiency of the substrate and the carbon sources to enhance the pigment production. The inoculated substrates were incubated at 28º±2C and examined for pigment production after 6, 12, 18 days.

## 6.9: EXTRACTION OF PIGMENTS:

After incubation of 6 days the fungal samples were autoclaved at 121ºC for15 min. The sample was dried in the tray drier at the temperature of 50ºC±2 for 1 hour. When its complete moisture was lost the samples were crushed and powdered by using mortar and pestle. The powders were stored from each setup that was placed in the previous section. this experiment was separated for samples incubated for 12 and 18 days.

0.1g of these powders was dissolved in 10ml of methanol and it was kept on rotatory shaker at 200rpm for 30 min. It was then centrifuged at 5000rpm for 30min. The supernatant was collected and the pellet was discarded. The obtained supernatant was further use for spectrophotometric analysis.

## 6.10: SPECTROPHOTOMETRIC ANALYSIS

The absorbance of the supernatant was read at 410 nm correspond to orange yellow colour by using an visible spectrophotometer. The colour value was determined by using the colour value formula given below

$$\text{Colour Value} = \frac{\text{OD X Dilution X Volume of Extract.}}{\text{Amount of sample(gm.)}}$$

The colour value was expressed as CVU/ml of the extracts.

## 6.11: PIGMENT STABILITY

### 6.11.1: Temperature stability:

The pigments were extracted by using the similar protocol mentioned in section 6.6. The absorbance of these extracts was taken and they were further subjected to various temperature treatments at 30, 40, 50, 60, 70, 80 and 90 maintaining in water bath After the incubation of half an hour absorbance is taken and finally both the absorbance values were compared to determine stability percentage by using the formula given below by Velmurgan *et al.*,(2011)

$$\text{Stability percentage} = \frac{\text{Initial Absorbance} - \text{Final Absorbance}}{\text{Initial Absorbance}} \times 100$$

### 6.11.2: pH STABILITY

The extracts were subjected to solvents of different pH values ranging from 4-9(4, 5,6,7,8,9). After an incubation of 30 minutes absorbance was read and their stability percentage was calculated. Velmurgan *et al.*,(2011)

### 6.11.3: SOLVENT STABILITY

The extracts were prepared with 4 different solvents including distilled water (Ethanol, Methanol, Chloroform, Acetone) to check their stability. After taking the initial absorbance the extracts were incubated at 30º±2C in dark. After incubation of 7 days, the final absorbance was recorded and the stability percentage was determined, as done by Wongsorn *et al.,* (2011).

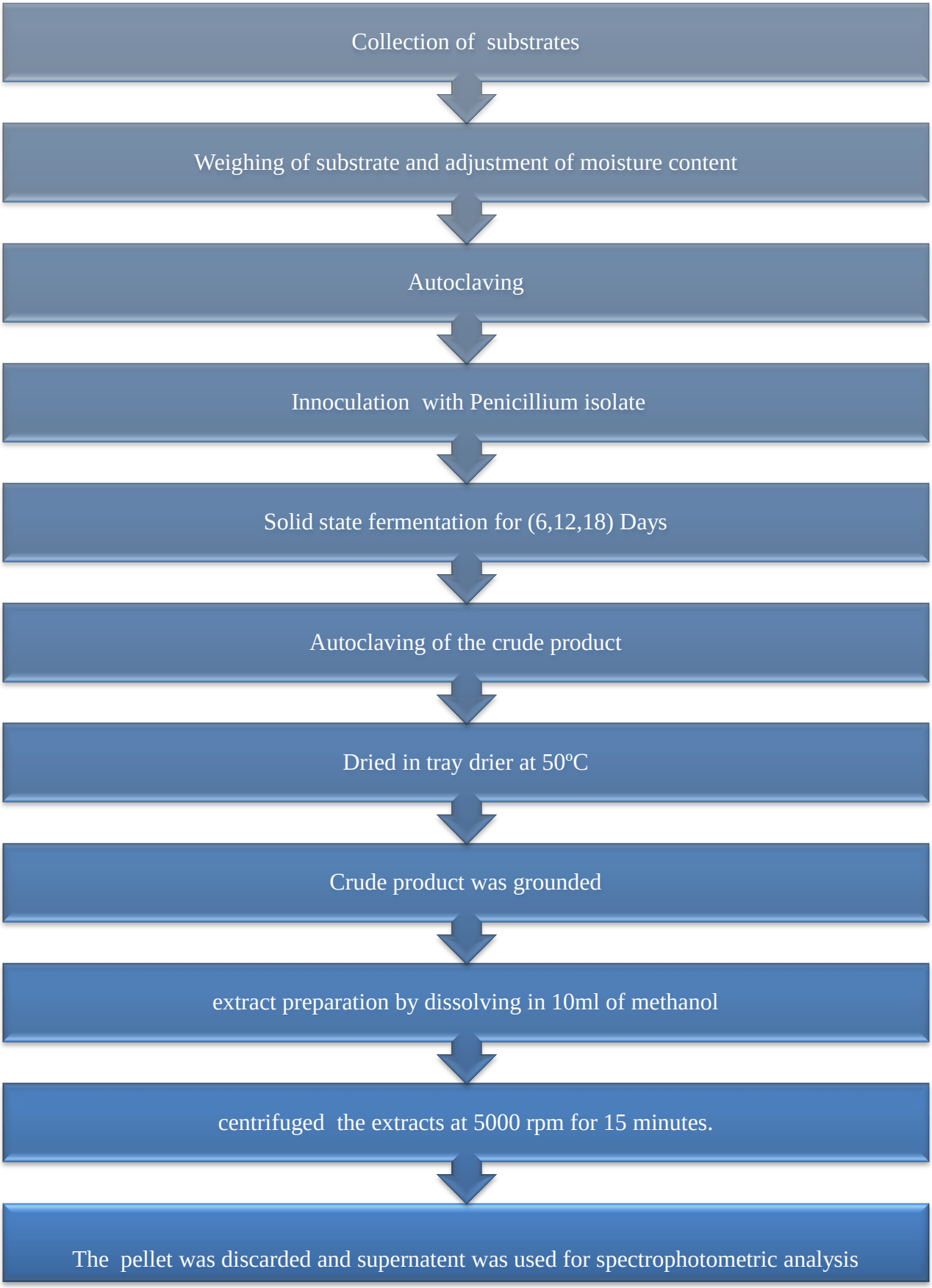
### 6.11.4: LIGHT STABILITY

The extracts were prepared in methanol and their initial absorbance was recorded and these extracts were kept in sunlight. The controls of the experiments were kept in dark. The final absorbance was observed at every interval of 24 hours. Further the stability percentage was calculated.

## 6.12: CHARACTERIZATION OF PIGMENTS:

The pigments were characterized by using FTIR (Fourier transform infrared spectra). Enough samples is required for the proper FTIR analysis. Firstly the background spectra are observed and then it was subtracted from the test spectra. The system is fully computerized and gives the information in the forms of different peaks which reveal different bonds (Lee *et al.,* 1984)

## FLOWCHART OF THE EXPERIMENTAL SETUP PLANNED (Fig: 1)



# CHAPTER-7
# RESULT AND DISCUSSION

## 7.1: COLONY MORPHOLOGY AND MICROSCOPICAL STUDY

**Plate 1: (a) Petri plate showing colony morphology:**

The *Penicillium sp.* was maintained on Potato Dextrose agar. The colonies were observed to have suppressed growth and were found to be whitish in colour at its very young stage of growth and later it turns into greenish colour at centre along with yellow colour pigment secreted onto the media. The culture was observed to be slow grower, as shown in plate1(a)

Plate1:(a): Colony morphology of *Penicillium sp.*

**(b) Microscopic view of *Penicillium* isolate**

On the microscopical analysis of culture it was found that they have brush like appearance with septate hyphae and aseptate conidiophore branched with metulate. Sterigmata were having flask shaped arrangement on metulae and unbranched chains of conidia present above the sterigmata, as shown in plate 1(b)

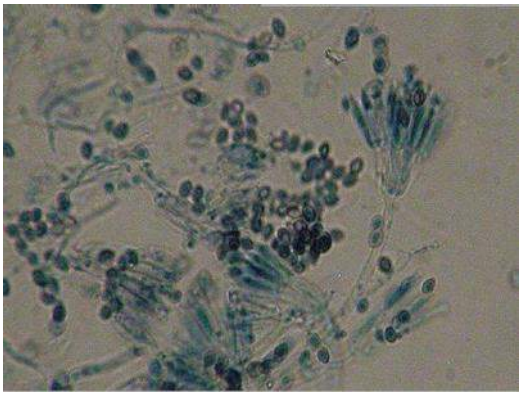


**Plate: 1(b) Microscopic observation of *Penicillium sp.***

### 7.2: RADIAL GROWTH:

The *Penicillium sp.* was cultured on potato dextrose agar at 28±2ºC and the colony diameter was recorded at an every interval of 24hours. Rapid growth was observed at initial 5 days of incubation having growth percentage of about 41.6%, 52.9%, 23.1 43.7% for 2, 3,4and 5 days of incubation respectively. Then the growth rate was declined showing the marginal growth 8.7%, 12%, 3.6%, 6.9%, 4.8% for 6, 7, 8, 9, 10 days of incubation respectively.(as shown in table:4)

Table: 2: Radial Growth:

| No. of days | Diameter (mm) * | Percentage increase/day |
|---|---|---|
| 1 | 12 | 0 |
| 2 | 17 | 41.6 |
| 3 | 26 | 52.9 |
| 4 | 32 | 23.1 |
| 5 | 46 | 43.7 |
| 6 | 50 | 8.7 |
| 7 | 56 | 12 |
| 8 | 58 | 3.6 |
| 9 | 62 | 6.9 |
| 10 | 65 | 4.8 |
| CD@ 5% | | 0.97 |

*****Average of three replicates
Temperature of incubation 28±2ºC

Medium of growth Potato dextrose agar

pH of incubation 6

The observed growth pattern may be because fungus were in the log phase and efficiently carrying out their metabolisms for their growth and development by up taking the nutrients from media components and hence leads to rapid growth while the later stages may have exhaustion of the nutrient sources or cell may have undergone stationary phase leading to marginal growth or slow growth rate.

## 7.3: Screening the agro industrial waste as a potential substrate for pigment production.

The below showed plates 2 were the photographs of *Penicillium sp.* on Broken Wheat, Wheat Bran and Wheat Straw. The initial growth for first three days was observed to have white color mycelia and as the day of incubation increased the mycelia color changes to greyish

color with green color at the center. After 6th day (plate:3) of incubation greyish color mycelia was observed matted over the surface of the substrates producing very less amount of extracellular pigments, on 12th day of incubation the substrates start turning brown with dense growth of *Penicillium* isolate. Relatively higher growth as well as pigment yield was observed in wheat bran then in comparison with wheat straw. The growth on wheat straw was observed to have the grey color mycelia attached to surfaces of straws of wheat. On the 18th day of incubation much dense growth was observed in comparison with the 6thand 12th day and hence the pigment yield was relatively higher after 18th day of incubation. Substrates was observed to be dried

## Screening the agro industrial waste as a potential substrate for pigment production.

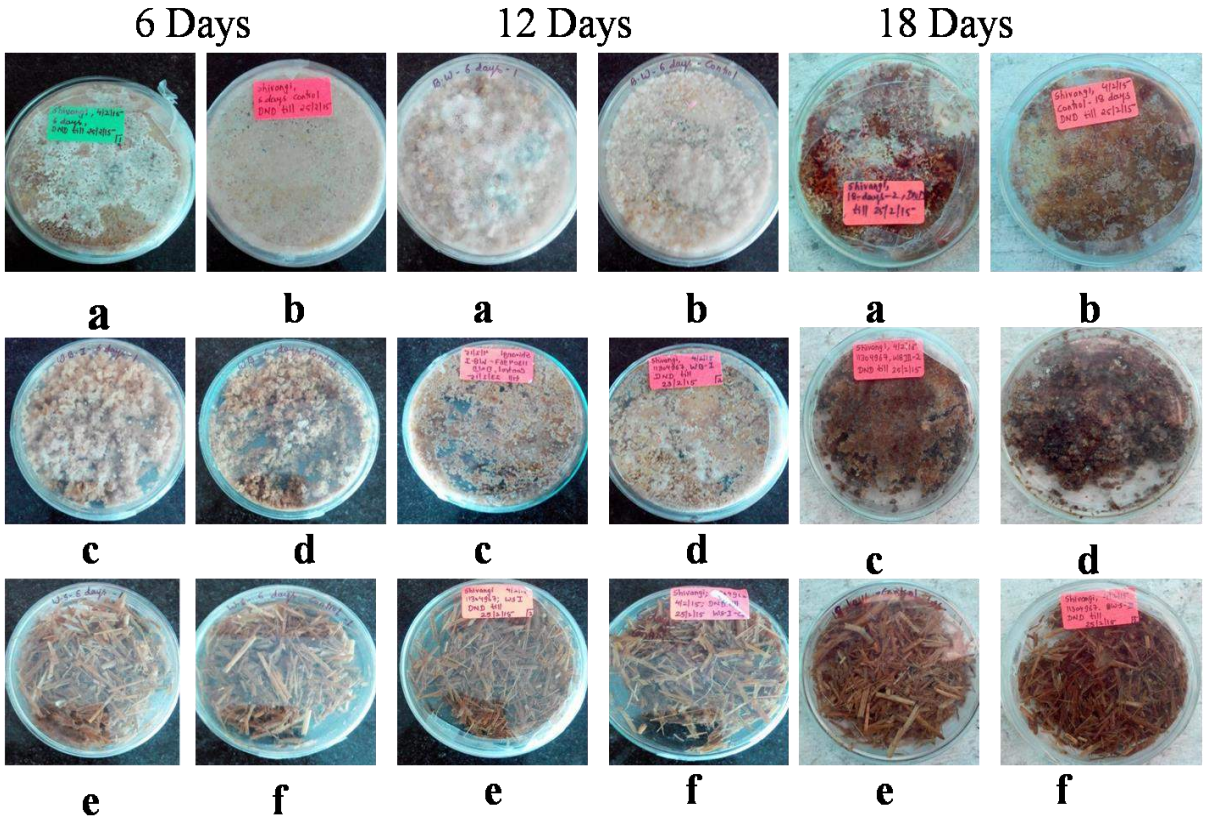



**Plate: 2:** Growth of the *Penicillium sp.* on (a) Broken wheat (b) Broken wheat Control (c) Wheat bran (c) Wheat bran Control (d) Wheat straw (e) Wheat straw Control for6,12 and 18 days

The experimental setup was planned in order to screen the potent agricultural wastes that can enhance the pigment production. In the present study efficiency of three agro industrial wastes were evaluated for supporting the pigment production which includes broken wheat, wheat bran and wheat straw. The maximum yield was obtained on the wheat bran as

compared to any other substrate that has been used. It was observed as the number of days of incubation was increased the pigment production also increased. Relatively Maximum yield of pigment was obtained on wheat bran on an incubation of 18 days that is of about 31.4± 0.43CVU/ml as compared to the pigment produced on 6 and 12 days that is 14.0±0.20CVU/ml and 26.9±0.11CVU/ml respectively. When Wheat straw was used as a substrate pigment production was significantly decreased. Wheat straw as a substrate gave the pigment yield of about 10.3±0.23CVU/ml,13.7±0.43CVU/ml,17.6± 0.60CVU/ml after 6, 12, 18 days of incubation respectively. Broken wheat has moderately supported the pigment production and gave the pigment yield in between the pigment yield of wheat straw and wheat bran that is of about 11.7±0.12CVU/ml, 15.9±0.89CVU/ml, 23.2±0.11CVU/ml for 6, 12, 18 days respectively.(as shown in table:3)

**Table: 3: Agro industrial wastes as a sole source of carbon**

| **Substrates** | **6 Days** | **12 Days** | **18 Days** |
|---|---|---|---|
| Wheat Straw | 10.3±0.23 | 13.7±0.43 | 17.6±0.60 |
| Wheat Bran | 14.0±0.20 | 26.9±0.11 | 31.4±0.43 |
| Broken Wheat | 11.7±0.12 | 15.9±0.89 | 23.2±0.11 |
| CD@ 5% | 0.84 | 2.00 | 1.51 |

Temperature of incubation 28±2ºC

pH of incubation 6

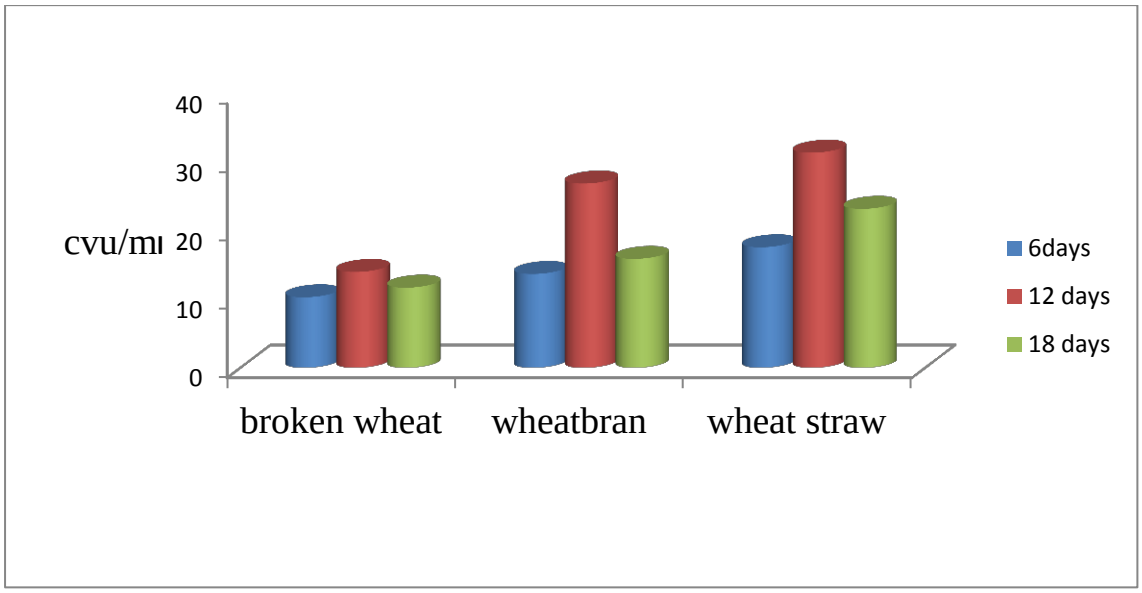



**Figure: 2: Graph showing yield of pigments produced using agro industrial wastes as a carbon sources**

**DISCUSSION:**

The high yield on wheat bran was obtained on 18$^{th}$ day of incubation of about 31.4±0.43 CVU/ml may be due to its water retaining capacity and the starchy content present in it which supports both growth of the *Penicillum sp.* and pigment production, whereas cellulose is rich in wheat straw which may not be easily degraded by the *Penicillium sp.* and hence the substrate does not support the growth and pigment production.

In the earlier studies Rosa *et al.*,(2003) has got similar results. The pigment production was enhance by using wheat flour as substrates for the red color pigment production by the *Monascus* species using submerged fermentation. Rajeswari *et al.*, (2014) has also found increase in pigment production by *Monascus species* using wheat bran as a substrate using solid state fermentation.

**7.4: Effect of inducers on pigment production by *Penicillium* Isolates :**

Plates 5, 6 and7 are the photograph of the setup planned with Wheat bran supplemented with inducers. Enhancement both growth of *Penicillium sp.* and yield of pigments were observed to be increase in comparison with non-supplemented substrates. On the 6$^{th}$ day the grey color mycelium suppressed growth on to substrates was observed and on later incubation dense

growth was observed with good amount of extracellular pigments produced. As the incubation type was increase the substrate was observed to be dry in nature.

**Effect of inducers on pigment production by *Penicillium sp.***

| 6 Days | 12 Days | 18 Days |
|---|---|---|

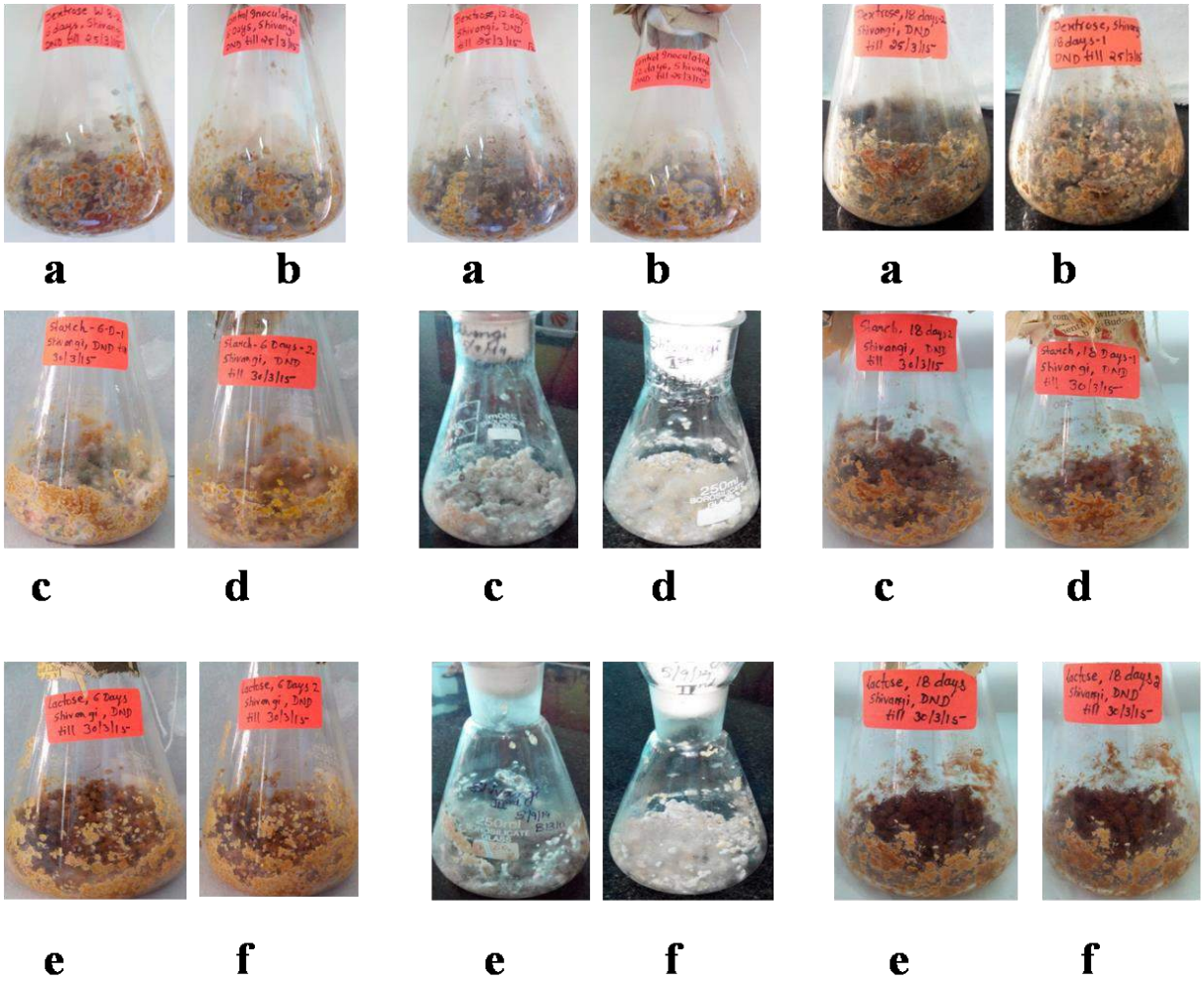



**Plate: 3:** Growth of *Penicillium sp.* after on (a) wheat bran +Dextrose (b) wheat bran(control) (c) wheat bran + Starch (d) wheat bran(control) (e) wheat bran +Lactose (f) wheat bran(control)

From the observation of the first setup wheat bran was found to be potentially enhancing the pigment production. Next setup was planned using wheat bran as a substrate supplemented with inducers *viz* dextrose, starch and lactose. Higher yield was obtained on the wheat bran

supplemented with starch as compared to any other inducers that have been used. On an incubation of 18 days that 122.4±4.07 CVU/ml of pigment could be obtained as compared to the pigment produced on 6 days (35.2±0.20CVU/ml)and 12 days (63.5±1.81CVU/ml). Starch was followed by dextrose as inducer 25.5±1.78 CVU/ml, 48.4±0.60 CVU/ml, 84.7±1.24 CVU/ml after 6, 12, 18 days of incubation respectively. Lactose was not found to an efficiently inducer of pigment production giving approximate yield of 18.6±0.63 CVU/ml, 31.0±1.53CVU/ml, 41.2±1.21 CVU/ml after 6, 12 and 18 days respectively. (as shown in table:4)

**Table: 4: Effect of inducers along with the carbon sources on pigment production by *Penicillium sp.* (in CVU/ml)**

| **Substrate** | **6 Days** | **12 Days** | **18 Days** |
|---|---|---|---|
| Wheat Bran (Control) | 11.3±0.17 | 23.9±0.28 | 28.8±0.008 |
| Wheat Bran +Dextrose | 25.5±1.78 | 48.4±0.60 | 84.7±1.24 |
| Wheat Bran + Starch | 35.2±0.20 | 63.5±1.81 | 122.4±4.07 |
| Wheat Bran + Lactose | 18.6±0.63 | 31.0±1.53 | 41.2±1.21 |
| CD@ 5% | 3.12 | 4.02 | 7.23 |

Temperature of incubation 28±2ºC

pH of incubation 6

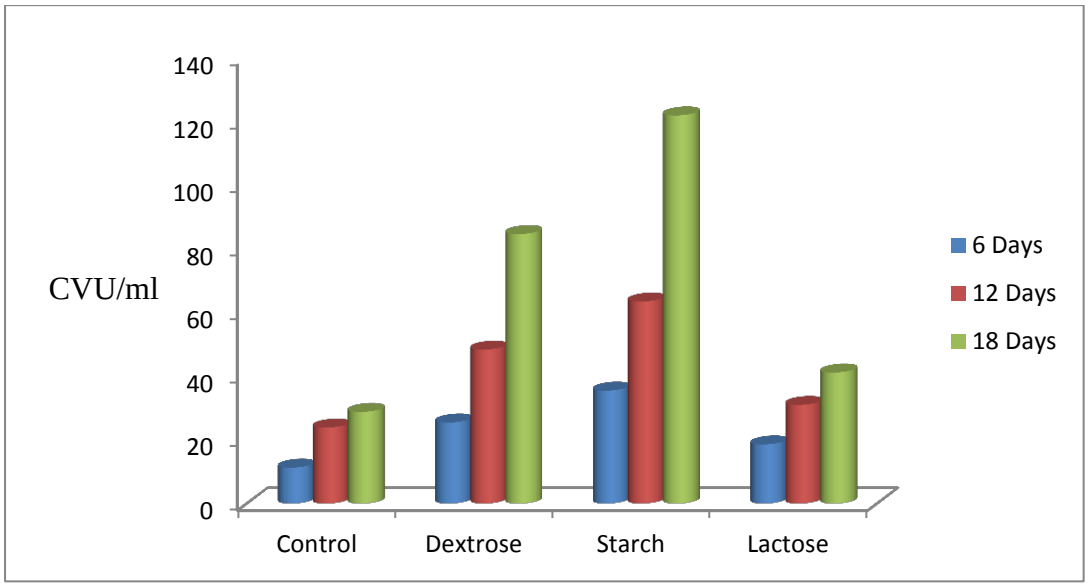



**Figure: 3: Graph showing the yield of pigments produced using wheat bran as substrate induced with inducers**

**Discussion:**

As it has been already discussed that wheat bran supports the pigment production due to its starchy content. Possible reasons for the maximum yield on substrate induced with starch may be starch can be rapidly metabolized by *Penicillium sp.*to produce pigments or it may act as a inducers to induce biosynthetic pathway for pigment production

Subashree *et al.*, (2011) obtained similar results using solid state fermentation for pigment production by *Monascus purpeureus* the where maximum yield of pigment was obtained using fructose as inducers and the second highest yield was obtained on starch .Lactose and manitol were not found to be supportive in pigment production.

## 7.5: Pigment stabilities:

## 7.5.1: Temperature stability:

The thermo stability of the pigment produced by the *Penicillium sp.* was determined by subjecting the methanol extracts of pigments to various temperature treatments at 30, 40, 50, 60, 70, 80 and 90. The pigment produced by *Penicillium sp.* was found to be stable up to 70ºC of about 94.4% and it was observed that as temperature increases the stability of the pigment decreases. The pigment stability at temperature of 80ºC was observed to be 89.2%and it was further decreased at 90ºC to about 75.6%.(as shown in Table 5)

Table: 5: Temperature stability of pigments produced by *Penicillium sp*:

| Temperature(ºC) | *Pigments Stability (%) |
|---|---|
| 30 | 99.8 |
| 40 | 98.4 |
| 50 | 99.1 |
| 60 | 92.0 |
| 70 | 94.4 |
| 80 | 89.2 |
| 90 | 75.3 |
| CD@5% | 0.89 |

*****Average of two replicates
Solvent : 80%Methanol
pH of incubation 6

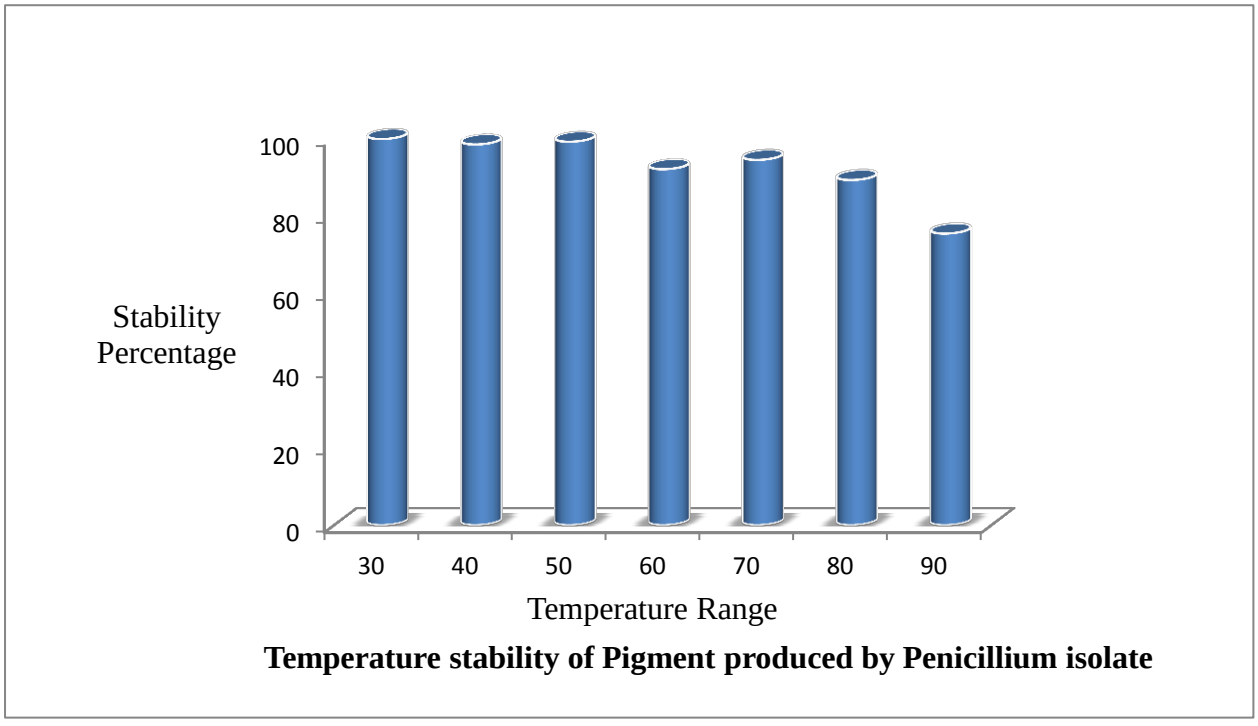



**Figure: 4: Temperature stability of pigments produced by *Penicillium sp.***

**Discussion:**

The pigment produced by *Penicillium sp.* was found to be stable upto70±2ºC. Decrease in stability was observed when it is subjected to further higher temperature this may be a reasons of due to certain chemical reaction favour by time and high temperatures that

facilitate the breakage of certain bonds leading to the degradation certain chemical compounds present in it and hence downing its stability. As the pigments were observed to be thermo stable it can be widely used in industrial application.

The results obtained were approximately similar to that of the results obtained by Velmurgan *et al.,* (2011).The pigment produced by *Monascus sp.* using corn cob as a substrate in a solid state fermentation was stable up to 60±2ºC.of about 86.2% .

### 7.5.2: pH stability

The pigment produced by *Penicillium species* was found to be stable at pH 5, 6, 7and 8. The stability percentages obtained are 97.9%, 98.6%, 98.1%, and 97.4% respectively. At higher acidic (pH4) and higher alkaline pH (pH 9) the stability of the pigment was observed to be decreased that is of about 88.5% and 87.8%.( as shown in Table:6)

**Table:6: pH stability of the pigments produced by *Penicillium* species**

| pH range | *Stability percentage (%) |
|---|---|
| 4 | 88.5 |
| 5 | 97.9 |
| 6 | 98.6 |
| 7 | 98.1 |
| 8 | 97.4 |
| 9 | 87.8 |
| CD@ 5% | 0.99 |

*****Average of two replicates
Solvent used: 80%Methanol
Temperature of incubation 30±2ºC

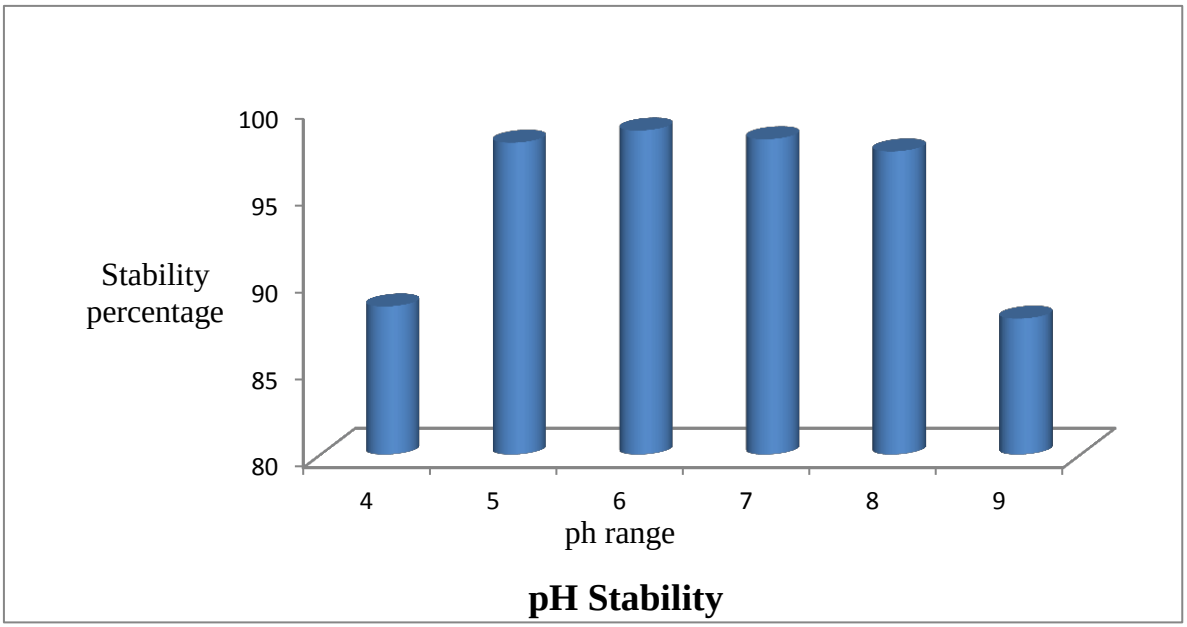



**Fig no 5: pH stability of pigment produce by *Penicillium sp.***

## Discussion:

The chemical characteristics of the pigments required certain specific range of pH to retain its activity and stability. The loss in stability may be due the protonation or change in pH has favour certain unwanted oxidation reactions that has given rise to an imbalance proportion in the chemical composition of the pigments produced by *Penicillium sp.* leads to the loss its stability at pH 9

The results got are approximately similar to that of the Velmurgan *et al.*, (2011).worked with *Monascus* pigments which was stable in pH 5, 6 and7.

## 7.5.3: Light stability

In order to check the light stability of the pigments produced by *Penicillium sp.* The methanol extracts of pigments were kept in sunlight for 5 days and the control for the same was kept under dark conditions it has been observed that the control has retained the stability that is 98.1%, 97.9%, 97.6%, 97.3% and 97.4% for day1, 2, 3, 4and 5 respectively. Whereas decrease in the stability was observed when the methanol extracts of pigments were exposed

to sunlight. The stability percentage obtained were about 96.2%, 93.4%, 93.2%, 90.9%and86.1% for day 1, 2, 3, 4 and 5 respectively.(as shown in Table:7)

**Table: 7: Light stability of the pigment produced by *Penicillium species***

| Number of days | Day1 | Day 2 | Day 3 | Day 4 | Day 5 |
|---|---|---|---|---|---|
| Control | 98.1% | 97.9% | 97.6% | 97.3% | 97.4% |
| *Pigment Stability | 96.2% | 93.4% | 93.2% | 90.9% | 86.1% |
| CD @ 5% | 0.57 | 1.25 | 1.93 | 2.07 | 2.15 |

*****Average of two replicates
Solvent used: 80% Methanol
pH of incubation 6
Temperature of incubation 30±2ºC

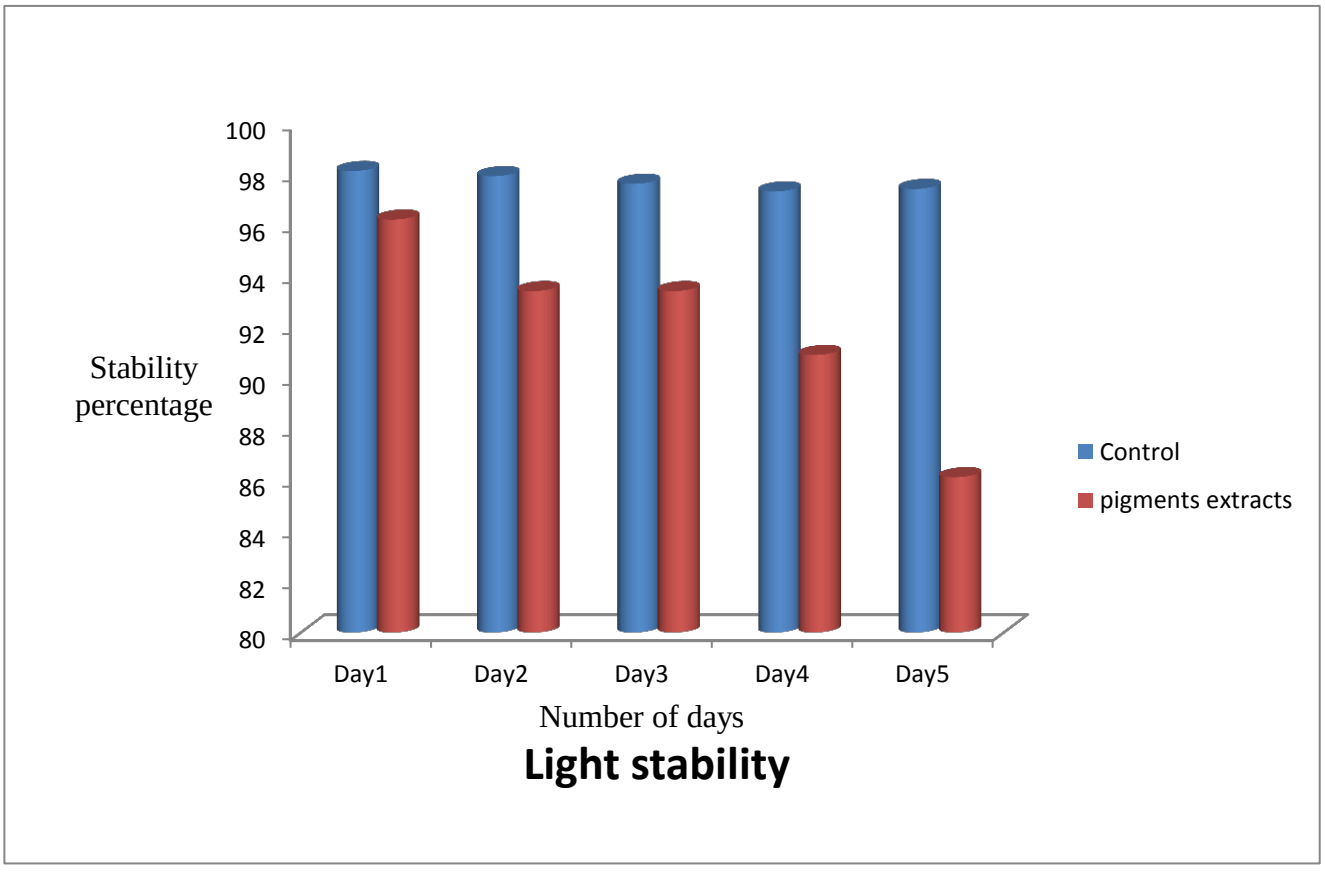



**Figure: 6: Light stability of the pigments produced by *Penicillium sp.***

**Discussion:**

As with increase in the time duration in the sunlight the absorption Of the pigments was observed to be decreased this may be due to the results of photo degradation of the chemical constituents of the pigments and hence changing its activity and stability as well.

Haieli *et al., (2011)* had co culture *Penicillium* sp. with *Candida tropicalis* it has been observed that the Pigment colour kept on dark for 30 days was not changed whereas change in color intensity was observed when kept in sunlight

## 7.5.4: Solvents stability:

The solvent stability of the pigments produced by *Penicillium sp.* was investigated and it was observed that the pigments was much more stable on methanol and ethanol that is of about 88% and83% respectively. The pigments were found to be least stable in chloroform and Acetone that is of about 50% and 44.5% respectively. (As shown in Table:8)

**Table: 8: Solvent stability of the pigment produced by *Penicillium species***

| Name of the Solvent | *Stability Percentage (%) |
|---|---|
| Ethanol | 80.25 |
| Methanol | 85.49 |
| Chloroform | 49.10 |
| Acetone | 44.35 |
| CD@ 5% | 0.29 |

*****Average of two replicates
Temperature of incubation: 30±2ºC
pH of incubation 6

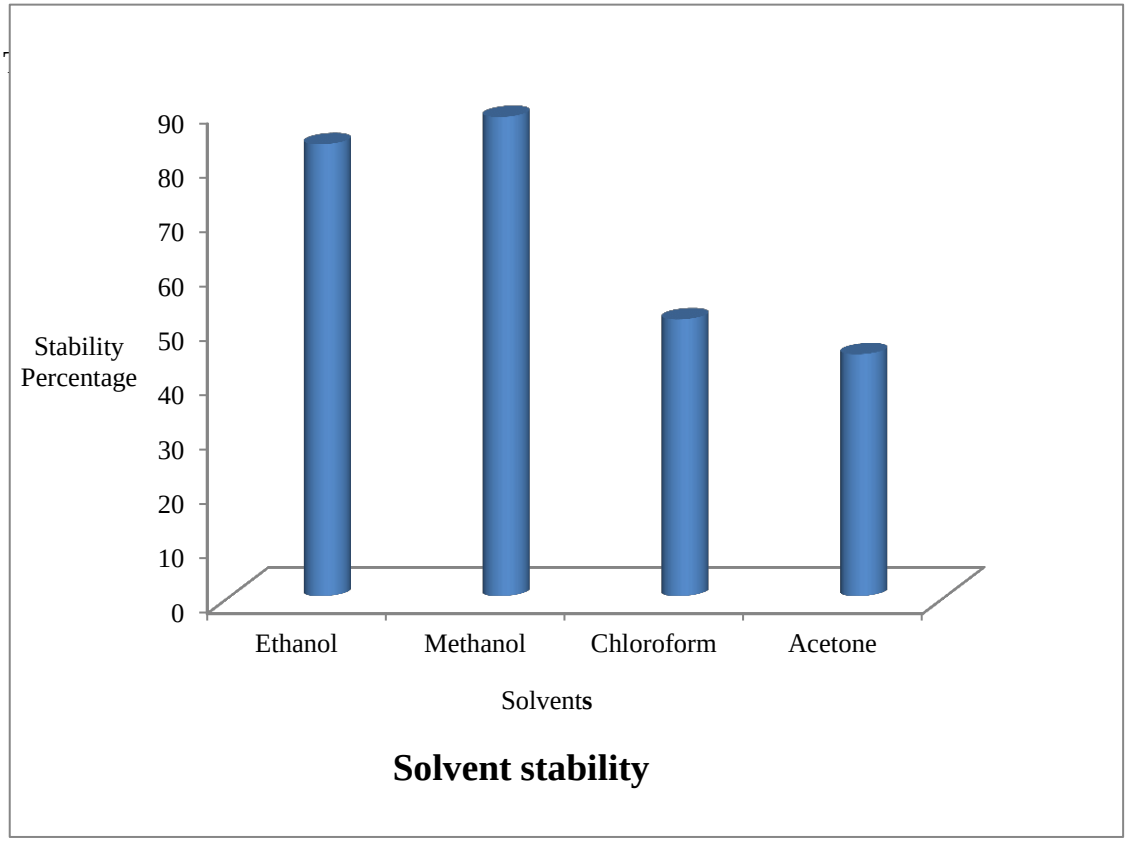


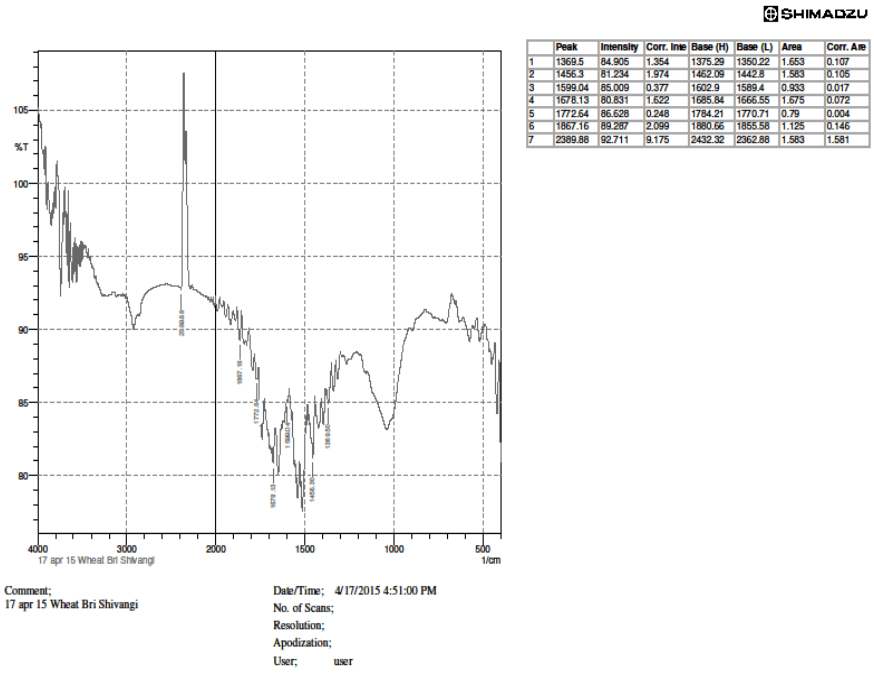

Figure: 7: Solvent stability of the pigments produced by *Penicillium sp.*

Wongsorn *et al.,* (2011) also got the similar results using methanol, ethanol, Propanol, diethyl ether, hexane the pigments was found to be maximum stable on ethanol and least stable was on methanol.

This observation may be due to the higher polarity index of methanol and ethanol in comparison with that of the Chloroform and Acetone .this properties of solvents maybe important to maintain the chemical integrity of the pigments and hence maintaining its stability.

## 7.6: Results of FTIR analysis:

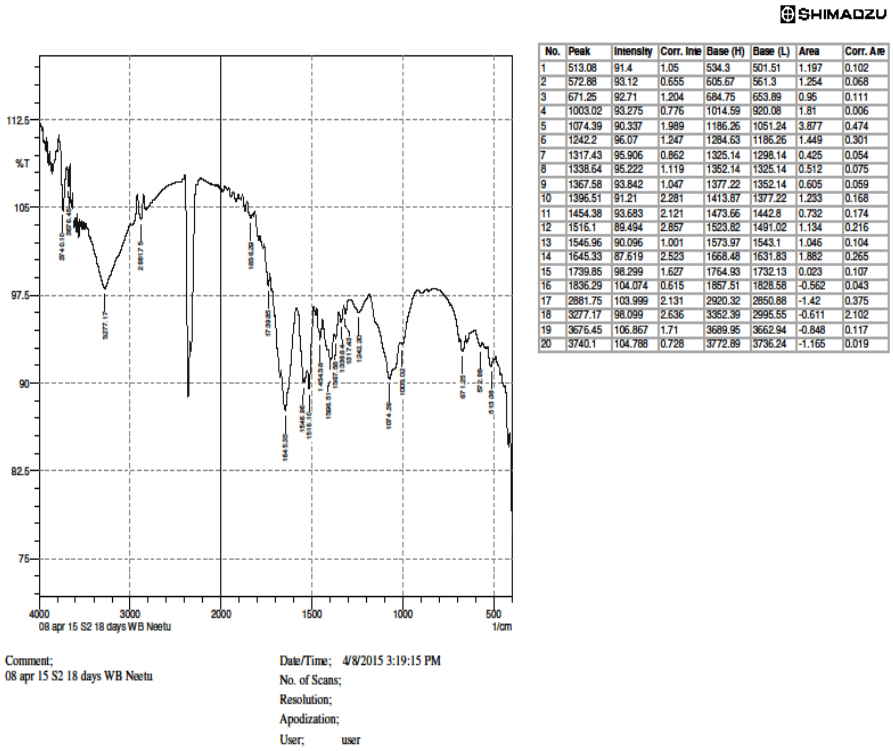
**Figure: 7.6.1: FTIR results of Control (Wheat Bran)**




| | Peak | Intensity | Corr. Inte | Base (H) | Base (L) | Area | Corr. Are |
|---|---|---|---|---|---|---|---|
| 1 | 1369.5 | 84.905 | 1.354 | 1375.29 | 1350.22 | 1.653 | 0.107 |
| 2 | 1456.3 | 81.234 | 1.974 | 1462.09 | 1442.8 | 1.583 | 0.105 |
| 3 | 1599.04 | 85.009 | 0.377 | 1602.9 | 1589.4 | 0.933 | 0.017 |
| 4 | 1678.13 | 80.831 | 1.622 | 1685.84 | 1666.55 | 1.675 | 0.072 |
| 5 | 1772.64 | 86.628 | 0.248 | 1784.21 | 1770.71 | 0.79 | 0.004 |
| 6 | 1867.16 | 89.287 | 2.099 | 1880.66 | 1855.58 | 1.125 | 0.146 |
| 7 | 2389.88 | 92.711 | 9.175 | 2432.32 | 2362.88 | 1.583 | 1.581 |

## Figure: 9: Infrared spectra of wheat bran (control)

The FTIR analysis of wheat Bran as a control gave the peaks in the range of 1369,1456, 1599,1678, 2389 which revealed that it contains C-H bonds, C-C stretch (in rings), N-H bonds bounded with primary amines, C=O stretch, C=H stretch, N-H wag bonding in them. It also indicates that wheat bran contains alkanes, aromatic compounds, primary amine, alpha and beta unsaturated aldehydes or ketones and carbonyls as a corresponding functional group (as shown in fig:8)




| No. | Peak | Intensity | Corr. Inte | Base (H) | Base (L) | Area | Corr. Are |
|---|---|---|---|---|---|---|---|
| 1 | 513.08 | 91.4 | 1.05 | 534.3 | 501.51 | 1.197 | 0.102 |
| 2 | 572.88 | 93.12 | 0.655 | 605.67 | 561.3 | 1.254 | 0.068 |
| 3 | 671.25 | 92.71 | 1.204 | 684.75 | 653.89 | 0.95 | 0.111 |
| 4 | 1003.02 | 93.275 | 0.776 | 1014.59 | 920.08 | 1.81 | 0.006 |
| 5 | 1074.39 | 90.337 | 1.989 | 1186.26 | 1051.24 | 3.877 | 0.474 |
| 6 | 1242.2 | 96.07 | 1.247 | 1284.63 | 1186.26 | 1.449 | 0.301 |
| 7 | 1317.43 | 96.906 | 0.862 | 1325.14 | 1298.14 | 0.425 | 0.054 |
| 8 | 1338.64 | 95.222 | 1.119 | 1352.14 | 1325.14 | 0.512 | 0.075 |
| 9 | 1367.58 | 93.842 | 1.047 | 1377.22 | 1352.14 | 0.605 | 0.059 |
| 10 | 1396.51 | 91.21 | 2.281 | 1413.87 | 1377.22 | 1.233 | 0.168 |
| 11 | 1454.38 | 93.683 | 2.121 | 1473.66 | 1442.8 | 0.732 | 0.174 |
| 12 | 1516.1 | 89.494 | 2.857 | 1523.82 | 1491.02 | 1.134 | 0.216 |
| 13 | 1546.96 | 90.096 | 1.001 | 1573.97 | 1543.1 | 1.046 | 0.104 |
| 14 | 1645.33 | 87.619 | 2.523 | 1668.48 | 1631.83 | 1.882 | 0.265 |
| 15 | 1739.85 | 98.299 | 1.627 | 1764.93 | 1732.13 | 0.023 | 0.107 |
| 16 | 1836.29 | 104.074 | 0.615 | 1857.51 | 1828.58 | -0.562 | 0.043 |
| 17 | 2881.75 | 103.999 | 2.131 | 2920.32 | 2850.88 | -1.42 | 0.375 |
| 18 | 3277.17 | 98.099 | 2.636 | 3352.39 | 2995.55 | -0.611 | 2.102 |
| 19 | 3676.45 | 106.867 | 1.71 | 3689.95 | 3662.94 | -0.848 | 0.117 |
| 20 | 3740.1 | 104.788 | 0.728 | 3772.89 | 3736.24 | -1.165 | 0.019 |

**Figure: 9: Infrared spectra of (Wheat Bran +Starch)**

The FTIR of the wheat bran induced with starch was analyzed in order to determine the chemical composition involve in pigments produced by *Penicillium sp.s* and the bonding present in between them it was observed that C-Br stretch, C-Cl stretch, N-H wag, C-O stretch, C-N stretch, N-O symmetric stretch, C-H stretch, C-H bend, N-O asymmetric, C=O, O-H stretch C-C stretch, C=H stretch bonds were present which indicates that wheat bran induced with starch and inoculated with *Penicillium* isolates consists of alkyl halide, primary amines, alcoholic and carboxylic acids, esters ,ethers, aliphatic amines, aromatic amines, nitro compounds, alkanes, alkenes, phenols as a corresponding functional groups.(as shown in fig9).

**DISCUSSION:**

*Penicillium sp.* cultured on wheat Bran induced with starch showed the presence of bonds such as alkanes (C-H stretch), alkynes(C=H stretch), and primary amines (C-C stretch), N-H wag. A similar bond was observed on wheat Brans as a control which indicates that these bonds are not a constituents of pigments but constituents of wheat bran.

Other bonds like C-Br stretch, C-Cl stretch, C-O stretch, C-N stretch, N-O symmetric stretch, N-O asymmetric, C=O, O-H stretch were not observed in the FTIR results of wheat Bran (control)so these components may be components of pigments.

# CHAPTER-8

# SUMMARY AND CONCLUSION

Pigments are those compounds which are significant to various kinds of industries. In Food industry they act as additives, colour intensifiers, antioxidants etc.Presently, the existing natural food colorants are of either animal or plant origin and they include various limitations such as instability against light, heat, adverse ph or low water solubility, and among all the main disadvantage is only limited sources are available.

In the present study the effect of carbon sources on the pigment production by *Penicillium* isolate was investigated using three different agro industrial wastes as a sole source of carbon. The maximum growth was obtained on wheat bran on its $18^{th}$ day of incubation (31.4.0±0.43)CVU/ml in comparison with the yield of pigments produced during $6^{th}$ and $12^{th}$ day that are14.0±0.20CVU/ml and 26.9±0.11.

The second highest yield was obtained by using broken wheat as a substrate that is of about 11.7±0.12CVU/ml, 15.9±0.89CVU/ml, 23.2±0.11CVU/ml on an incubation of 6 days, 12 days, 18days.respectively. Wheat straw was not found to be much supportive in pigment production. And hence the yield was not much higher 10.3±0.23CVU/ml, 13.7±0.43CVU/ml, 17.6±0.60CVU/ml at 6days, 12 days, 18days

As wheat Bran was much more supportive in pigment production. Hence the wheat bran was induced with purified carbon sources such as starch, lactose, dextrose. The maximum yield was obtained on starch as a inducers that is 35.5±0.20CVU/ml,63.5±1.81CVU/ml, 122±2.03 CVU/ml at $6^{th}$, $12^{th}$, $18^{th}$ days of incubation. Dextrose has moderately induced the pigment production process and gave the yield of about 25.5±1.78CVU/ml, 48.4±0.60 CVU/ml, 84.7±1.24CVU/ml.at $6^{th}$, $12^{th}$, 18th days respectively. Lactose was observed to be least inducing the pigments production about and hence gave less yield of pigments that is 18.6±0.63CVU/ml,31.0±1.53CVU/ml, 41.2±1.21CVU/ml. The pigments produced by *Penicillium sp.* was found to be stable upto 70ºC and at an ph5,6,7 and 8 The pigment was quite stable in methanol showed high stability in methanol.

The *Penicillium sp.* are the potent pigment producers. As the pigments are quite stable in the different environmental conditions therefore it can be used for a wide range of industrial applications. The yield of these pigments can be increased by using wheat bran as a substrate or by inducing the substrate with starch.

# Chapter-9

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