STUDY OF PIGMENT PRODUCTION FROM *Penicillium sp.* BY SOLID STATE FERMENTATION

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ABSTRACT

Many industries like the food, textiles, cosmetic and pharmaceutical industries extensively use various kinds of pigments including natural and synthetic pigments. However with the growing public concern regarding the detrimental health and environmental impacts of synthetic dyes, there is a growing preference for natural colorants over synthetic colorants among the consumers. The aim of this study was to evaluate the potential of *Penicillium sp* for the synthesis of pigments by solid state fermentation using agro industrial residues such as rice straw, wheat straw, okara, sugarcane bagasse, broken rice, broken wheat and sweet potato. This work also aimed at evaluating the stability of the produced pigments. as substrates for the culture of *Penicillium sp*. It was found that after an incubation for specific 15 days *Penicillium sp* exhibited highest pigment yield on broken rice (109.9 CVU/gds) while least yield was obtained from rice straw (4.26 CVU/gds) and wheat straw did not produce any pigments. Enhancement of pigment yield was achieved by incorporation of different organic and inorganic nitrogen sources among which yeast extract when added to broken rice supported maximum pigment yield of 157.2 CVU/gds. Pigment stability studies revealed that the pigment was stable within a temperature range of $40^{o}C$ – $70^{o}C$, it was stable at neutral pH (6-7) and degradation was observed when exposed to light and methanol (179 CVU/gds) was found to be the best solvent for the pigment extraction followed by acetone (150.2 CVU/gds).

Keywords: Pigments, *Penicillium sp*, solid state fermentation (SSF), yeast extract, and methanol.

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Satarupa Gogoi

CERTIFICATE

This is to certify that **Satarupa Gogoi** (11302195) has completed Dissertation project report (BTY 731), entitled "**Study of pigment production from *Penicillium sp* by solid state fermentation**" under my guidance and supervision. To the best of my knowledge, the present work is the result of her original investigation and study. No part of the report has ever been submitted for any other degree at any University.

The report is fit for submission and the partial fulfillment of the conditions for the award of M. Tech. in Biotechnology.

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DECLARATION

I hereby declare that this thesis entitled "Study of pigment production from *Penicillium sp* by solid state fermentation" 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 Er. Robinka Khajuria, 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.

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CHAPTER 1

INTRODUCTION

Microbes when tamed are the most promising benefactors with their applications varying from therapeutics to industrial processes. Bacteria have been used directly or indirectly in many household practices such as curd formation, baking etc. Filamentous fungi are known to produce primary and secondary metabolites like pigments, peptides, enzymes, heterologous proteins, antibiotics, and pigments (Lopes *et al*., 2013).

Another potential application of fungus is their use in pigment production. Fungal species are rich ecological source of pigments and stable colorants. Pigments are used extensively in food and textile factories. In food factories they are used as supplements, colour enhancers, anti-oxidants etc. Many of these compounds have been reported. Fungus produces pigments in variety of colours where most of these pigments are soluble in water (Duran *et al*., 2002). Natural colorants are better than artificial colorants regarding both health and environmental aspects (Duran *et al*., 2006). A growing interest in natural dyes throughout the world has been observed as some synthetic dyes are known to have toxic, carcinogenic and polluting nature (Singh, 2000). So, microbial pigments are considered as better alternative in relation to other additives extracted from animals and plant parts like roots, stems leaves, flowers, fruits etc and dried bodies of certain insects and shellfish etc as they undergo degradation on exposure to heat, light or varying pH conditions (Singh, 2000).

Microorganisms are mostly preferred for pigment production because they grows rapidly thus ensuring availability throughout the year. Also microbial pigments are very stable with water soluble characteristics giving rise to colours of different shades. Another advantage of using microbes for production of pigments is that inexpensive substrates like agro-industrial residues can be used as carbon sources. Huge amounts of agro-industrial wastes are mainly originated from different commercial sources which increases the pursuit for research on the maximum usage of these waste products as inexpensive sources to support the growth of microorganisms (Rajesekaran *et al*., 2008).

A number of fungus has been reported to produced pigments are *Aspergillus niger, Aspergillus terreus, Phymatotrichum sp, P. vasconiae, P. italicum, Fusarium sp, Alternaria sp, Monascus rubum, Monascus purpureus.* Some of the *Penicillium sp*

mentioned in the report by Tiwari *et al* (2011), are *P.citrinum, P.frequentans, P.funiculosum, P.notatum, P.variabile, P.purpurogenum, P. multicolour, P.lilacinum and P.rubrum P. oxalicum, P. regulosum, P.chrysogenum,* etc. Among these fungi, *Penicillium sp* has been reported to produce red and yellowish red coloured pigments. These *Penicillium* strains are used for the testing of pigment production naturally. The colour of the pigments that have been mentioned in many experimental studies are red, yellow, brown, orange, magenta, blue, yellow-red, pink etc (Lopes *et al.*,2013).

Naturally fungus grows on wet moist ground or soil, spoiled vegetables and fruits under natural aeration (Babitha *et al*; 2006). *Penicilium sp,* commonly found in temperate and subtropical regions and on salted food products, but it is mostly found in indoor environment like damp or water-damaged area and most commonly found naturally in moist soils or on fruit causing decay with huge amount of carbon and nitrogen supplement for miccohrizal growth. Therefore, solid state fermentation is best suited for the development of fungi, where the mycelium gets enough space to extend on the surface of solid substrates with sufficient aeration and moreover this process includes very less moisture content which is favourable for the growth of mold. After the substrates are inoculated with the strains, they are needed to be maintained as proper cultures and the screening of the strains are to be done for various carbon and nitrogen content for optimizing the pigment yield. Hence, solid state fermentation has proved to be a better alternative for liquid, culture-based fermentation process. It is required to isolate and screen the efficient pigment producers as a potential role in food, cosmetics, textile and pharmaceutical factories (Engstrom *et al*., 19822; Suhr *et al*., 2002; Dufossé, 2006; Méndez-Zavala *et al.*, 2007; Hernández-Rivera *et al.*, 2008). The carbon sources used in this process provides the microorganisms the required nutrients that support their cell growth and development (Velmurugan *et al*; 2011).

The objective of this study was to evaluate the pigment producing potential of *penicillium sp* by solid state fermentation using agro-industrial wastes. The study also aimed at evaluating the stability of pigments produced.

CHAPTER 2

TERMINOLOGY

Fungal Pigments: Pigments are substances which absorb lights of different frequency and transmit them. Fungal pigments are secondary metabolites produced by different classes of fungi in their log phase.

Solid state fermentation: It is a process used to grow microbes on a solid media. It can be termed as a bio-molecule manufacturing process and the bio-molecules produced are generally microbial metabolites.

Submerged fermentation: submerged fermentation is also a bio-molecule manufacturing process in which the microbes are grown in a liquid media instead of a solid one.

Penicillium sp: *Penicillium sp* is one of the most common causes of fungal spoilage in fruits and vegetables.

Solvent extraction: It is a process of separating a substance from one or more others having different phases using a solvent.

Agro-industrial residues: They are the organic materials that produced as by-products in agro-industries.

CHAPTER 3

REVIEW OF LITERATURE

3.1 NATURAL PIGMENTS FROM MICROBES OVER SYNTHETIC PIGMENTS

In later part of 19th century synthetic pigments or dyes replaced the natural colorants or pigments from plant and animal extracts (Singh, 2000). But gradually with time it was found that synthetic colorants are environmental toxins and have hazardous impact on ecosystem. It has been reported that most of the produced dyes goes into the water bodies during dyeing process (Hur and Balachandar, 2011).

Nowadays, interest in natural dyes is seen throughout the world as some western countries due to their toxic, carcinogenic and polluting nature (Singh, 2000). So there is an augmentation of processes to extract pigments from natural sources due to harmful effects of artificial synthetic dyes, which have been commonly used in food, cosmetics, textiles and drug production (Kim *et al.* 1995). In the food factories they are used as supplements, colour enhancers, anti-oxidants, etc. Pigments can be found in different colours where most of them are soluble in water. So, many of these secondary metabolites are exploited and further produced, purified, isolated,studied and characterized (Duran *et al.*, 2002) as quoted by Mendez *et al*., (2011).

Recent growing hazardous threats from artificial food colourants by the new generations have encouraged the production of natural colouring options (Dufossé, 2006). Many factories have started to use natural colours extracted from plant and animal sources. There were several drawbacks in pigment produced from either ways, plants or animal extracts, that they undergo degradation on exposure to light, high temperatures or varying pH (Sameer *et al*, 2006).

Microbial pigments are often stable and soluble than those from plant or animal sources and hence mostly preferred and are of commercial interest (Gunasekaran and Poorniammal, 2008). Fungus grows fast without depending on weather conditions leading to high productivity and it is very stable with water soluble characteristics giving rise to pigments of different colours, and grows on cheap substrates like agro-industrial residues (Jiang *et al.*, 2005). Huge amounts of agro-industrial residues are mainly produced from different commercial sources which encourages the research on usage of such residues as cheap sources to support the growth of microorganisms.

Microorganisms have been traditionally used to produce several substances with industrial importance. Filamentous fungi have found to be useful, because of their ability to produce primary and secondary metabolites such as peptides, enzymes, organic acids, heterologous proteins, antibiotics, and pigments (Lopes, 2013). Most of the microbes are capable of producing pigments in huge amount, including species of *Monascus, Paecilomyces, Serratia, Streptomyces* and yellowish-red and blue pigments produced by *Penicillium sp*. Among all microbes, fungi have been reported to produce pigments that are chemically stable (Hajjaj *et al*, 2000). The evaluation and production of microbial pigments as natural textile colorants has been reported (Hamlyn, 1998).

Fungi are more available source of pigments as some fungal species produces a plenty of pigments or colorants which are stable in nature like anthraquinone (Gill and Steglich, 1987, Raisanen, 2001). Various studies reported that fungus of genus *Monascus* produce red colourants, which are used as colouring agents in food and textiles. Hence filamentous fungi are used as an alternative source for natural pigment production rather than plants, animals and other synthetic sources mainly because of the toxic effects caused by artificial dyes used in food, textile, pharmaceutical and cosmetic factories.

The fungi *penicillium chrysoenum* IFL1 and IFL2, *Fusarium graminearum* IFL3, *Monascus purpureus* NRRL 1992 were grown on agro industrial residues or PDA and *Penicillium vasconiae* IFL4 on PDB have been selected as pigment producers to finally produce water-soluble pigments. It was repoted that the production of yellow pigments was predominant and two strains of *Penicillium sp*. proved to be the most potential producer. These above mentioned pigments or filtrates after isolation and purification have the potential to be used in various food, textile and pharmaceutical industries (Lopes *et.al*, 2013). *Penicillium* strain produces red coloured chromophore of anthraquinone type, which can be used in food and cosmetic industries. Some strains of *Penicillium sp* are very effective biological controlling agent, for example few of them produces an enzyme that induces clotting of milk (Sardaryan *et al*., 2004).

Thus natural dyes or pigments from microbial sources are preferred as an important alternative to potentially harmful synthetics (Balachandar *et al*, 2011). Mostly fungi have been reported as the suitable candidate for natural pigment production above all other sources.

3.2 BIOLOGICAL PIGMENTS

Pigments refer to any kind of colorant or dye may be synthetic or natural. Biological pigments are those pigments which are derived from living organisms or natural source like plants, insects, animals and micro organisms like fungi yeast bacteria etc. Recently, microbial pigments are in demand and microbes mainly bacteria; yeast and fungus are responsible for pigment production. Some of naturally producing pigments are carotenoids, chlorophyll, b-carotene, xanthophyll, phycobilins, riboflavin etc (Kushwaha *et al*, 2014).

3.2.1 SOURCES OF PIGMENT PRODUCTION

Pigments are naturally produced from plants, animals, insects and even microbes mainly bacteria, yeast and fungi. Nature is rich in colours or pigments derived from fruits, vegetables, roots, plants, algae etc and microorganisms such as fungi, yeast, bacteria that are quite in vogue and hence known as 'biocolours' as reported by Kushwaha *et al*, (2014). Some of the pigments that are produced by biological sources are carotenoids, melanins, flavins, quinones and more specially monascins, violacein or indigo (Dufosse *et al.*, 2005).

Pigments like chlorophyll and carotenoids can be extracted from plant leaves (Pavia *et al.*, 1999). Any part of a plant like flowers, leaves etc that depicts colour is capable of producing pigments. Parts like roots, fruits, such as beet, grapes, paprika etc gives pigment in various colours. Some of the pigments are chlorophyll, carotenoids, b-carotene, xanthophylls, phycobilins, etc produced by plants, algae or cyanobacteria (Joshi *et.al* 2003).

Mainly microbes are used to produce pigments nowadays because of their availability and non complicated productivity, to overcome the harmful effects of the synthetic dyes. Microbes like bacteria, yeast and fungus are capable of producing pigments using different cheap agro-industrial wastes. It was reported that *Monascus* pigment, astaxanthin from *Xanthophyllomyces dendrorhous*, arpink red from *P. oxalicum*, riboflavin from *Ashbya gossypii*, b-carotene from *Blakeslea trispora* are used as fermentative food grade pigments (Duffose *et al.*, 2006). *Monascus sp, Penicillium sp, Paecylomices sp, Aspergillius sp* produces pigments (Blanc *et al*., 1994; Mendez-Zvala *et al*., 2007; Mapari *et al*., 2006, yeast *Paffia rhodozyma* and microalgae

Porphyridium cruentum and *Haematoccocus pluvialis* as stated by Boussiba, 2000; Yoshimura *et al*., 2006) as quoted by Espinoza-Hernandez et al., (2013)

As reported by Karpagam *et al*., (2013), bacteria like *Pseudomonas aeruginosa* produces soluble pigments like pyocyanin, fluoesceins and exo-polysaccharides. Meinicke *et al*, 2012 reported that fungus has the power of producing secondary metabolites as the biopigments with connected polyketide nature. For example *Monascus sp* produces six primary pigments, the colour of which is yellow (anka flavin, monascine), orange (rubropunctatin, monascorubrine) and red (rubropuntantamine, monascorubramine). Similarly, *Fusarium sp*, *Aspergillus sp*, *Penicillium sp* are also a potent pigment producer (Rashmi *et al.*, 2013, Akinyele *et al.*, 2013, Premalatha *et al.*, 2012)**.** Different microbial sources of pigments are enlisted in table1 (a) and 1(b).

Table 1(a): Pigments produced from microbes used as natural colorants as adapted from Dufosse, 2006)

Molecule	Colour	Microorganism	Status*
Ankaflavin	yellow	*Monascus* sp. (fungus)	IP
Anthraquinone	red	*Penicillium oxalicum* (fungus)	IP
Astaxanthin	pink-red	*Xanthophyllomyces dendrorhous* (yeast), formerly *Phaffia rhodozyma*	DS
Astaxanthin	pink-red	*Agrobacterium aurantiacum* (bacteria)	RP
Astaxanthin	pink-red	*Paracoccus carotinifaciens* (bacteria)	RP
Canthaxanthin	dark red	*Bradyrhizobium* sp. (bacteria)	RP
Lycopene	red	*Blakeslea trispora* (fungus)	DS
Lycopene	red	*Fusarium sporotrichioides* (fungus)	RP
Melanin	black	*Saccharomyces neoformans* var. *nigricans* (yeast)	RP
Monascorubramin	red	*Monascus* sp. (fungus)	IP
Naphtoquinone	deep blood-red	*Cordyceps unilateralis* (fungus)	RP
Riboflavin	yellow	*Ashbya gossypi* (fungus)	IP
Rubrolone	red	*Streptomyces echinoruber* (bacteria)	DS
Rubropunctatin	orange	*Monascus* sp. (fungus)	IP
Torularhodin	orange-red	*Rhodotorula* sp. (yeast)	DS
Zeaxanthin	yellow	*Flavobacterium* sp. (bacteria)	DS
Zeaxanthin	yellow	*Paracoccus zeaxanthinifaciens* (bacteria)	RP
β-carotene	yellow-orange	*Blakeslea trispora* (fungus)	IP
β-carotene	yellow-orange	*Fusarium sporotrichioides* (fungus)	RP
β-carotene	yellow-orange	*Mucor circinelloides* (fungus)	DS
β-carotene	yellow-orange	*Neurospora crassa* (fungus)	RP
β-carotene	yellow-orange	*Phycomyces blakesleeanus* (fungus)	RP
Unknown	red	*Penicillium purpurogenum* (fungus)	DS
Unknown	red	*Paecilomyces sinclairii* (fungus)	RP

Table 1 (b): Microbial sources for natural pigment as adapted from Malik *et al*., 2012.

Microorganism(s)	Pigments/Molecule	Colour/appearance
Bacteria		
Agrobacterium aurantiacum	Astaxanthin	Pink-red
Paracoccus carotinifaciens	Astaxanthin	Pink-red
Bradyrhizobium sp.	Canthaxanthin	Dark- red
Flavobacterium sp., *Paracoccus zeaxanthinifaciens*	Zeaxanthin	Yellow
Achromobacter		Creamy
Bacillus		Brown
Brevibacterium sp.		Orange yellow
Corynebacterium michigannise		Greyish to creamish
Corynebacterium insidiosum	Indigoidine	Blue
Rugamonas rubra , Streptoverticillium rubrireticul, Vibrio gaogenes, Alteromonas rubra	Prodigiosin	Red
Rhodococcus maris		Bluish- red
Xanthophyllomyces dendrorhous	Astaxanthin	Pink –red
Haloferax alexandrines	Canthaxanthin	Dark Red
Staphylococcus aureus	Staphyloxanthin Zeaxanthin	Golden Yellow
Chromobacterium violaceum	Violacein	Purple
Serratia marcescens, Serratia rubidaea,	Prodigiosin	Red
Pseudomonas aeruginosa	Pyocyanin	Blue-green
Xanthomonas oryzae	Xanthomonadin	Yellow
Janthinobacterium lividum	Violacein	Purple
Algae		
Dunaliella salina	β-carotene	Red
Chlorococcum	Lutein	
Hematococcus	Canthaxanthin	
Fungi		
Aspergillus sp.		Orange-red
Aspergillus galucus		Dark –red
Blakeslea trispora	β –carotene	Cream
Helminthosporium catenarium		Red
Helminthosporium avenae		Bronze
Penicilllum cyclopium		Orange
Penicilllum nalgeovensis		Yellow
Fusarium sporotrichioides	Lycopene	Red
Haematococcus Pluvialis	Astaxanthin	Red
Monascus sp.	Monascorubramin	Red Orange
Monascus purpureus	Monascin Ankaflavin	Red-yellow
Monascus roseus	Canthaxanthin	Orange-Pink
Monascus sp.	Ankaflavin	Yellow
Penicillium oxalicum	Anthraquinone	Red
Blakeslea trispora	Lycopene	Red
Cordyceps unilateralis	Naphtoquinone	Deep blood-red
Ashbya gossypi	Riboflavin	Yellow
Mucor circinelloides, Neurospora crassa and Phycomyces	β-carotene	Yellow-orange
Penicillium purpurogenum , Paecilomyces sinclairii		Red
Pacilomyces farinosus	Anthraquinone	Red
Yeast		
Cryptococus sp.		Red
Saccharomyces neoformans var. *Nigricans*		Melanin black
Phaffia rhodozyma	Astaxanthin	Pink-red
Rhodotorula sp. *Rhodotorula glutinis*	Torularhodin	Orange-red

3.2.2 *PENICILLIUM* SPECIES AS A POTENT SOURCE FOR PIGMENT PRODUCTION:

Penicillium sp is a class of filamentous fungi under the microbial category. Species of *penicillium* are mostly soil fungi that grow on cool and moderate climates wherever organic material is available such as organic biodegradable substances. *Penicillium,* commonly found in temperate and subtropical regions and on salted food products, but it is mostly found in indoor environment like damp or water-damaged area and most commonly found naturally in moist soils or on decayed fruit having available carbon and nitrogen supplement for miccohrizal growth. During in-vitro cultivation, *Penicillium* develops into a colony that starts to grow as a white fluffy mass that later turns green then black and finally yellow colour appears after several days of incubation that spreads all over the solid support provided or the medium. These *penicillium* strains are used for the testing of pigment production naturally. The colour of the pigments that have been mentioned in many experimental studies are regrd, yellow, brown, orange, magenta, blue, yellow-red etc. It was reported that the production of yellow pigments was predominant (Lopes *et al.,* 2013).

The production of *Monascus*-like pigments or compounds from *Penicillium* strains have a potential use in the food industries because they are not associated with any citrinin or mycotoxin production as reported by Sameer *et al.,* (2009). They are homologues of pigments produced by *Monascus sp* which have similar chromophore polyketides (Mapari *et al.,* 2008). It has been reported that the isolation and characterization studies of three *Penicillium* strains revealed the capability of producing red pigments (Espinoza-Hernandez *et al.,* 2004, 2011 and Duran *et al*., 2002). As reported by Mendez *et al*., (2011), *P. Purpurogenum* GH2 is capable of producing a potential pigment that is water soluble which gains popularity among the food industries where it's used as a natural colorant and further studies revealed that this particular strain when undergoes optimization processes then the combined effect of pH and temperature leads to high production of red pigment.

As reported by Dhale et al., (2009) a potential *Penicillium sp* that produces red pigment was isolated from marine sediment and further studies revealed that it naturally possesses radical scavenging property which draws the attention of the pharmaceutical, food and neutriceutical industries. Hence *Penicillium sp* of various strains like *P. chrysogenum, Penicillium italicium, Penicillium oxalicum, Penicillium regulosum, Penicillium nalgiovense, Penicillium notatum, Penicillium herqueri* and

Penicillium atrovenetum etc are being used for various pigment production and it has been reported as one of the most reliable and potent pigment producing microbe and a high yielding source of pigment by Tiwari *et a.l,* (2011) and Lopes *et a.l*, (2013).

3.3 SOLID STATE FERMENTATION

Fermentation was traditionally performed in order to produce a variety of compounds that are advantageous to individuals and commercial manufacturers. Fermentation processes have gained popularity as they provide economic and environmental advantages. Old processes have been further revised and polished to increase the productivity. Fermentation can be defined as the process of biological conversion of complex compounds into simple components by various microorganisms such as bacteria and fungi where metabolic breakdown occurs to release several additional components apart from the regular by-products produced during fermentation, such as carbon dioxide and alcohol and the additional compounds are called secondary metabolites such as antibiotic, peptides, enzymes, pigments, growth factors etc as reported by Balakrishnan and Pandey (1996); Machado *et al*., (2004); Robinson *et al*., (2001).

In a report published by Babitha *et al*., (2007) Solid state fermentation (SSF) has proved to be an advantageous process as compared to submerged liquid culture based fermentation process. The residues or carbon sources used in SSF provides the essential supplements like nutrients and supports the growth and development of the cells. SSF allows a more adequate habitat for fungi which results in increased pigment production by a relatively cost effective way of manufacturing where agro-industrial products are used as growth supporting carbon source. Agro-industrial residues like rice bran, wheat bran, coconut oil cake, sesame oil cake, palm kernel cake, groundnut oil cake, cassava powder, spent brewing grain, and jackfruit seed powder have been put to various analytical processes in order to select the most suitable carbon source for specific microbes for pigment production as seen in reports published by Sivaramakrishnan, (2007), Velmurugan,(2011).

Solid state fermentation has proved to be the most cost effective and environment friendly process as compared to submerged liquid fermentation for manufacturing various industrial by-products like enzymes, bio-fuels, pigments etc. Solid State Fermentation (SSF) is a process of growing and cultivating micro-organisms under

optimized conditions in the absence of moisture to produce the desired products of interest as reported by Mienda *et al.,* (2011).

Filamentous fungi are capable of producing many primary and secondary metabolites such as peptides, enzymes, organic acids, heterologous proteins, antibiotics and pigments (Radzio R and Kuck U, 1997). Many natural pigment colorants are produced in various colours like red, brown, blue, yellow etc. These pigments are more potential than any pigments from other sources other than fungi. Mainly many research reported that among fungi, *Penicillium* is one of the highest yielding type, to give different range of pigments which are very stable and reliable (Lopes *et al,* 2013).

Pigment production from *Penicillium* species by solid state fermentation was included in many works for its better yield. In a recent work by Espinoza-Hernandez *et al*., (2013) a study was made on three fungal strains isolated from *Quercus sp* and *Larrea tridentaa* and later on the strains were found to be *Penicillium purpurogenum*(GH2) and *Penicillium pinophilum* (EH2 and EH3). Here solid state fermentation and submerged fermentation was done for the pigment production. It was found that *penicillium sp* that were isolated from the Mexican semi-desert were a potential pigment producer.

Taskin *et al*., (2010), stated that solid state fermentation when enhanced by inert support or controlling few factors like moisture level, nutrients etc can increase the growth of *Penicillium G* using sugar- beet pulp as a substrate. As compared to submerged fermentation it gave better results with high yield of pigment.

Thus the secondary metabolites obtained from such fungal strains are beneficial to mankind. Most microbes mainly fungi are the booming objects for future research as the recent development in biotechnology has showed a path for utilizing and taming microbes for mankind as a benefactor.

3.4 PIGMENT PRODUCTION USING AGRO-INDUSTRIAL WASTES AS SUBSTRATES

As comparable to pigment production by *penicillium sp*, in another report published by Babitha *et al*., (2006), jackfruit seed was used as a carbon source to produce pigments from *Monascus purpureus* by Solid-state fermentation using jackfruit seed in powder form and the jackfruit seed is known to have a buffering nature, due to which colour intensity of pigments produced was stable under specific range of pH,

temperature, light etc whereas these seed powder with a particle size between 0.4 and 0.6 mm without any added carbon source was reported as the best for pigment production. It was also found that when the jackfruit seed powder was infused with soybean meal, peptone, chitin powder or monosodium glutamate the particular species of *Monascus* gave water soluble pigments. In a similar manner many works have been done to establish a suitable substrate for efficient pigment production from *Monascus* species. In the case of *Penicillium sp* while using submerged fermentation culture media like potato dextrose agar (PDA) was used to yield pigments.

It was reported by Carvalho *et al*., (2007) that solid state fermentation of *Monascus sp* was done on rice and it proved to be the best carbon source for pigment production from microbes. Several agro-crops such as barley, wheat, rice, cassava, and agro-industrial residues such as wheat bran, rice bran, sugarcane bagasse, cassava bagasse, various oil cakes like coconut oil cake, palm kernel cake, soybean cake, fruit pulps like apple, pomace, corn cobs, saw dust, seeds like tamarind and jack fruit, coffee husk and coffee pulp, tea waste, and spent brewing grains are the carbon sources that are commonly used as substrate for solid state fermentation as reported by Shazwani et al., (2012). There are many other natural substrates which act as very good sources of carbon and nitrogen for the growth and development of fungus.

Taskin *et al.*, (2010) in the review reported that sugar-beet pulp, an agro- industrial waste from the sugar factories, has been selected as a substrate for solid state fermentation of *Penicillium G.* In another work done by Velmurugan *et al.*, (2011) pigment from *Monascus sp* was produced by using corn cob as substrate and it was found that the usage of corn cob waste as a carbon source for pigment production gave better results than many other forms of substrates. This was evaluated using *M. purpureus* by soaking the corn cob powder at 80°C for 48 hours resulted in higher production of pigments and the fermentation resulted in a high concentration of red pigment using corn cob compared to other agro industrial residues like coconut-oil cake, groundnut oil-cake, sesame oil cake, tamarind powder, cassava flour, wheat bran, spent brewing grain, palm kernel cake, and jackfruit seed-powder. Finally the report stated that the spectrophotometric reading showed high absorbance at 490nm.

3.5 FACTORS AFFECTING THE PIGMENT PRODUCTION:

There are many chemical and physical factors such as pH, temperature, gaseous environments, agitation, aeration, carbon sources, nitrogen sources and solvents etc

that affects the production of pigments by microorganisms and these includes moisture, pH, size of inoculums, and nutrient supplements (Lee *et al*., 2002). As reported by Suhr *et .al* (2002) that the most affecting factor for the yellow colour formation is pH from the statistical analysis in the studies related to factors affecting growth and pigment production of *Penicillium caseifulvum*. Mendez *et al*., (2011) reported that the pigment produced by the *Penicillium sp*, when treated with combined effect of pH and temperature, showed a decline in intensity of colour at 24^{o} C with alkaline pH and increase in colour with acidic pH. Similarly, at 34^{o} C with alkaline ph there is a increase in colour intensity and with acidic ph there is a decrease in colour.

3.6 NITROGEN SUPPLEMENTATION:

Different kinds of nitrogen sources available are monosodium glutamate, sodium nitrite, ammonium nitrite, ammonium sulphate, yeast extract, beef extract etc. According to (Shepherd and Carels, 1983), nitrogen sources tremendously affect the pigment production. As reported by Silvana *et al*, (2006) amount of peptone, monosodium glutamate, grape waste used as the substrate or carbon source plays an important role in pigment production by *Monascus sp*. The supplementation of glutamate added with peptone showed a high increase in pigment production. In work done by Babitha *et al*., (2006) it was found that monosodium glutamate proved to be an excellent nitrogen source for red pigment production, followed by peptone, soybean meal and chitin powder and further stated that alone carbon source i.e. jackfruit seed powder with no added nitrogen source was not able to produce any pigment that is soluble in water as quoted by Shazwani, (2012).

Lovastatin production by *Monascus pilosus* was increased highly with the addition of nitrogen sources like peptone, sodium nitrite, glycerol etc to the carbon sources (Tsuyoshi *et al* 2005). As reported by Anaashari M *et.al* (2012) addition of yeast extract and sucrose increased production of *Penicillium G* by *Penicillium chrysogenum* at 28-26 ^{o}C.

3.7 PIGMENT PRODUCTION AND EXTRACTION:

In the report published by Henrique *et.al*, (2007), extraction of chlorophyll pigments was done from marine micro algae using methanol as a solvent and methanol was reported to be the most preferable solvent for extracting chlorophyll from this strain,

using Lichententhaler correlation, after 24 hours extraction. The extraction was enhanced by cell disruption, time variation, application of correlations and furthermore the lysis ability of the solvent itself favours the extraction. The extraction of polar pigments, such as carotenoids, hydrophilic solvents like methanol play a significant role in extraction, which is not so interferred by the application of any kind of external influence such as disruption etc.

Extraction of the pigments from microbial sources by solid state fermentation was done as cited in the research work of Babitha *et al*., (2005) where the dried product after fermentation was mixed in 90% ethanol and kept on a rotary shaker for a particular period of time. This was then rested for nearly 15 minutes which finally gave pure pigments. Then these pigments were ready for further characterization and quantification.

Extraction of pigments chlorophyll a, chlorophyll b and carotenoid pigments from four algal species was made by using solvents like acetone, ethanol and ethyl acetate and finally the spectrophotometric observation of pigments determined that ethyl acetate showed higher significance than ethanol and acetone as reported in the works of P. Kumar *et al.,* (2010). Karpagam *et al.,* (2013) reported the extraction of pigments from the *Pseudomonas sp* by chloroform which gave a very satisfactory result. Here pigments like pyocyanins, flouresceins, exo-polysaccharide were extracted from bacterial strain using chloroform. In a report published by Espinoza-Hernandez *et al*., 2013 the pigments produced by penicillium sp was extracted by distilled water giving satisfactory results.

Therefore in the above mentioned papers it is evident that solvents like ethanol, methanol, ethyl acetate, acetone, chloroform etc can be used for extraction of various pigments from microbial, plant etc sources. Most commonly used solvents are methanol and ethanol for extraction of pigments.

3.8 PROSPECT OF NATURAL PIGMENTS

As its been proved in many general studies that natural pigments rule over artificial synthetic pigments. With growing intensity of pollution in the environment at an alarming rate, because of deposition of harmful chemicals into the water bodies and soil it's better to go the natural way, especially in context of pigment production. In the works done by Velmurugan *et al*., (2005), they have stated that pigment production from fungi has better impact than synthetic pigments. Also it is found that

the dye stuff factories were suffering from the rising cost price of the feed stock and power supply for dye synthesis and hence they are subjected to a lot of pressure to decrease the harmful effects on the environment (Ajay Kumar *et al*., 2012). The growing hazardous threats from artificial food colorants by the new generations encouraged the production and usage of natural dyes and colour options (Dufossé, (2006). Therefore, the growing awareness among mankind regarding the health and environment has lowered the interest on the use of artificial colorants. Thus there is a rise in demand for natural colorants in food and textile manufacture. Hence the safest option that is left out is the use of various classes of fungi to produce pigments that will be natural. The greatest advantage of natural pigment production is that they can be produced all around the year with equal efficiency, and they won't pose any climatic or production constraints. Thus natural pigments should be accepted for the betterment of mankind and the environment.

CHAPTER 4

SCOPE OF STUDY

The artificial dyes used in various food and textiles industries have hazardous effect on the human and the environment. The presence of artificial colorants in the living world poses a threat to mankind as it recycles into the water bodies, atmosphere, soil and give rise to various environmental pollutions. Thus in order to maintain a balance in the nature, natural ways of meeting the human needs, calls for an immediate notice. Production of natural colorants is an interesting area of research. The main aim of the study is to analyse the amount of pigments produced by solid state fermentation from *Penicillium sp* using various cheap agro-crop or wastes and study the stability of pigments regarding various factors. The substrates used here are natural products like sweet potato, broken rice, broken wheat, rice straw etc for culturing the *Penicillium sp*. The production was cost effective in order to maintain the economic balance. Thus the study paved a path towards the remedy of overcoming the usage of artificial colorants.

CHAPTER 5

OBJECTIVE OF STUDY

This particular *Penicillium sp* was isolated from soil (agricultural field of LPU) and the report comprises of evaluation of pigments produced from this strain using agro-industrial waste by solid state fermentation. These are the following objectives of the study:

1. To evaluate the potential of *Penicillium sp* for producing pigments using agro-industrial wastes as carbon sources.
2. To study the effect of nitrogen supplementation on pigment production.
3. To study the effect of temperature, pH, visible light and solvent on pigment stability.

CHAPTER 6

EQUIPMENTS, MATERIALS AND EXPERIMENTAL SET UP

6.1 EQUIPMENTS

6.1.1 INSTRUMENTS AND EQUIPMENTS

Table 2: Instruments and Equipments

S.No.	Materials	Company
1.	Autoclave	NSW Pvt. Ltd.,India
2.	Face Mask	Smart Care
3.	Glass-wares	Borosil Glass
4.	Hot-air oven	NSW Pvt. Ltd., India
5.	Incubator	Yorco Incubator Bacteriological
6.	Laminar-air flow	Rescholar Equipment
7.	Microwave	INALSA
8.	Microscope	Magnus
9.	Micro-pipette	P'Fact A
10.	Micro tips	TARSONS
11.	Orbital shaker	REMI
12.	Centrifuge	REMI
13.	Plastic-wares	Poly lab
14.	Refrigerator	LG
15.	Weighing-balance	Adventurer, DHAVS
16.	Spectrophotometer	ELICO double beam

6.2 MATERIALS:

6.2.1 Glass wares:

Conical flasks, Petri plates, test tubes, glass beaker, streaking rod, glass rod.

6.2.2 Chemicals:

Potato dextrose agar (PDA), tween 80, acetone, diethyl ether, chloroform, methanol, ethanol, antibiotic (streptomycin sulphate), tween 80, ammonium sulphate, ammonium nitrate, sodium nitrate, peptone, yeast extract, MSG (monosodium glutamate) .

The above chemicals were procured from “Loba chemi” and “Himedia”

6.2.3 Miscellaneous:

Cotton, muslin cloth, inoculation loop, measuring cylinder, pipette, distilled water, butter paper, brown paper, thread, microtips, burner, and testube stand.

6.3 EXPERIMENTAL SET UP:

All the experimental works were carried out in the project lab of Lovely Professional University, Punjab (28- 301) under controlled aseptic conditions.

CHAPTER 7

RESEARCH METHODOLOGY

7.1 *PENICILLIUM SP* CULTURE

The *Penicillium sp* strain was previously isolated from soil (the soil was collected from the agricultural field around LPU, Phagwara, Punjab) and then the culture was maintained on potato dextrose agar (PDA) at 28-30 ^{o}C and preserved at 4^{o}C for further use and sub-cultured every thrice a week (Babitha *et.al*, 2006).

7.2 MAINTANANCE OF CULTURE AND INOCULUM PREPARATION

Penicillium sp grown on PDA was allowed to fully sporulate in an incubator for 6 to 8 days in the agar slant culture at 28-30 ^{o}C. 8-10 ml of sterile distilled water mixed with tween-80 solution (100ml sterile distilled water + 0.5 ml tween-80) was added and with the help of an inoculation loop the spores were scrapped off under aseptic conditions. The spore suspensions obtained were used as the inoculums.

7.3 PREPARATION OF SUBSTRATES (CARBON SOURCES)

Substrates like broken rice, broken wheat, rice straw, wheat straw, bagasse, okara (soya bean extract) etc were obtained from local market and substrate like sweet potato was washed, shredded and dried in tray dryer and powdered using mortar-pestle.

7.4 SOLID STATE FERMENTATION

10g of each dried substrates as carbon sources was taken and 10 ml of distilled water was added to the flask and moisture content was set at 56-60% according to the following equation (Dikshit and Tallapragada, 2012):

Moisture content (%) = 100 × (wet weight – dry weight) / wet weight

The contents in the flask was blended well and then autoclaved at 121°C for 20 min and then cooled to room temperature. It was then inoculated with the prepared 1ml of *Penicillium sp* spore suspension and incubated at 30°C for 5, 10, 15 and 20 days respectively.

7.5 PIGMENT EXTRACTION:

After incubation for desired days, the substrate was kept for drying on aluminium foil for 24 hours at 50 degree Celsius in a tray dryer and ground to fine powder in a mortar and pestle. Pigment extraction was done by using 10ml of 80% methanol per 0.1gram of dry fermented substrate. Then the mixture was placed on rotary shaker at 200rpm for 1 hour and allowed to stand for 15 minutes and centrifuged at 5000rpm for 30 minutes and the supernatant was taken for pigment analysis by spectrophotometer. Methanol extract of non-fermented substrate was taken as a blank for the analysis so that the impurities in the substrates were subtracted from the pigment produced by the *penicillium sp*.

7.6 ESTIMATION OF PIGMENTS

The analysis of extracted pigments was done using SL 210 UV-VIS spectrophotometer (ELICO double beam), measuring absorbance (OD) at specific wavelengths 410, 500 nm and taking into consideration the dilution factor of the samples. Finally pigment yield was measured in OD/gdfs expressed as A_{max} at corresponding wavelengths and colour value was calculated to study the effect of these sole carbon sources and to select the best one for pigment production, taking into consideration the dilution factor of the sample OD at its maxima, per gram dry fermented matter (Dikshit and Tallapragada, 2012).

Color value = O.D × dilution ×volume of extracts / Amount of sample

7.7 NITROGEN SUPPLEMENTATION

Various organic and inorganic nitrogen sources were used to enhance the growth of pigments like MSG (Na-L-Glutamate monohydrate), yeast extract, peptone, ammonium nitrate, ammonium sulphate, sodium nitrate. Vidyalakshmi *et al*., (2009) (0.5%) 0.05gm of nitrogen sources were added to 10gm of carbon source which gave the high pigment yield separately. Further these sources were inoculated with the sample and incubated for 15 days and then powdered after autoclaving and drying at 50^{o}C. Then extraction was done by 80% methanol and finally spectrophotometric reading was taken for the analysis.

7.8 STABILITY STUDIES OF PIGMENTS

There are various factors affecting the stability of pigments such as temperature, pH, light, various solvents etc. Here the effect of temperature, pH, light and different solvents on the pigments was checked.

7.8.1 Effect of temperature:

10ml of methanolic extract of pigment was taken in each test tube separately and incubated in a hot water-bath at temperatures varying from 40 to 90 ^{o}C for 15 minutes in separate test tubes (10ml each) followed by OD measurement at 410 and 500 wavelengths (nm) (Kaur *et.al*, 2008).

7.8.2 Effect of pH:

5ml of extracted pigments was taken in e separately and then pH was adjusted from 4 to 9 using 0.1N NaOH or dil HCL kept at room temperature followed by OD measurement at 410 and 500 nm (Kaur *et.al*, 2008).

7.8.3 Effect of visible light:

A set of 5 test tubes having 5ml of methanolic extracted pigment in each were kept in open light for 1 to 5 days (1^{st}, 2^{nd}, 3^{rd}, 4^{th}, and 5^{th}). At an interval of 24 hours the spectrophotometric reading was taken in OD (410, 500 nm). Here, the samples kept in dark were considered as control.

The color value was calculated using the formula:

Color value = (OD × dilution ×volume of extract) / amount of sample

7.8.4 Effect of different solvents:

This experiment was done to check the stability of pigment in various solvents. Different solvents considered were methanol, ethanol, distilled water, acetone, diethyl ether and chloroform. 0.1gm of powdered pigment was dissolved in 10ml of each

solvent and kept undisturbed for 7 days. Then after 7 days, OD measurement was taken at 410 and 500 nm. Similar work can be seen in the report published by Wongsorn *et al*., 2011.

CHAPTER 8

RESULT AND DISCUSSION

8.1 MORPHOLOGICAL STUDIES:

The *Penicillium sp* strain isolated from soil (taken from the agricultural field around LPU, Phagwara, Punjab) was inoculated in Potato dextrose medium and incubated at 28 ± 2 ^{O}C till confluent growth was observed. The colonies were slow growing , intially forming white cottony fluffy growth which eventually turned green, mostly consisting of a dense felt of conidiophores as shown in Fig1 (a). It was observed that the strain used was a slow growing strain showing growth after incubation of 5 days. Microscopic analysis of the fungus revealed phialides produced in groups giving a brush-like appearance**.** As seen in Fig1 (b) the strain has septate mycelium with globose and ellipsoidal conidia. Similar morphological characteristics for *Penicillium sp* were reported by Pitt *et al* (1979), Chen *et. al* (2002) and Demain,2006.

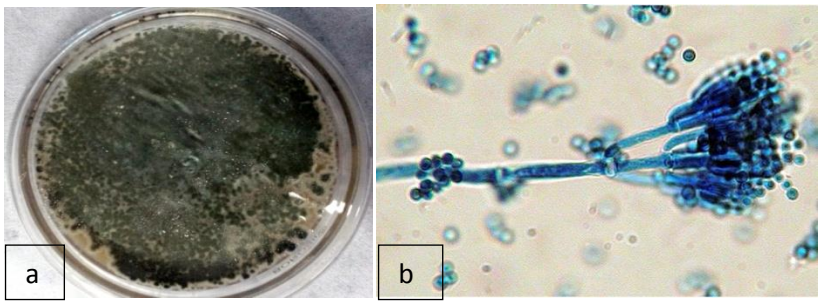

Fig1:a) Petri plate showing colony morphology of *Penicillium* isolate and, b) fungal hyphae bearing brush shaped conidiophores and conidia.

8.2 MAINTENANCE OF CULTURE:

The *Penicillium sp* culture was isolated from soil (agricultural field) which was later sub-cultured and grown on potato dextrose agar and made into slants. A required amount of antibiotic (0.01g in 50ml DW) was also introduced to the slants before inoculation and the slants were incubated at 28-30^{o}C till confluent growth was observed.

Fig 2: *Penicillium sp* on slants.

8.3 SCREENING OF CARBON SOURCE FOR PIGMENT PRODUCTION

The substrates used as carbon sources included broken rice, broken wheat, okara, sweet potato, wheat straw, rice straw, and sugarcane bagasse. Production of yellowish red pigment reached its maximum on 15th day of incubation and the high absorbance (colour value) at 410 nm indicated more production of yellow, yellowish red or orange pigment as compared to wine red colour. Among all substrates broken rice (109.9 CVU/gds) gave the highest yield of pigment followed by sweet potato (81.1 CVU/gds) and broken wheat (45.02 CVU/gds) at 410 nm when incubated for 15 days at 28-30^{o}C (Table 3, 4 and Fig 3, 4). Okara, sugarcane bagasse, rice straw comparatively very meagre amount of pigment and wheat straw gave no pigment at all. As reported by Rashmi and Padmavati, (2013) *Monascus* strain was grown on various solid substrates like local polished rice (*Oryza sp*), finger millet flour (*Eleusine sp*), sweet potato, (*Manihot sp*) tapioca and screening for the best substrate was done. It was found that maximum pigment production occurred with local rice followed by sweet potato and tapioca whereas flour was found to be the weakest one.

Dhale and Raj (2009) reported that a red pigment producing *Penicillium sp* NIOM-02 was isolated from marine sediment and solid state fermentation was done on wheat whereas on sugarcane bagasse it showed less pigment production and more scavenging radicals. As reported by Evelyne *et.al* (2000), methanol treatment produced the best recoveries of micro algal pigments when compared to acetone.

Hence from the above observation it can be concluded that among all the selected substrates broken rice (local rice) has the highest colour value and yield. Therefore broken rice was taken forward for further studies in pigment production and stability.

Table3: Effect of carbon sources on pigment production (at 410 nm)

Substrates / Incubation	COLOR Value(CVU/gds)			
	5 Days	10 Days	15 Days	20 Days
Broken rice	-	43.3±0.4	109.9±0.3	83.7±0.6
Sweet potato	-	38.7±0.2	81.1±0.1	40.2±0.9
Broken wheat	-	26.4±0.3	45.2±0.3	31.3±0.5
Okara	-	-	30.3±0.1	12.6±0.7
Rice straw	-	-	4.26±0.4	0.97±0.3
Sugarcane bagasse	-	-	14.87±0.2	3.06±0.3
Wheat straw	-	-	-	-

Table4: Effect of carbon sources on pigment production (at 500 nm)

Substrates / Incubation	COLOR Value(CVU/gds)			
	5 Days	10 Days	15 Days	20 Days
Broken rice	-	39.9±0.3	96±0.3	64±0.1
Sweet potato	-	19.4±0.5	73.2±0.3	39.2±0.4
Broken wheat	-	12.7±0.4	35±0.3	43.4±0.2
Okara	-	-	8.5±0.2	0.232±0.6
Rice straw	-	-	0.725±0.2	-
Sugarcane bagasse	-	-	-	-
Wheat straw	-	-	-	-

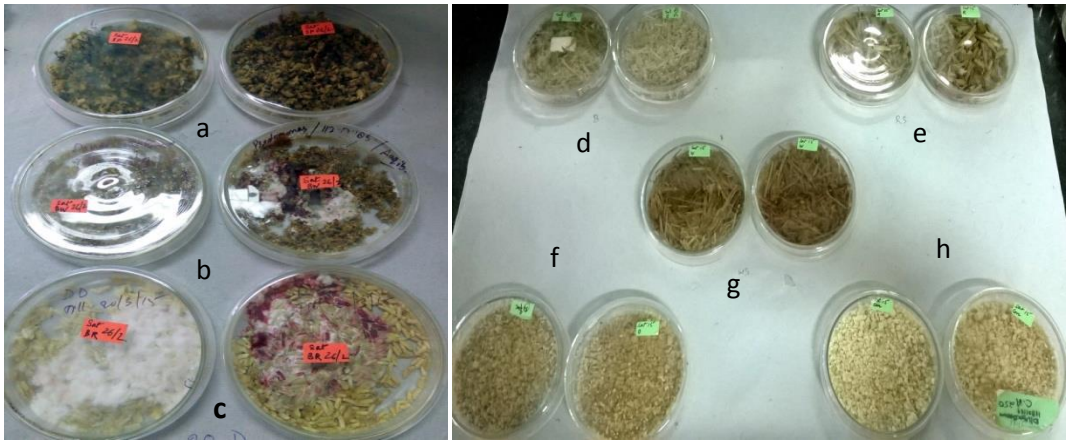

Fig3: Carbon sources for SSF a) sweet potato b) broken wheat c) broken rice d) bagasse e) rice straw f) okara g) wheat straw h) control.

Fig4: * 15 day old extract a) broken rice b) sweet potato c) broken wheat d) okara e) sugarcane bagasse f) rice straw g) wheat straw ; ** Extracts from broken rice h) control i) 10 days incubation j) 15 days incubation k) 20 days incubation.

8.4 EFFECT OF NITROGEN SUPPLEMENTATION

Nitrogen sources used were yeast extract, monosodium glutamate, peptone, ammonium sulphate, sodium nitrate, ammonium nitrate. Among all the nitrogen sources organic sources showed better results than the inorganic sources. Yeast extract was found to be more efficient as it enhanced the pigment production when added to the suitable carbon source (broken rice). Yeast when added to broken rice gave highest colour value of 157.2 CVU/gds followed by peptone (150.6 CVU/gds), sodium nitrate, MSG and ammonium sulphate. Therefore organic supplementation gave satisfactory results (Table 5 and Fig 5, 6). Nitrogen sources tremendously affect the pigment production (Shepherd and Carels, 1983). As reported by Anaashari M

et.al (2012) addition of yeast extract and sucrose increased production of *Penicillium G* by *Penicillium chrysogenum* at 28-26 ^{O}C. As reported by (Tsuyoshi *et al* 2005) lovastatin production by *Monascus pilosus* was increased highly with the addition of nitrogen sources like peptone, sodium nitrite, glycerol etc to the carbon sources. Under optimized conditions, the addition of salts NaCl and Na_2SO_4, at concentrations of 0.1 and 0.5M in specific buffer (Ph 8.0), the red pigments showed good stability as reported by Santos-Ebinuma *et al*, (2013).

Table5: Effect of nitrogen supplementation on pigment production

Wavelength (nm)	**Colour value CVU/gds**					
	Organic sources			**Inorganic sources**		
	Yeast extract	**MSG**	**Peptone**	**Ammonium nitrate**	**Ammonium sulphate**	**Sodium nitrate**
410	157.2±0.1	106.6±0.3	150.6±0.1	143±0.2	65.4±0.4	120.4±0.1
500	99.82±0.2	53.62±0.2	85.42±	66.6±0.2	28.62±0.5	85.42±0.1

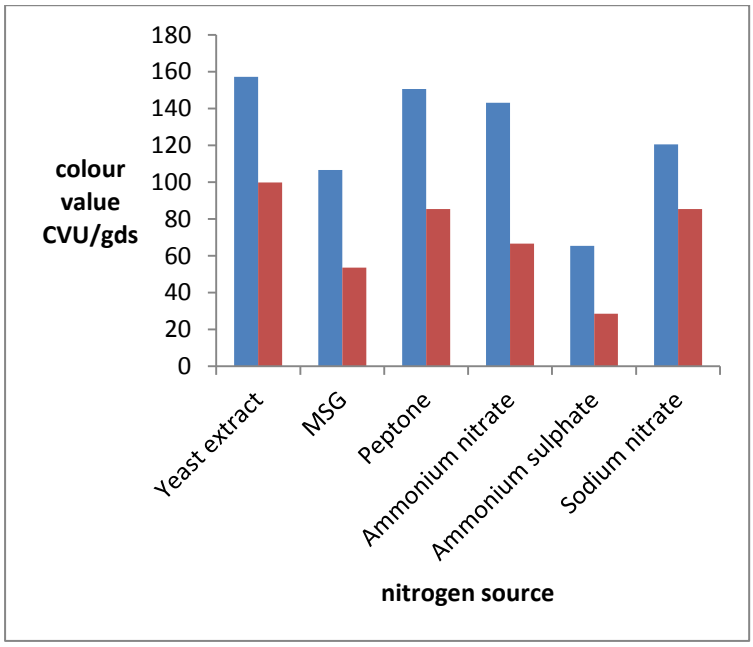

Fig5(a): Effect of supplementation of organic and inorganic nitrogen sources.

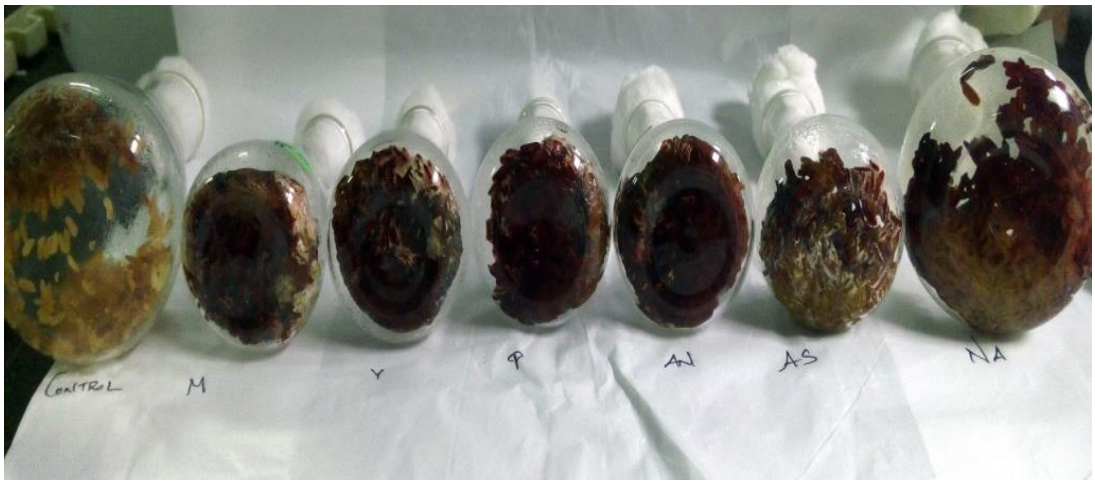

Fig5 b: Effect of nitrogen sources; M-monosodium glutamate, Y-yeast extract, P-peptone, AN-ammonium nitrate, AS-ammonium sulphate, NA-sodium nitrate.

Fig6: Pigment extract obtained from Broken rice (a) Control (b) 10 days incubation (c) 15 days incubation (d) 20 days incubation (e) Broken rice with yeast extract supplementation

8.5 PIGMENT STABILITY STUDIES

The stability of the extracted pigments was studied by considering various physical and chemical factors like temperature, visible light, solvents and pH and effect of these factors was observed on the pigments. As commented by Joshi *et.al*, (2003) the pigments produced by the Microbes are heat labile and colour intensity changes with varying acidity, exposure to light, air, water-activity etc and hence not stable and sometimes get degraded.

8.5.1 EFFECT OF TEMPERATURE

The effect of temperature was checked within the range of 40 to 90 ^{O}C. It was observed that pigments were stable when incubated within the range of 40-70^{O}C, after which a decrease in the colour intensity was observed. As reported by Kaur et.al (2008), heat stability of the pigments produced by *Monascus sp* was determined by incubation of the extracts at different temperatures viz, 70, 80, 90 and 100^{o}C and it was observed that pigments were thermo-labile above 70^{o}C and showed degradation at 100^{o}C. Another report of Kaur *et al.,* (2008), stated that *Rhodotorula rubra* showed more stability in higher temperature when cultured by solid state fermentation than submerged fermentation. In a report published by Santos-Ebinuma et al, (2013) it was seen that red pigments produced by *Penicillium purpurogenum* were stable at 70 ^{o}C and showed gradual increase in red colorants. As reported by Babitha *et al*, (2007) the production of red pigment was maximum at 30^{o}C and gradual decrease was seen in colour intensity at temperature higher than 40^{o}C whereas there was seen a rise in the yellow pigment production.

Table6: Effect of temperature on pigment

Wavelength (nm) / **temperature (in ^{O}C)**	**Colour value (CVU/gds)**					
	40	50	60	70	80	90
410nm	143	148.2	156.1	155	130	96
500nm	88	93.7	101	118.7	70	55.8

8.5.2 EFFECT OF SOLVENTS

Solubility of the extracted pigment was tested with various solvents like ethanol, methanol, distilled water, chloroform, acetone, and diethyl ether. This pigment that has been produced by *peniciilium sp* was found to be water soluble and not soluble in other organic solvents like diethyl ether. Among the solvents, the maximum stability was seen with distilled water followed by methanol, ethanol, and acetone and chloroform showing poor results whereas it was found that pigment was not soluble in Diethyl ether (As shown in Table 7 and Fig 7).

In a report published by Wongsorn *et.al*, (2011), the effect of solvent on yellow, orange and red pigments produced by *Monascus purpureus* was investigated by dissolving the pigment in six solvents namely hexane, diethyl ether, propanol, methanol, ethanol and distilled water and incubated at 30^{o}C in dark for 7 days. The spectrophotometric results showed that pigments were most stable in diethyl ether followed by distilled water and ethanol whereas it was seen that pigments were not stable in hexane, propanol and methanol.

Carvalho *et al.*, (2006), reported that pigments produced on rice from *Monascus sp* were extracted by methanol followed by DMSO and methanol gave the better result.

Table7: Effect of different solvents on pigment

Wavelength (nm) / **Solvents**	**Colour value CVU/gds**					
	Ethanol	Distilled water	Methanol	Acetone	Chloroform	Diethyl ether
410	98.5	29.4	179	150.02	11.81	-
500	65.4	17.35	152.1	137	-	-

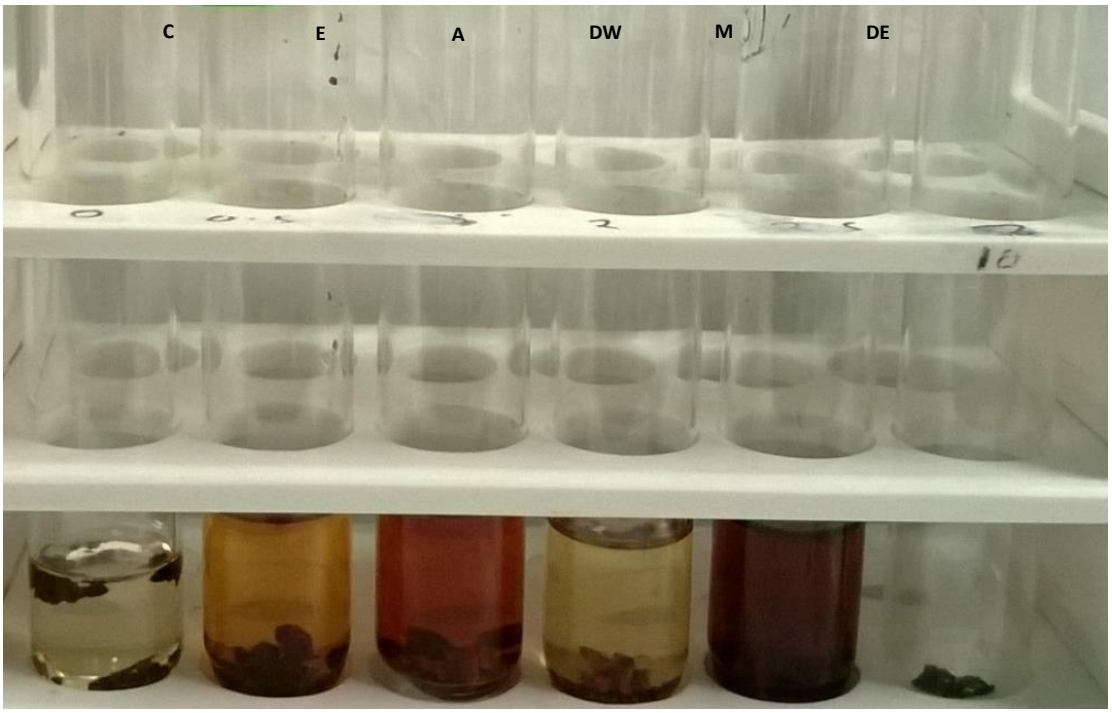

Fig 7: Effect of solvents on pigments, C-chloroform, E-ethanol, DW-distilled water, A-acetone, M-methanol, DE-diethyl ether.

8.5.3 EFFECT OF pH

The stability of the extracted pigments was checked for various pH ranges from 4 to 9. Stability was achieved on pigments when these were adjusted to neutral pH within 6 to 7 and showed decrease in colour intensity below pH 6 and above pH 7 (As shown in Table 8). As reported by Kaur *et.al* (2008), raw alcoholic extracts of pigments produced by *Monascus sp* were checked for stability by adjusting pH from 5 to 7 and the pigments showed stability pigments at neutral pH. The pigments produced from *Epicoccum nigrum* showed stability at alkaline pHs and sudden decrease of colour intensity when adjusted to acidic pHs (Bleoju *et.al* 2007).. In another report by Kaur *et.al*, (2008), the methanolic solution of extracted yellowish pink pigments produced from Rhodotorula rubra were found to be more stable at neutral pH than acidic or alkaline pH. Therefore, it can be concluded that different microbial pigments showed different ph stability. Hernandez-Rivera *et al.*, (2008), reported that the satisfactory results for the production of yellowish pigments were obtained at pH values from 3.0

to 3.5 while best results for the production of red pigments were obtained at pH levels between 7.0 and 7.5.

Table8: Effect of pH on pigment production.

Wavelength (nm) / pH	Colour value(CVU/gds)					
	4	**5**	**6**	**7**	**8**	**9**
410	74.4	84.02	152.1	144	90.9	83.01
500	62.1	66.5	111.3	96.04	73	56.23

8.5.4 EFFECT OF VISIBLE LIGHT:

The pigments showed light sensitivity when exposed to visible light. The effect of visible light was being observed for 5 days and it was found with the increase in exposure time, the colour intensity decreased. The colour intensity reduced when exposed for 120 hours. Therefore, indicating that the dye is unstable due to longer period of light exposure (As shown in Table 9). As reported by Velmurugan *et al*., 2009, pigments produced by *Monascus* sp degraded when exposed to visible light for a longer duration. Joshi et al., (2003) reported that pigments produced from microorganisms are not stable at varying temperatures, light effect, acidity, air, water-activity and sometimes prone to degradation.

Table9: Effect of visible light on pigment production.

Wavelength (nm) / Time (Hrs)	Colour value (CVU/gds)				
	24	**48**	**72**	**96**	**120**
410	155.04	145	132.24	114.2	94.15
500	164.3	150.04	163.2	130.8	110

CHAPTER 9

CONCLUSION AND FUTURE SCOPE

The synthetic dyes used in various field like food and textiles industries have hazardous effect on the human and the environment. The objective of the study was to evaluate the pigment producing potential of *penicillium sp* by solid state fermentation using agro-industrial wastes. The study also aimed at evaluating the stability of pigments produced. The study on pigment production and their optimization by *Penicillium sp* revealed that among all the agro industrial residues, broken rice gave highest yield of 109.9 CVU/gds after 15 days of incubation. Supplementation of nitrogen enhanced pigment production with yeast extract supporting it gave the maximum yield (157.2 CVU/gds). Pigment stability studies revealed that the pigment was stable in between 6-7 pH and temperature (40-70ºC). It was also found that pigment degraded gradually on increase in the exposure to visible light. Methanol was found to be the best solvent (179 CVU/gds). Natural pigments from *Penicillium* are of practical interest in food and textile industries as a wide range of edible pigments which are safe and show many beneficial properties.

REFERENCES

Atalla, Mabrouk M, khrisy E, Youssef E, Y. A. and Asem M (2011). Production of textile reddish brown dyes by fungi. Malaysian Journal of Microbiology 7(1): 33-40.

Babitha S, Soccol C R and Pandey A (2006). Jackfruit seed- a novel substrate for the production of *Monascus* pigment through solid state fermentation. Food Technology and Biotechnology 44(4) 465-471.

Balakrishnan K. and Pandey, A. (1996), Production of Biologically Active Secondary Metabolites in Solid State Fermentation, Journal of Scientific and Industrial Research, 55: 365-372.

Carvalho J C, Oishi B O, Pandey A and Soccol CR (2005). Biopigment from *Monascus*: strain selection, citrinin production and color stability ., Brazilian Archives of Biology and Technology, 48(6):885-894.

Carvalho J C, Oishi B O, Woiciechowski, Adenise A, Pandey, Ashok, Sumathy B and Carlos S R (2006). Effect of substrates on the production of *Monascus* biopigments by solid-state fermentation and pigment extraction using different solvents. Indian Journal of Biotechnology, **6:**194-199.

Dhale M A and Vijay R A S (2009). Pigment and amylase production in *Penicillium sp* NIOM-02 and its radical scavenging activity. International Journal of Food Science and Technology, 44(12):2424-2430

Dikshit R and Tallapragada P (2011). *Monascus purpureus*: A potential source for natural pigment production. Journal of Microbiology and Biotechnology. 1(4): 164-174

Dikshit R and Tallapragada P (2012). Comparative study of *Monascus sanguineus* and *Monascus purpureus* as potential source for red pigment production. International Journal of Pharma and Bio Sciences 3(4):(B) 885-895

Dikshit R and Tallapragada P (2013). Exploring *Monascus sanguineus* as a potential natural source for pigment production. International Research Journal of Biological Sciences 2(5): 59-67

Dufosse L (2006). Microbial Production of Food Grade Pigments .Food Technology and Biotechnology. 44 (3) 313–321

Dufosse L (2009). Pigments, Microbial. Encyclopedia of Microbiology. 4: 457-471

Durán N, Teixeira MFS, de Conti R, Esposito E (2002). Ecological-friendly pigments from fungi. Critical Reviews in Food Science and Nutrition.42(1):53–66.

Engstrom GW, Stenkamp RE, McDorman DJ, Jensen LH (1982). Spectral identification, X-ray structure determination, and iron chelating capability of erythroglaucin, a red pigment from *Aspergillu sruber*. Journal of Agriculture and Food Chemistry, 30(2):304–307.

Espinoza-Hernández T C, Rodríguez-Herrera R, Aguilar- González C N, Lara-Victoriano F, Reyes-Valdés M H and Castillo- Reyes F (2013). Characterization of three novel pigment-producing *Penicillium* strains isolated from the Mexican semi-desert, African Journal of Biotechnology, 12 (22): 3405-3413.

General T, Kim J H, Prasad B, Ngo H T A, Vadakedath N and Cho G M (2013). Fungal utilization of a known and safe microalgae for pigment production using solid state fermentation.

Gill M. and Steglich W (1987). Pigments of fungi (macromycetes). Progress in the Chemistry of Organic Natural Products. Springer-Verlag, Wien, New York publisher, 51:S1-317.

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

Hajjaj H, Blanc PJ, Groussac E, Goma G, Uribelarrea JL, Loubiere P (1996) Improvement of red pigment/citrinin production ratio as a function of environmental conditions by *Monascusruber* . Biotechnology and Bioengineering. 64(4):497–501.

Hernández-Rivera JS, Méndez-Zavala A, Pérez-Berúmen C, *et al*. (2008). Culture Conditions to Improve the Red Pigment Production by *Penicilliumpurpurogenum* GH2. In: Soto-Cruz O, Angel PM, Gallegos-Infante A, *et al*., editors. Advance in Food Science and Food Biotechnology in Developing Countries. Saltillo, Mexico: MexAsoc Food Science Editions, 108–112.

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

Joshi V K, Devender A, Anju B and Shashi B (2003). Microbial Pigments,.Indian Journal of Biotechnology 2:362-369.

Lee D W (2007). Nature's palette-the science of plant color. University of Chicago press.

Lopes C. F, Tichota D M, Pereira J Q, Segalin J, Rios A O &Brandelli A (2013) Pigment Production by Filamentous Fungi on Agro-Industrial Byproducts: an Eco-Friendly Alternative, Application Biochemical Biotechnology.

Machado C.M, Oishi B.O, Pandey A. and Soccol C.R (2004) Kinetics of Gibberellafujikori Growth and Gibberellic Acid Production by Solid State Fermentation in a Packed-Bed Column Bioreactor. Biotechnology Progress, 20: 1449-1453.

Mapari SAS, Meyer AS, Thrane U (2008) Evaluation of *Epicoccumnigrum* for growth, morphology and production of natural colorants in liquid media and on solid rice medium. Biotechnology Letters. 30(12):2183–2190.

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 Ingeneiria Química.6:267–273

Mendez A, Perez C, Cesar J, Martinez G and Noe C (2011). Red pigment production by *Penicillium purpurogenum* GH2 is influenced by pH and temperature. J Zhejiang University-Science B (Biomedical & Biotechnology), 12(12):961-968.

Nelson D, Maria F. S, Roseli D, and Elisa E, (2002). Ecological friendly pigments from fungi. Criteria and Review of Food Science Nutrition 42(1): 53-66.

Osman M E, Khattab O H, Zaghlol G M and El-Hameed R M (2011). Screening for the Production of Cholesterol Lowering Drugs (Lovastatin) by Some Fungi. Australian Journal of Basic and Applied Sciences 5(6): 698-703

Pandey A, Soccol C R, Rodriguez J A and Nigam P (2001). Solid state fermentation in biotechnology, fundamentals and application. New Delhi: Asiatech Publisher p.221

Robinson T, Singh D and Nigam P, (2001). Solid-statefermentation: a promising microbial technology for secondary metabolite production. Applied Microbiology and Biotechnology, 55: 284-289.

Sajo B, Idi A and Umar A (2011). Microbiological Features of Solid State Fermentation and its Applications - An overview. Research in Biotechnology, 2(6): 21-26

Sameer A, Mapari A and Meyer A (2008). Evaluation of *Epicoccum nigrum* for growth, morphology and production of natural colorants in liquid media and on a solid rice medium. Biotechnology Letters, 30:2183–2190

Sardaryan , E.*et al*., Arpink Red, (2004). Meet new natural red food colour out of microbial origin, in pigment in food, More Than Colour, Dufosse L., Ed., Universite de Bretagne occidantale Quimper,207

Shazwani N (2012). The optimization of red pigment by *monascus purpureus* FTC 5356 in solid state fermentation A thesis, faculty of chemical and natural resources engineering. University of Malaysia Pahang.

Subramaniyam R. and Vimala R, (2012). solid state and submerged fermentation for the production of bioactive substances: a comparative study. International journal of science and nature, 3(3): 480-486.

Suhr KI, Haasum I, Streenstrup LD, Larsen TO. (2002).Factors affecting growth and pigmentation of *Penicillium caseifulvum* . Journal of Dairy Science, 85(11):2786–2794.

Subramaniyam R and Vimala R (2012). Solid state and submerged fermentation for the production of bioactive substances: a comparative study. Iternational Journal Of Security And Networks, 3(3): 480-486

Sutton D A, Fothergill W A and Rinaldi G M (1998). Guide to clinically significant fungi. William & Wilkins, Baltimore 1st edition.

Taskin *et al.*, (2010), penicillium solid state fermentation with sugerbeet pulp bioresourses 5(1) – 268- 275.

Velmurugan P, Hur H, Balachandar V, Kannan S K and Lee K J (2011). *Monascus* pigment production by solid-state fermentation with corn cob substrate. Journal of Biosciences and Bioengineerig. 112(6): 590–594

Velmurugan P, Kamala S, Balachandar V , Lakshmanaperumalsamy P , Chan J and Taek B (2009). Natural pigment extraction from five filamentous fungi for industrial applications and dyeing of leather. Carbohydrate Polymers (79)2: 262–268

Velmurugan P, Kim J M, Park S J, Karthikeyan K, Lakshmanaperumalsamy P, Lee J K, Park J H and Oh T B (2010). Dyeing of cotton yarn with five water soluble fungal pigments obtained from five fungi. Fibers and polymers 11(4): 598-605

Vidyalakshmi R, Paranthaman R, Murugesh S and Singaravadivel K (2009). Microbial bioconversion of rice broken to food grade pigments. Global Journal of Biotechnology & Biochemistry 4 (2): 84-87

Wongsorn H, Wongjewboot I and Kongruang S (2011). Solvent stability of ultrasonic mutant of *Monascus purpureus* pigments. International Journal of Bioscience. Biochemistry and Bioinformatics 1(3):206-210

Zeelandiae N, Ping L,Yufeng L ,Zhifang R ,Gang W(2012). Overproduction of a potential red pigment by a specific self-immobilization biomembrane-surface liquid culture of *Penicillium*. Bioprocess and Biosystem Engineering 35:1407–1416

APPENDIX

LIST OF ABBREVATIONS

PDA: Potato dextrose agar

SSF: Solid state fermentation

SF: Submerged fermentation

MSG: Monosodium glutamate

OD: Optical density

UV: Ultra violet

CVU/gds: Colour value unit/ gram dry substrate