



LOVELY
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**EVALUATION OF PIGMENT PRODUCTION FROM
Aspergillus terreus MTCC 7600 BY SOLID STATE
FERMENTATION**

**SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENT
FOR THE AWARD OF THE DEGREE OF**

**MASTER OF TECHNOLOGY
IN
BIOTECHNOLOGY**

SUBMITTED BY:

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UNDER THE SUPERVISION OF

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Annexure I



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2. Isolation of pigment producing bacteria
3. Extraction & quantification of pigment from fungi

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*Guide should finally encircle one topic out of three proposed topics and put up for approval before Project Approval Committee (PAC)

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ABSTRACT

Pigments including natural and synthetic pigments are used extensively in many industries like the food, textiles, cosmetic and pharmaceutical industries. 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 *Aspergillus terreus* MTCC 7600 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. It was found after an incubation of 15 days *Aspergillus terreus* MTCC 7600 exhibited highest pigment yield on broken rice (110 CVU/gds) while least yield was obtained by sugarcane bagasse (11.21 CVU/gds) and okara (9.24 CVU/gds). Enhancement of pigment yield was achieved by incorporation of different organic and inorganic nitrogen sources among which monosodium glutamate (MSG) supported maximum pigment yield of 152.7 CVU/gds while least pigment production was given by ammonium sulphate (18.3 CVU/gds). Pigment stability studies revealed that the pigment was stable between 40°C- 60°C, it was stable at acidic pH, the pigments were stable in the dark and it was found that chloroform was the best solvent for pigment extraction (399.2 CVU/gds) followed by methanol (398.32 CVU/gds).

Keywords: *Aspergillus terreus* MTCC 7600, Chloroform, Monosodium glutamate (MSG), Pigments, Solid state fermentation (SSF)

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Pursenla I Pongen

CERTIFICATE

This is to certify that **Pursenla.I.Pongen** (11307069) has completed Dissertation project report (BTY 731), entitled “**Evaluation of pigment production from *Aspergillus terreus* 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 “Evaluation of pigment production from *Aspergillus terreus* 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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M.Tech (Biotechnology)

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Pigments including natural and synthetic pigments are being used extensively in many industries like the food, textiles, cosmetic and pharmaceutical industries. Due to health and environmental aspects there is a thriving desire among the customers to opt for colorants obtained natural over synthetic colorants (Frenanda *et al.*, 2013). Natural pigments were obtained from the insect tissues and the flowering plants. However, such extraction process have several shortcomings like dependency on the quantity of organic matter, batch-to-batch variation of coloring profile, light, pH and heat affectability (Wang *et al.*, 2012). It is due to these shortcomings of conventional pigment sources that microbial production of pigments is gaining a lot of attention. Microbial pigments have several advantages like their self-reliance against climate settings, different colors, growth on cheap agro-industrial residues and rapid growth of microorganisms leading to higher productivity (Fernanda *et al.*, 2013).

Microbes have been reported to reproduce a huge quantity of stable pigments like anthraquinones, carotenoids, flavonoids, quinines and rubramines (Nagia *et al.*, 2007). Fungi also contain secondary metabolites like anthraquinones (Deepshikha *et al.*, 2012). Hence, fungus can be exploited for the commercial production of pigments. However, a major hindrance in doing so is the fact that of some fungi such as *Monascus* species are known to produce mycotoxins in combination with industrially applicable colorants (Sumathy *et al.*, 2006). Thus, it is important to search for fungal strains capable of producing non-toxic pigments. *Aspergillus terreus* is one such fungus that is known to synthesize pigments without producing mycotoxins. Also known as *Aspergillus terrestris*, it originates in the land worldwide. It is brown in tint and becomes darkened as it matures on cultivation media (Samson *et al.*, 2004). It also produces a drug mevinolin (lovastatin) used for reducing serum cholesterol (Alberts *et al.*, 1980). It is a thermo tolerant species as it have ideal proliferation in thermal reading within 35-40°C and maximal maturation between 45-48°C (Rokem, 1999). Pigments produced by *Aspergillus terreus* on potato dextrose agar are beige to buff to

cinnamon, reverse is yellow (Hoog *et al.*, 2000). Colonies of *Aspergillus terreus* on czapek solution agar produced light yellow to dark orange yellow with light yellow on the reverse, whereas on malt extract agar it produced orange brown with yellow brown on the reverse (Hina *et al.*, 2013).

There are different strategies employed for cultivation of fungus viz. solid state fermentation (SSF) and submerged fermentation (SF). However, solid state fermentation is a favored approach since it is perfect for fermentation procedure comprising of fungi and microorganisms that demand limited water content (Atta *et al.*, 2013). Solid state fermentation exploits solid substrates like bagasse, rag paper, broken rice and wheat etc. which are nutrient-rich waste materials that can be easily recycled as substrates. In solid state fermentation, the substrates are very gradually employed, in order to utilize the same substrate for longer duration of fermentation (Subramaniyam *et al.*, 2012). Convenience such as greater fermentation yield, improved stability of output, reduced catabolic suppression and power requirement over the submerged cultivation has led to abounding application of solid state fermentation (Ashok *et al.*, 2012). The cheap and readily available substrates can play as a backup for generation of pigment by fungus (Wei *et al.*, 2013). Another major advantage of solid state fermentation is that the products can be used directly as food colorant or as a material for pigment extraction (Liu *et al.*, 2010) whereas pigments produced through submerged culture must be extracted before used as a colorant (Gong *et al.*, 2002).

Many agro- industrial residues such as like cassava, coffee husk ,wheat bran, ground nut oil cake, tea waste , sugarcane bagasse, , various oil cakes, palm kernel cake, , fruit pulps like, bagasse, corn cobs, saw dust, rice ,apple pomace, seeds like tamarind and jack fruit, and coffee pulp and spent brewing grains are the most often and frequently utilized substrates for solid state fermentation. Agro wastes are readily available and therefore it makes the solid state fermentation cheap and wide acceptance in industries.

The aim of this study was to evaluate the potential of *Aspergillus terreus* for the synthesis of pigments by solid state fermentation using agro industrial residues. This work also aimed at evaluating the stability of the produced pigments.

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 by which microbes are grown on a solid media. It can be termed as a biomolecule manufacturing process and the biomolecules produced are generally microbial metabolites.

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

Apergillus terreus: It is a fungus (mold) which is found abundantly in the soil, also known as *Aspergillus terrestris*.

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

Agro industrial residue: Agro industrial residues are the organic materials that are produced as byproducts in agro industries.

INTRODUCTION

According to Dufosse , (2006) the synthetic dyes utilized in food industry has many dispute therefore intense attentions have been shown by the modern consumers for natural coloring alternatives. Nature is wealthy in colors and microorganisms that produce pigment like fungi, yeast and bacteria. Accomplishment and acceptability of pigment yielded by fermentation lean on regulatory agreement, consent and the overall cost of the production process.

Bashir *et al.*, (2011) states that manufacture of valuable industry based products like pigments, enzymes, biofuels etc. solid state fermentation is regarded as the most economic and nature loving as compared to other fermentation process. According to Wang *et al.*, (2011) pigment production cost as well as raw materials can be recovered by large-scale fermentation of microbes.

3.1 BIOLOGICAL PIGMENTS

Biological pigments also known as pigments or bio chromes, comprises of plant and flower pigments, they are generated by living organisms and show fussy color absorption. Example, the wings of butterfly contains anatomical color, nevertheless bountiful butterflies have cells that contains pigment as well (Stavenga *et al.*, 2014). Specific pigments absorb light at different wavelengths at the same time reflecting others (Lee, 2007).

3.2 PIGMENT PRODUCTION BY PLANTS AND ANIMALS

Photosynthesis is the primary function of pigments in plants, other functions include attraction of insects to flowers to encourage pollination. Pigments of plant include a variety of different kinds of molecules like carotenoids, anthocyanins, betalains and porphyrins (Esteban *et al.*, 2004).

Table 1: Types of pigments produced from plants (Lee, 2007 & Esteban *et al.*, 2014)

Plant Pigments	Properties
Flavonoids	-A major group of compound in plant, comprising numerous, mostly yellow dyes. In Latin flavus means yellow
Anthocyanins	-It may be considered to be a subset of flavonoids, and its structure is very similar to flavonoids. They are water soluble and are blue, violet, purple or red.
Betalains	-They are like anthocyanins and are water soluble, they are red or yellow pigments. The deep red color of beetroot is due to betalains.
Carotenoids	-It is fat-soluble and mostly yellow, orange and red. It functions as an antioxidant and promotes healthy eyesight in humans.

The ability to synthesize dyes in animal is less developed as compared to plants. In animals the tissues are protected from sunburn by ultraviolet radiations by a skin pigment known as melanin.

Table 2: Types of pigment produced from animals (Pete *et al.*, 2007 & Margarita)

Animal pigments	Properties
White	-Feathers and mammal hair comprises of keratin which is colorless transparent.

Melanins	-It can be synthesized by all animals. The furs of mammals, feathers of birds, exoskeletons of many insects and black areas on wings of butterfly are colored by melanin. Our body synthesizes melanin; tan of skin and hair color is due to it.
Pterins	-It was first discovered in the pigments of butterfly wings. It gives white and yellow pigment.
Papiliochromes	-It is a group of pale yellow pigments found in Papilionidae (swallowtail butterflies).
Flavonoids	-It is a plant pigment which can't be synthesized by Lepidoptera (an order of insect), but consumed in the diet of the caterpillar. In almost all families of butterflies, white and yellow flavonoids have been found
Carotenoids	-It is responsible for the yellow to red color of many bird feathers, ladybugs, Colorado beetles and many other insects. However it has not been detected in butterfly wings.
Green	-Green coloration of many insects is due to chlorophyll taken from plant food. Combination of a blue pigment, biliverdin, yellow pigment and carotenoids results in the green color.

3.3 MICROBIAL PIGMENTS

Microbial pigments are a promising alternative to other color additives extracted from animals or vegetables, as they are considered as natural, pose no seasonal production problems and have high productivity. Microorganisms produce a variety of pigments like carotenoids, melanins, flavins, indigo, monascins and violacein (Dufosse, 2009). They have been used in the food industry to pigment salmon, trout and poultry flesh or to identify the color of egg yolk (Johnson *et al.*, 1995).

Table 3: List of pigment producing microorganisms (Malik *et al.*, 2012)

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

Table 4: Production of pigments from microbes (Dufosse, 2006)

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

* IP- Industrial production, DS- Development stage, RP- Research project

3.3.1 Pigment production by bacteria

Pigments are produced from bacteria for various reasons which play a significant part. Few examples of pigment-producing bacterial strains include *Serratia marcescens* that produces prodigiosin, *Streptomyces coelicolor*, *Thiobacillus versutus* and *Chromobacterium violaceum*. These bacteria are obtained from soil, water bodies, plant, insects, animals and man (Ahmad *et al.*, 2012). An example of a water soluble, non-fluorescent blue-green pigment produced by *Ps. Aeruginosa* is pyocyanin, it crystallises as beautiful blue needles and have a role in respiration. A number of *Pseudomonas* species produce a yellow water soluble fluorescent pigment, under conditions of iron limitation, known as pyoverdinin, pyoverdinin or simply (Moss, 2002)

3.3.2 Pigment production by fungi

Many species of higher fungi (*Basidiomycetes* and *Ascomycetes*) produce brightly colored fruiting bodies in a range of pink, red, orange, yellow, purple, grey and olive red. Under natural conditions, when supplies of vital nutrients become depleted or once the growth of a fungal colony is well established, parts of the mycelium may switch biochemical activity to secondary metabolism pathway (Susan, 1994). Fungi have a physiological mechanism for repairing some forms of radiation damage to vital biochemical compounds and these systems may supplement the protection afforded by pigments. Some spores are released from the parent fungus in large quantities of mucilage (extracellular matrix). This mucilaginous matrix also provides protection against harmful ultraviolet radiations.

According to Fernanda *et al.*, (2013) synthetic dyes are toxic therefore filamentous fungi grown on agro-industrial byproduct are utilized to yield natural pigments. It was also reported by Dikshit and Tallapragada, (2013) that *Monascus sanguineus* strain isolated from pomegranate produced red pigment, and at a pH range of 6.5 maximum red pigment yield was observed.

Palanivel *et al.*, (2009) studied on natural pigments extracted from five filamentous water soluble fungi (*Monascus purpureus*, *Isaria spp.*, *Emericella spp.*, *Fusarium spp.*, and *Penicillium spp.*) for dyeing of pre-tanned leather samples. The results obtained revealed that the pigment concentration was 6% on weight of leather, and the best pH and temperature

for dye stuff was 5 and 70°C for a time duration of 120 min. The shift in shades of the samples was immense in *M. purpureus* yielding red pigment. Water soluble pigments were also isolated from five filamentous fungus viz. *Fusarium verticilloides*, *Penicillium purpurogenum*, *Monascus purpureus*, *Emericella nidulans* and *Isaria farinose*, and the isolated pigments were utilized for cotton yarn dyeing (Palanivel *et al.*, 2010).

Vidyalakshmi *et al.*, (2009) conducted a study to determine the yield of pigment by *Monascus. ruber* under solid state fermentation using rice. The *Monascus. sp.* is cultivated on steamed non-glutinous rice and the fermented product is used as food colorant. Among the various nitrogen sources incorporated, mono sodium glutamate (MSG) gave a higher pigment yield of 0.464(OD) and 1.314(OD) U/g. Julio C *et al.*, (2006) studied the potential of natural substrates for bio pigment production using a strain of *Monascus sp.* and to compare different solvents for the extraction of the pigments. He found that rice was the best substrate for cultivation under solid state fermentation. The comparison of several solvents used for extraction showed that methanol was the best solvent, followed by DMSO and ethanol.

Six major pigments produced by *Monascus* has been categorized into three groups based on their color and are both polyketetides and azaphilones. They are yellow pigment of monascin and ankaflavin, orange pigments of rubropunctain and monascoubirin and red pigments of rubropunctaminea and monascorubramine (Wang *et al.*, 1979; Zhou *et al.*, 2008).

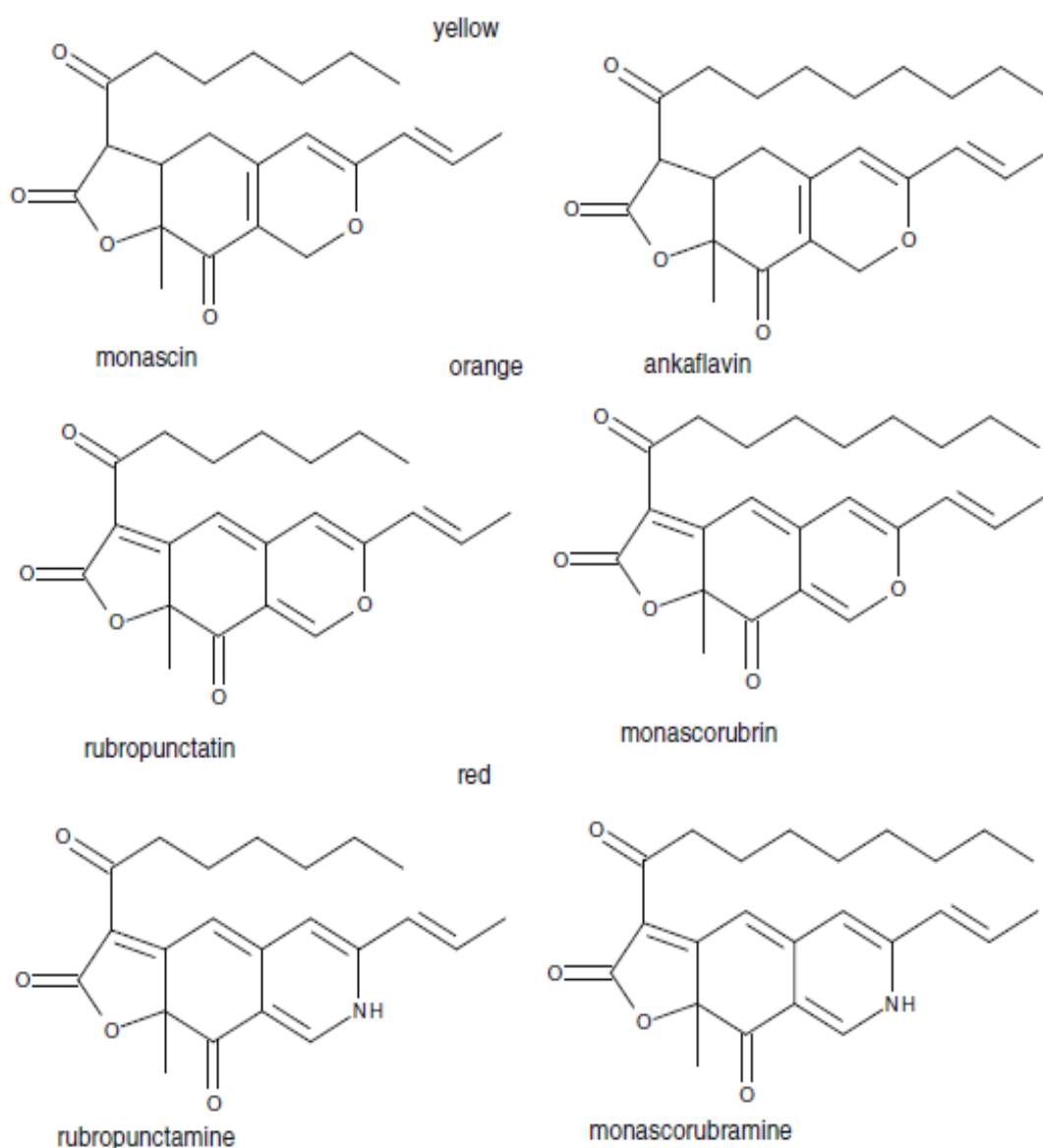


Fig 1: Major *Monascus* pigments (Petakova, 2013)

According to Dhale M *et al.*, (2009) *Penicillium. sp* NIOM-02 isolated from marine sediment, grown in media containing corn steep liquor scavenged 72-88 % of DPPH radical. During the solid state fermentation on wheat, the fungus produced more pigment (9.232 OD Units). The production of pigment and radical scavenging activity suggested its application in food, pharmaceutical and nutraceutical industries. Joshi *et al.*, (2003) reviewed the potential of pigment producing microorganisms such as *Monascus*, *Rhodotorula*, *Bacillus*,

Achromobacter, *Yarrowia* and *Phaffia*. According to them ideal pigment producing microorganisms should be capable of utilizing a wide range of carbon and nitrogen sources, have tolerance to pH, temperature and mineral and also give reasonable color yield.

3.4 PIGMENT PRODUCTION BY *Aspergillus terreus*

Aspergillus terreus also known as *Aspergillus terrestris* belong to the family Trichocomaceae, is a fungus (mold) and found worldwide in the soil. It was reported by Atalla *et al.*, (2012) that *Aspergillus niger* yielded brown dye at the end of the incubation period, which was then utilized in textile industry to dye wools. Colonies of *Aspergillus* usually inoculated on potato dextrose agar and Czapek dox agar are incubated at 25°C and are usually fast growing with distinct white, yellow, yellow-brown, brown to black or shades of green, and they consist mostly of packed protective covering of raised conidiophores. Colonies of *Aspergillus terreus* on czapek dox agar had compact conidial heads, columnar (upto 500 ×30-50 µm in diameter) and biseriate. Conidiophores are smooth-walled and hyaline, Conidia are globose to ellipsoidal (1.5 – 2.5 µm in diameter), hyaline to slightly yellow and smooth-walled.

Sutton *et al.*, (1998) and Hoog *et al.*, (2000) studied the microscopic morphology of *Aspergillus terreus* and found that the conidial head are columnar and biseriate, and the conidiophores were smooth-walled and hyaline usually 70 to 300 µm long. Conidia were smooth, globose and small (2-2.5 µm). *Aspergillus terreus* is used in industries for the yield of important organic acids, such as itaconic acid and cis-aconitic acid as well as enzymes like xylanase. It was also the initial source for the drug mevastatin (lovastatin), a drug used for lowering serum cholesterol. According to Soma *et al.*, (2011) *Aspergillus* produced cellulose under submerged and solid state fermentation utilizing coir waste as a substrate.

Barrios *et al.*, (2008) reported that *Aspergillus terreus* expressed lovastatin biosynthetic genes more in solid state fermentation as compared to liquid state fermentation. *Aspergillus terreus* are mostly isolated from soil but it can also be isolated from the textile industry effluent (Vinod *et al.*, 2012). According to Osman *et al.*, 2011 bagasse was the most suitable substrate for lovastatin production (50µg/ml). Lovastatin is a competitive inhibitor of 3-hydroxy-3-methylglutaryl-CoA reductase, the rate limiting enzyme of cholesterol biosynthesis. The

fungal isolates were cultivated in a two stage submerged fermentation followed by testing for the presence of lovastatin. *Aspergillus terreus* was the best lovastatin producing isolate with a level of 52.9µg lovastatin per ml of screening production medium. Different substrates were tested for lovastatin production viz. molasses, apple waste, strawberry waste, bagasse, wheat bran, corn meal and whey.

3.5 FACTORS INFLUENCING PIGMENT PRODUCTION

There are several factors like sources of substrate (carbon and nitrogen), fermentation environment, agitation, aeration that influence the production of pigment. Optimization of the conditions required for culture is critical in order to maximize and sustain the productivity of *Monascus* pigments. Evaluation of the effects of various environmental and nutritional factors needs to be done to determine their influence on pigment production in solid state fermentation, which includes the pH, moisture content, nutrient supplement, sample size, inoculums size etc. (Lee *et al.*, 2002)

An extracellular pigment-producing ascomycetous filamentous fungi belonging to the genera *Penicillium* was obtained from soil and its optimal culture conditions were investigated. The optimal culture conditions for pigment production were found to be; pH 9.0, temperature 30°C and inoculums age 4 days. The effect of physico-chemical conditions like sunlight, fluorescent light, UV light, high temperature and preservatives (sodium bisulfate, ascorbic acid and citric acid) are also investigated.

Supavej *et al.*, (2012) explored and successfully used three reactor types for pigment production by *Monascus*: shake flasks, and shaken and stirred miniaturized reactors. Shake flasks gave good pigment yields, but scale up was difficult, and they cannot be automated. The availability of oxygen appeared to affect biomass levels less than pigment production; red pigment production in particular needed very high oxygen levels. Mendez *et al.*, (2011) studied the influence of pH and temperature for the red pigment production by *Penicillium purpurogenum* GH2. In this study it was shown that the growth of *P. purpurogenum* and red pigment production under submerged culture can be controlled by the pH and temperature of the medium. *P. purpurogenum* GH2 was able to produce a water soluble red pigment, with maximal production (2.46 g/L) with treatment of pH 5 and 24°C. This study showed the

potential of fungus for red pigment production for use in the food industry. According to Shepherd *et al.*, (1983) nitrogen sources had a tremendous effect on the pigment production.

3.6 SOLID STATE FERMENTATION (SSF)

According to Bashir *et al.*, (2011) solid state fermentation is the growth or/and cultivation of microorganisms under controlled conditions in the absence of free water for the production of desired products of interest. Comparative studies between solid state fermentation and submerged liquid fermentation have proven higher yields and reduced contamination by fungi, yeast and bacteria since low water availability reduces possible chances of contamination. This allows working in aseptic condition in some cases. Production cost is also low at industrial level as solid state fermentation in most cases is characterized with low energy requirement, often in some cases autoclaving or vapour treatment, mechanical agitation and aeration are not necessary.

Subramaniam *et al.*, (2012) studied solid state fermentation by utilizing solid substrates like barn, bagasse and paper pulp as these substrates are nutrient rich waste materials that can be recycled easily as substrates. In solid state fermentation the same substrates are utilized very slowly and steadily. For fermentation involving fungi and microorganisms solid state fermentation is the best technique, but it cannot be used for fermentation process involving organisms that require high water activity.

It was reported by Maria *et al.*, (2008) that solid materials employed as supports in solid state fermentation can be classified into two categories: non-inert and inert materials. Non-inert materials are divided and humidified solids (e.g. cereal rains, flour, bran, saw dust) that behaves as support as well as nutrient source, while inert materials are nutritionally inert solids(synthetic form) that acts exclusively as a support, is soaked in a nutrient solution. It may be noted that using agro-industrial wastes as non-inert solid state fermentation support is much economical and therefore becoming popular.

Solid state fermentation has numerous advantages over submerged fermentation, such as simple fermentation equipment, less effluents and high volumetric productivity. Solid state fermentation is mostly done using agro-industrial residues, therefore the fermentation media is quite cheap and simple as compared to submerged fermentation (Pandey *et al.*, 2001).

Table 5: Pros and cons of solid state fermentation over submerged fermentation (from Couto and Sanroman, 2006)

Pros	Cons
- Superior yield	- Problem in scale up
- Media cost is less	- Problematic to regulate process parameters (pH, heat, moisture, nutrient composition etc.)
- Less attempt in downstream processing	- Product impurity is high
- Reduced energy and cost requirement	- Increasing recovery product cost
- Simple technology	
- It resembles the natural habitat for several microorganisms.	

3.7 AGRO-INDUSTRIAL PRODUCT AS SUBSTRATES FOR SOLID STATE FERMENTATION

Several agro-industrial residues such as wheat bran, rice, sugarcane bagasse, cassava bagasse, various oil cake, palm kernel cake, ground nut oil 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 most often and commonly used substrates for solid state fermentation processes. Since these substrates are readily available it makes the solid state fermentation cheap and wide acceptance in industries. Corn cob powder was used as substrate for the production of pigments by *Monascus purpureus* in solid state fermentation (Palanivel *et al.*, 2011) and a pigment yield was achieved by optimizing the various process parameters .It was also found that the pigments produced were stable at

acidic pH, high temperatures and salt solutions. The corn cob is also environmental friendly and economical to the end users.

Saccharina japonica, a readily available macroalgae (a type of sea weed) which is cheap and safe was used as substrate for pigment production by fungus *Talaromyces amestolkiae* GT 11 under solid state fermentation. Subsequently this led to natural pigment production that could be utilized for food, pharmaceutical industries and cosmetics (Thiyam *et al.*, 2013).

RATIONALE AND SCOPE OF THE STUDY

Natural colorants have several advantages over the synthetic colorants used in different textile and food industries. Synthetic pigments are known to have a detrimental impact on environment and human health in general. Besides this, there is always a demand for developing low cost process of product production. This project was aimed at testing different conditions required for the maximum pigment production using easily available agro-industrial residue such as rice straw, wheat straw, okara, sugarcane bagasse, broken rice, broken wheat and sweet potato.

OBJECTIVES OF THE STUDY

Aspergillus terreus MTCC 7600 was obtained from Institute of Microbial Technology, Chandigarh to evaluate its pigment production potential under solid state fermentation using agro-industrial waste. The following objectives were met to carry out this work.

1. To evaluate the potential of *Aspergillus terreus* MTCC 7600 for pigment production using agro-industrial residues as carbon source
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.

EQUIPMENTS, MATERIALS AND EXPERIMENTAL SET UP**6.1 EQUIPMENTS****Table 6: 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.	Micropipette	P'Fact A
10.	Microtips	TARSONS
11.	Orbital shaker	REMI
12.	Centrifuge	REMI
13.	Plastic wares	Poly lab

14.	Refrigerator	LG
15.	Weighing balance	Adventurer, DHA VS
16.	UV VIS spectrophotometer	“ELICO” double beam

6.2 MATERIALS

6.2.1 Glass wares

Conical flask (250 and 100 ml), petriplates, test-tubes, streaking rod, glass rod, glass beakers and inoculation loop.

6.2.2 Chemicals

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

The above chemicals used were procured from “Loba Chemi” and “Himedia”.

6.2.3 Miscellaneous

Cotton, muslin cloth, butter paper, brown paper, thread, microtips, burner, scissors, test tube stands, aluminum foils, micropipette and distilled water.

6.3 EXPERIMENTAL SETUP

All the experimental works were carried out in the project lab of Department of Biotechnology and Biosciences (room no: 28-301) at Lovely Professional University, Punjab under controlled aseptic conditions.

7.1 *Aspergillus terrus* CULTURE

Aspergillus terrus MTCC 7600 procured from Institute of Microbial Technology (IMTECH), Chandigarh was used in the present study.

7.2 MAINTENANCE OF CULTURE

Aspergillus terrus MTCC 7600 was maintained on potato dextrose agar (PDA) slant and incubated at 28±2 °C in an incubator for 7 days and it was sub-cultured once in three weeks (Babitha *et al.*, 2006).

7.3 PREPARATION OF INOCULUM

Aspergillus terrus MTCC 7600 inoculated on potato dextrose agar slants was allowed to fully sporulate for 6-8 days at 28±2 °C in an incubator. 10 ml of sterile distilled water mixed with tween80 solution (100ml sterile distilled water + 0.5 ml tween80) was added to the slant and with the help of an inoculation loop the spores were scrapped off under aseptic conditions. The spore suspensions obtained were used as inoculum (Velmurugan *et al.*, 2011).

7.4 PREPARATION OF SUBSTRATES

Different carbon sources used include Rice straw, Wheat straw, Sugarcane bagasse, Okara, Broken rice, Broken wheat and Sweet potato, Substrate preparation was done as specified by Babitha *et al.*, (2006) with some variation. The carbon source like sweet potato was prepared by washing, shredding and drying them in a tray dryer for 12 hours at 55-60°C. Then the dried substrates were blended to powder form with the help of pastel and mortar.

7.5 SOLID STATE FERMENTATION (SSF)

10g of the dried carbon substrates was taken in a 50 ml conical flask and moisture content was set at 56-60% according to the following equation (Dikshit and Tallapragada, 2012):

$$\text{Moisture content (\%)} = 100 \times (\text{wet weight} - \text{dry weight}) / \text{wet weight}$$

The contents in the flask were blended well and then autoclaved at 121°C at 15 psi, it was cooled to room temperature. It was then inoculated with the prepared spore suspension(1000 µl) and incubated at 28±2 °C in the BOD incubator for 5, 10, 15 and 20 days respectively.

7.6 EXTRACTION OF PIGMENTS

For pigment extraction, the fermented solids were dried in tray drier for 24 h at 55-60°C, and grinded by pestle and mortar to form powder. 0.1 gm of the powdered sample was mixed with 80% ethanol in a 50 ml conical flask and kept on a rotary shaker for 1 h at 200 rpm, followed by centrifugation for 30 minutes at 1000 rpm. The supernatant was collected for pigment analysis. Ethanol extract of non-fermented substrates was used as blank (Dhale *et al.*, 2009).

7.7 ESTIMATION OF PIGMENTS

Pigment estimation was done by following the method of Velmurugan *et al.*, 2011 in which the optical density at its absorbance maxima were expressed as the concentration of pigment produced. The extracellular pigment was quantified by measuring OD at 410nm using a SL 210 UV-VIS spectrophotometer (ELICO double beam), taking into consideration the dilution factor of the sample OD at its maxima, per gram dry fermented matter (Dikshit and Tallapragada, 2012).

$$\text{Color value} = \text{O.D} \times \text{dilution} \times \text{volume of extracts} / \text{Amount of sample}$$

7.8 NITROGEN SUPPLEMENTATION

Various nitrogen sources both organic and inorganic viz. MSG, yeast extract, peptone, ammonium nitrate, ammonium sulphate and sodium nitrate were added to the carbon source(0.5 %) which gave the highest pigment yield (Vidyalakshmi *et al.*, 2009). Next, the culture inoculum was added to the different nitrogen sources in combination with the best carbon source. Estimation of pigment OD was measured at 410nm using a SL 210 UV-VIS spectrophotometer (ELICO double beam), taking into consideration the dilution factor of the sample OD at its maxima, per gram dry fermented matter.

7.9 STABILITY STUDIES OF PIGMENTS

The following stability studies on pigments were conducted.

7.9.1 Effect of temperature

Temperature study was done by heating methanolic extract of *A. terreus* MTCC 7600 pigment in a hot water bath at different temperature viz. 40°C, 50°C, 60°C, 70°C, 80°C and 90° C respectively for 15 min in separate test tubes (10 ml each) , followed by absorbance measurement at 410nm (Kaur *et al.*, 2008)

7.9.2 Effect of pH

5 ml each of methanolic extract of *A. terreus* MTCC 7600 pigment were taken in separate test tubes. Then pH was adjusted from 4 to 9, using 0.1 N NaOH or dil HCL. absorbance of the *A. terreus* MTCC 7600pigment was measured at 410nm (Cesar *et al.*, 2005)

7.9.3 Effect of visible light

To investigate the effect of light on pigments, methanolic extract of *A. terreus* MTCC 7600 pigment were poured onto 10 test tubes (5 ml each). After covering them properly with cotton plugs they were kept exposed to visible

light. Extracted pigments kept in dark served as the control. The color value was calculated using the formula:

$$\text{Color value} = (\text{OD} \times \text{dilution} \times \text{volume of extract}) / \text{amount of sample}$$

7.9.4 Effect of different solvents

Solvent stability study was done by dissolving the samples in six different solvents viz. ethanol, chloroform, diethyl ether, distilled water, methanol and acetone. Then the absorbance of the samples dissolved in different solvents was measure, then after an interval of 7 days the absorbance was measured again (Wongsorn *et al.*, 2011).

8.1 MAINTENANCE OF CULTURE

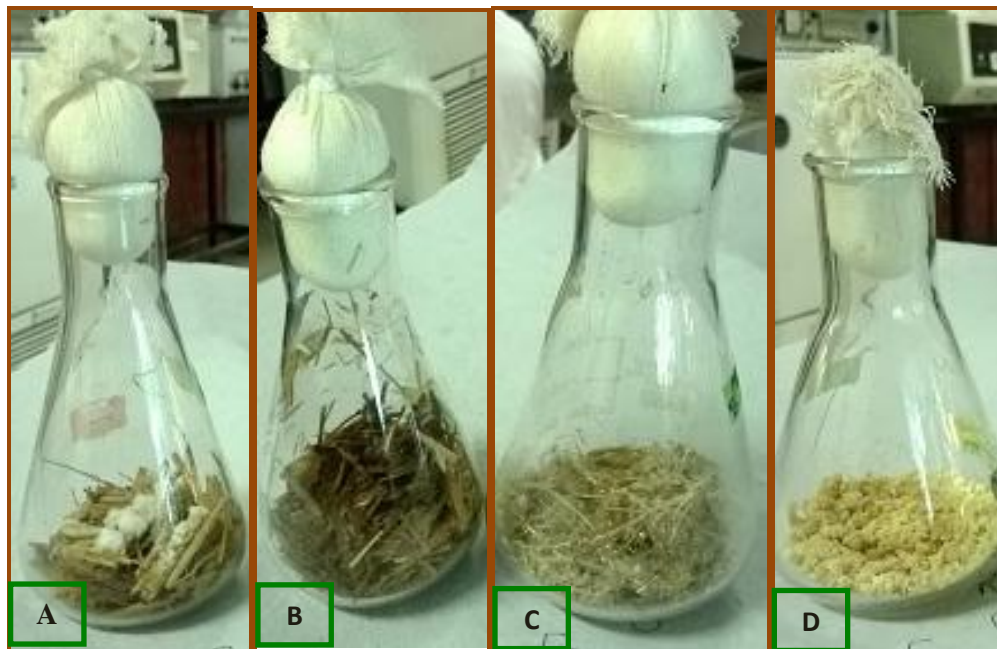
Culture of *Aspergillus terreus* MTCC 7600 obtained from Institute of Microbial Technology (IMTECH), Chandigarh was maintained in potato dextrose agar (PDA) at 28 ± 2 °C till confluent growth was observed. It was sub cultured once every week aseptically. The color of the culture turned brownish in color and as it matured over time, the color darkened (Fig : 2).



Fig 2: Front and back view of growth of *Aspergillus terreus* MTCC 7600

8.2 SCREENING OF AGRO-INDUSTRIAL RESIDUES FOR PIGMENT PRODUCTION

Different substrates viz. rice straw, wheat straw, sugarcane bagasse, okara, broken rice, broken wheat and sweet potato were inoculated with *A. terreus* MTCC 7600 and incubated at $28\pm 2^{\circ}\text{C}$ for 20 days (Fig: 3). Maximum pigment production was observed in case of broken rice (110.7 CVU/gds), followed by sweet potato (98.05 CVU/gds) and broken wheat (81.6 CVU/gds) while least pigment was produced in okara and sugarcane bagasse (Table: 7). Since maximum pigment was produced in broken rice, it was chosen as the substrate for further optimization studies. In addition to this, it was also observed that highest pigment production was obtained after an incubation of 15 days followed by a decrease in the activity on increasing the incubation (Table: 7). Similar productions patterns have been reported by Dikshit and Tallapragada (2013). They reported that *oryza spp.* (local polished rice) was the best substrate among the different substrates used for pigment production by *Monascus sanguineus*. Sameer *et al.*, (2008) also reported that *Epicoccum nigrum* inoculated onto rice gave 6.2 folds higher pigment production. This result is also in consistent with the studies that reported *oryza spp.* (local polished rice) to be the best solid substrate for both *Monascus sanguineus* and *Monascus purpureus* (Dikshit and Tallapragada, 2012)



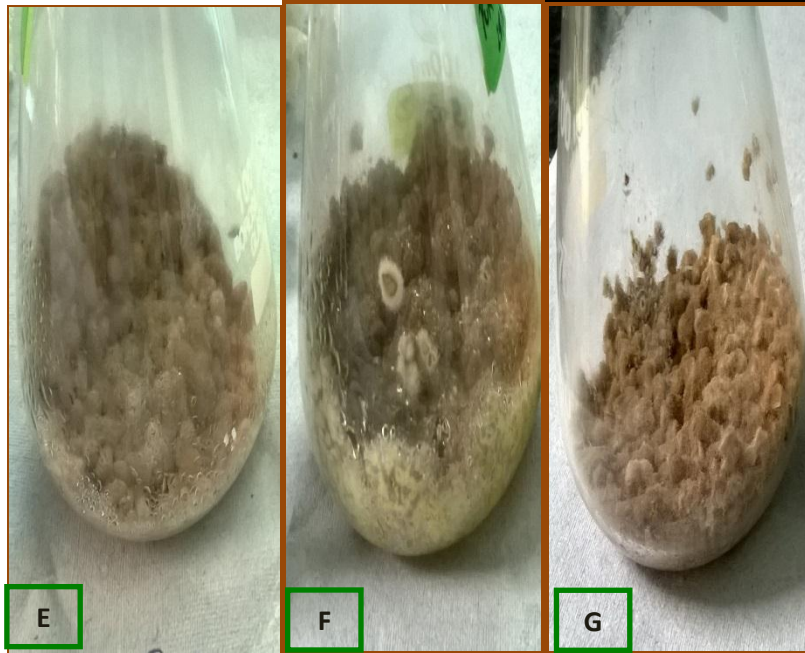


Fig 3: Pigment production on different carbon sources A) rice straw B) wheat straw C) sugarcane bagasse D) okara E) broken rice F) broken wheat G) sweet potato

Table 7: Color values of different carbon substrates at 410 nm

No. of days	Color value (CVU/gds)						
	Rice Straw	Wheat Straw	Okara	Sugarcane Bagasse	Broken Rice	Broken Wheat	Sweet Potato
5	NIL	NIL	NIL	NIL	NIL	NIL	NIL
10	11.17 ± 0.191	21.86 ± 0.537	2.16 ± 0.742	5.28 ± 0.304	88.215 ± 0.903	48.591 ± 0.115	58.51 ± 0.174
15	21.84 ± 0.418	42.25 ± 0.407	9.24 ± 0.768	11.21± 0.566	110.7 ± 0.912	81.6 ± 0.054	98.05 ± 0.626
20	16.55 ± 0.209	37.25 ± 0.749	5.59 ± 0.368	8.27 ± 0.629	96.35 ± 0.70	72.24 ± 0.919	80.01 ± 0.912

8.3 EFFECT OF NITROGEN SUPPLEMENTATION ON PIGMENT PRODUCTION

The carbon substrate i.e., broken rice giving the best yield was selected and it was supplemented with various nitrogen sources, both organic and inorganic (Fig: 4). It was observed that monosodium glutamate (MSG) gave the maximum pigment production with a color value of 152.7 CVU/gds while least pigment production was seen on supplementation with ammonium sulphate (Fig: 6). It was seen that on addition of organic nitrogen sources, higher pigment production was achieved as compared to inorganic sources. These results are also similar to the findings of Babitha *et al.*, (2006) who reported a positive impact on pigment production due to addition of MSG as a nitrogen compound. This result is also consistent with the studies that reported MSG as the most suitable nitrogen source for pigment production (Lin *et al.*, 1993). Vidyalakshmi *et al.*, (2009) also reported a maximum pigment (131.4 CVU/gds) at 410 on supplementation of MSG by *Monascus ruber* followed by ammonium nitrate (89 CVU/gds).

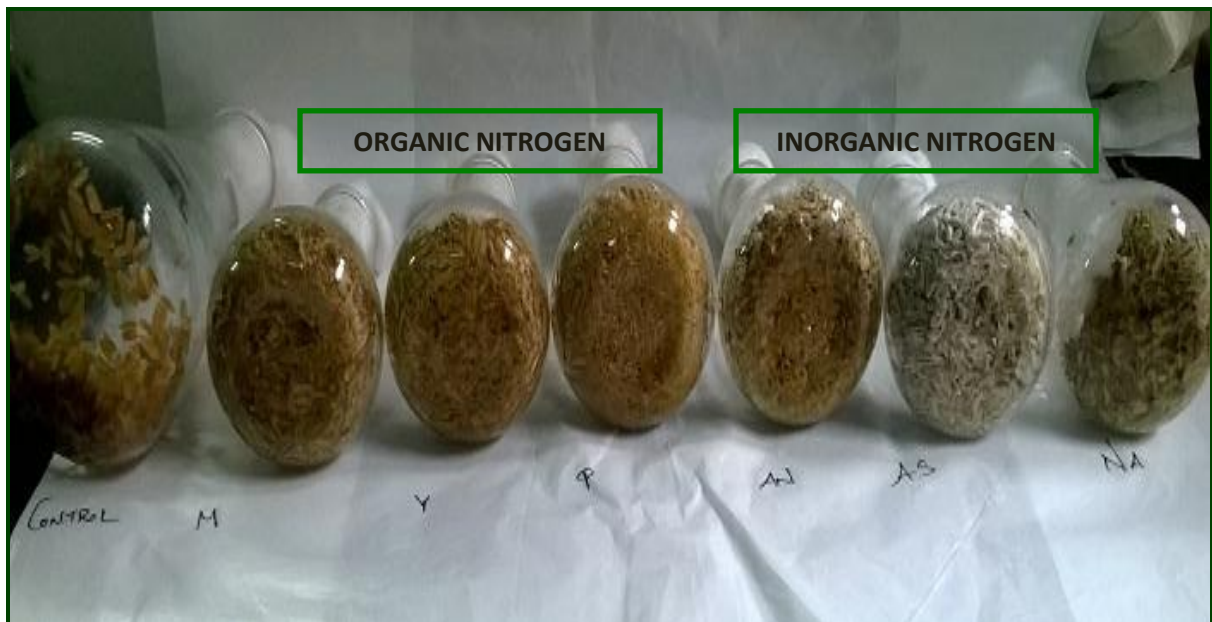


Fig 4: SSF using broken rice supplemented with organic and inorganic nitrogen sources (Organic- MSG, yeast, peptone; Inorganic- ammonium nitrate, ammonium sulphate, sodium nitrate)

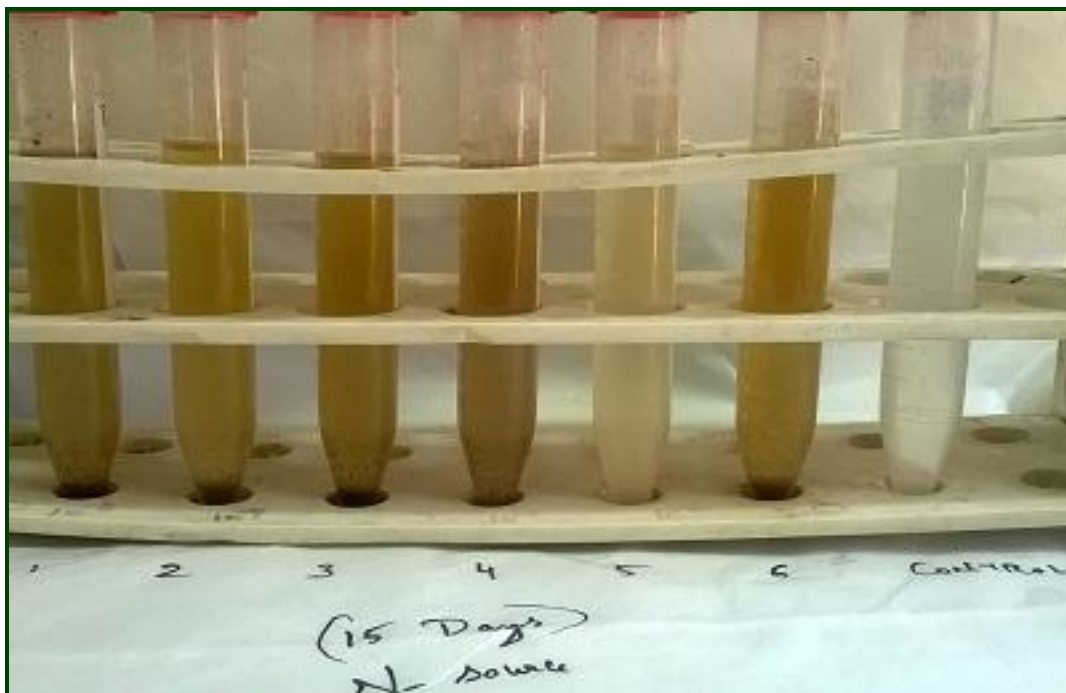


Fig 5: Pigment extraction using 80% methanol 1) Yeast; 2) Peptone; 3) MSG; 4) Ammonium nitrate; 5) Ammonium sulphate; 6) Sodium nitrate

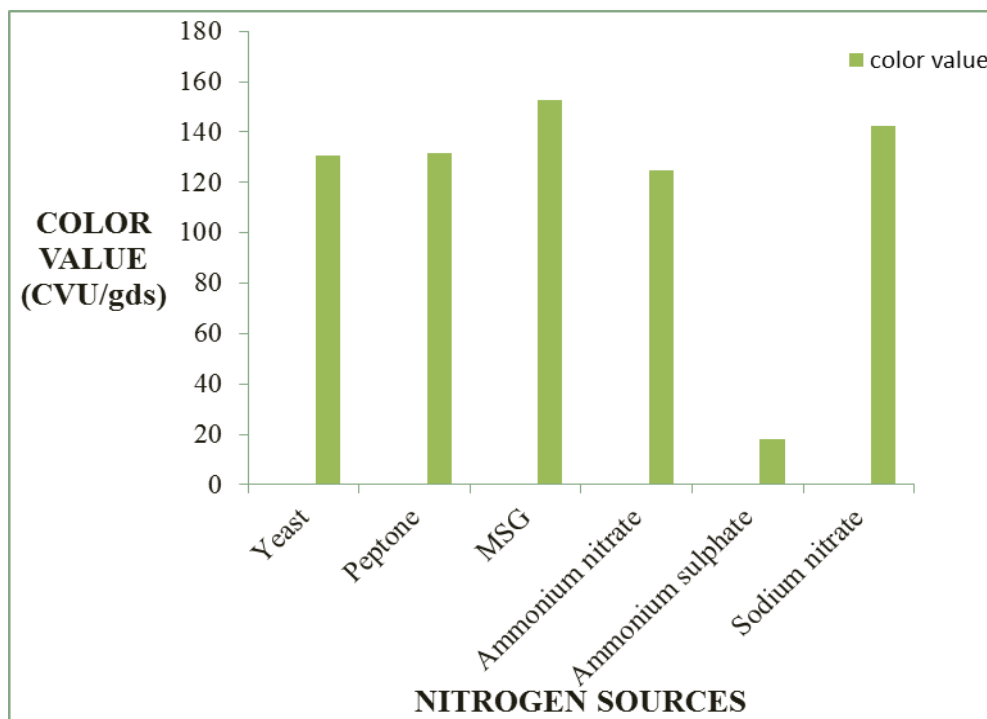


Fig 6: Effect of supplementation of organic and inorganic nitrogen source

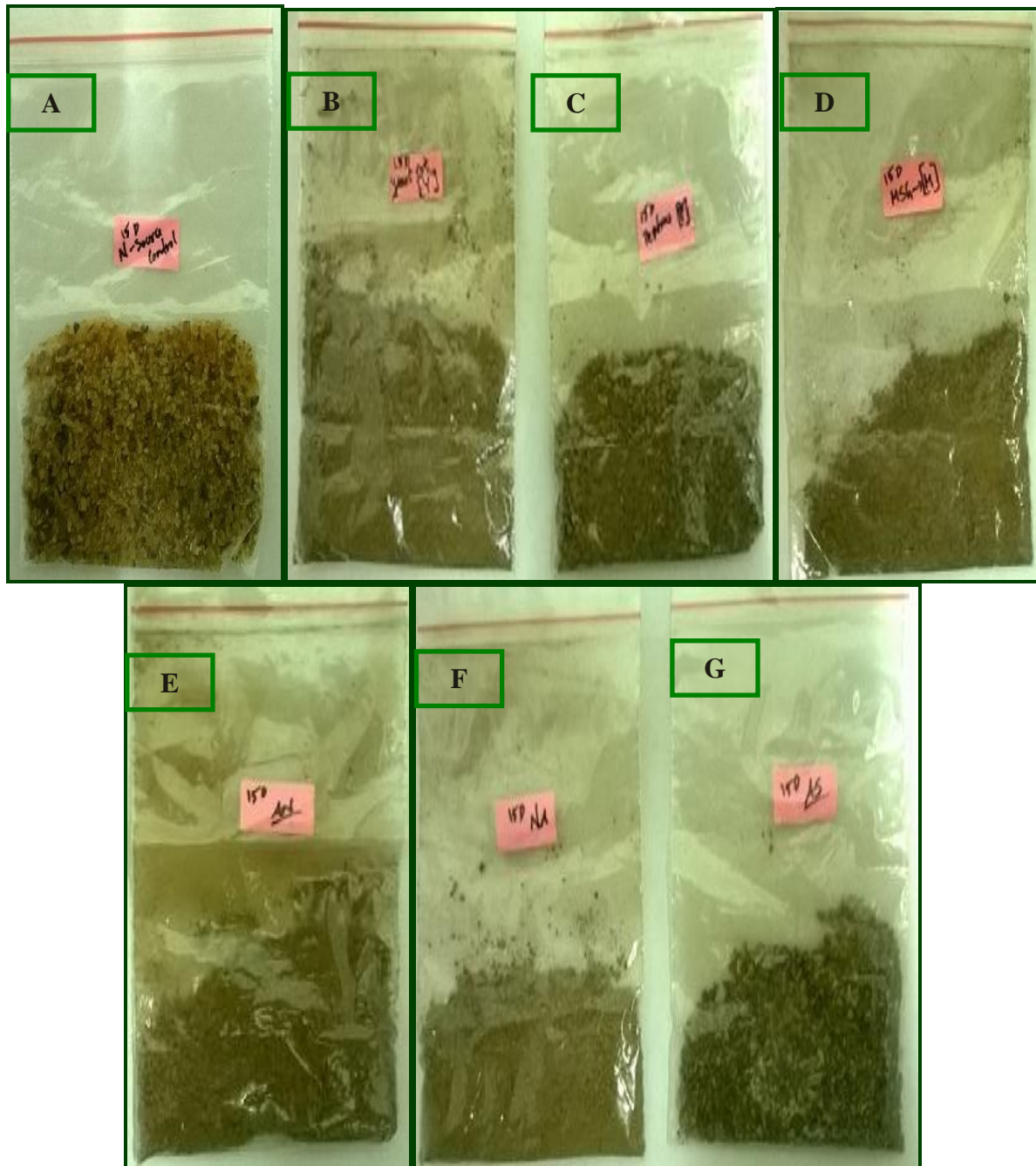


Fig 7: Pigment extracted after an incubation of 15 days A) Control B) Yeast C) Peptone D) MSG E) Ammonium nitrate F) Sodium nitrate G) Ammonium sulphate

8.4 PIGMENT STABILITY STUDIES

8.4.1 Effect of temperature

The pigments produced by *Aspergillus terreus* MTCC 7600 was observed to be stable at 40°C, 50°C and 60°C respectively. But as temperature increased from 70-90°C the pigments were unstable (Table: 8). These results are also similar to the findings of Kaur *et al.*,(2008) who reported that pigment produced by *Rhodotorula rubrua* MTCC 1446 when heated from 70-100°C showed a rapid decrease in color. Bhosale *et al.*, 2003 also reported that pigments produced by *Rhodotorula* get denatured on treatment with temperature.

Table 8: Color value of the pigment at different temperatures

Temp. (°C) Wave length	Color value (CVU/gds)					
	40	50	60	70	80	90
410 nm	384.2 ± 0.687	364.06 ± 0.890	355.34 ± 0.262	325.71± 0.615	248.03± 0.256	216.09 ± 0.128

8.4.2 Effect of pH

With increase in pH the color value of the pigments decreased gradually thereby indicating that the pigment is stable at acidic pH (Table: 9). It was also reported that *Monascus sanguineus* and *Monascus purpureus* produced maximum pigment at pH 6.5 and pH 5.5 respectively (Dikshit and Tallapragada, 2012). In an experiment conducted by (Soumya *et al.*, 2014) it was reported that the pigments *Chaetomium cupreum* showed maximum absorption in alkaline condition with a color difference, it turned from yellow to orange at alkaline pH and it remained deep red in a pH range of 6-9

Table 9: Color value of the pigment at different pH

pH Wave Length	Color value (CVU/gds)					
	4	5	6	7	8	9
410 nm	183.4 ± 0.918	152.81 ± 0.488	120.2 ± 0.307	116.09 ± 0.841	111.11 ± 0.054	105.94 ± 0.230

8.4.3 Effect of visible light

The pigments produced by *Aspergillus terreus* MTCC 7600 was observed to degrade under visible light (Table 10). Many work has not been done on the stability of pigment produced by *Aspergillus terreus* but reference can be done to the work of Velmurugan *et al.*, 2009 where it was reported that *Monascus purpureus* produced maximum pigment in darkness. This result is also in consistent to the studies of Joshi *et al.*, 2003 that the major hurdle of microbial pigment is their instability to heat, light, acidity, air and water activity.

Table 10: Color value of the pigment after 24 hrs interval

Time Wave Length	Color value (CVU/gds)					
	24 hrs	48 hrs	72 hrs	96 hrs	120 hrs	144 hrs
410nm	158.22 ± 0.933	140.42 ± 0.0748	131.72 ± 0.338	97.08 ± 0.146	71.13 ± 0.393	67.31 ± 0.431

8.4.4 Effect of different solvents

Aspergillus terreus MTCC 7600 grown on broken rice with supplemented MSG was dissolved in six different solvents viz. ethanol, chloroform, acetone, distilled water, methanol and diethyl ether (Fig: 8). Out of the different solvents used chloroform was the best solvent for pigment extraction (399.2 CVU/gds) followed by methanol (398.32 CVU/gds) and it was found that the pigment has low water solubility (Table: 11) thus we can conclude that the pigment produced is water-insoluble pigment. Work done by Carvalho *et al.*, 2006 for pigment production by *Monascus* strain, it was found that methanol was the best solvent followed by DMSO.

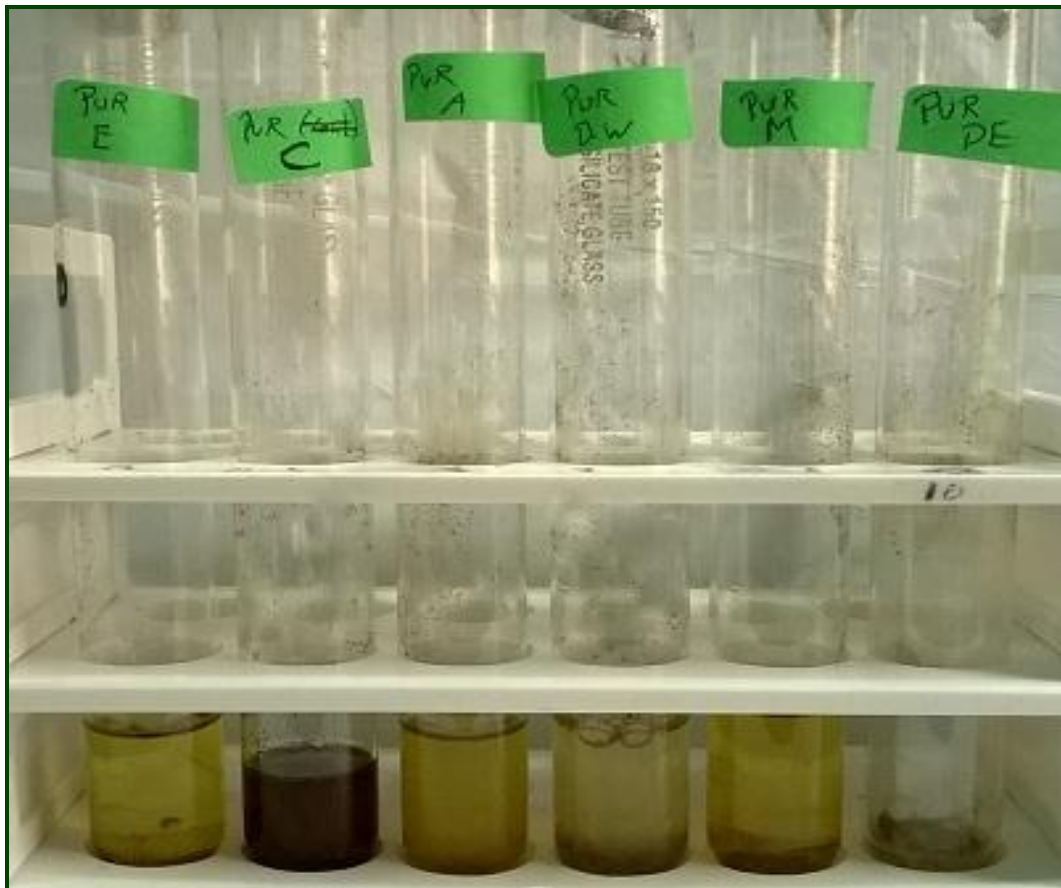


Fig 8: Effect of different solvents, E-ethanol; C-chloroform; A-acetone; DW- distilled water; M-methanol; DE-diethyl ether

Table 11: Color value of the pigment in different solvents

Solvents Wavelength	Color value (CVU/gds)					
	Ethanol	Chloroform	Diethyl Ether	Distilled Water	Methanol	Acetone
410 nm	355.57 ± 1.434	399.2 ± 0.706	385.26 ± 0.396	128.7 ± 1.414	398.32 ± 0.516	386.16 ± 0.801

CONCLUSION AND FUTURE SCOPE

From the experimental studies done on evaluation of pigment production by *Aspergillus terreus* MTCC 7600 it can be concluded that among seven agro industrial residues used, broken rice (110 CVU/gds) could be an effective substrate for pigment. Other than broken rice good pigment yield was also observed in sweet potato (98.05 CVU/gds) and broken wheat (81.6 CVU/gds). Enhancement of yield was achieved by incorporation of different organic and inorganic nitrogen sources among which monosodium glutamate (MSG) supported maximum pigment yield of 152.7 CVU/gds followed by sodium nitrate (142.6 CVU/gds) and peptone 131.9 CVU/gds and ammonium sulphate gave the least pigment production (18.3 CVU/gds). Pigment stability studies revealed that the pigment was stable 40°C- 60°C, it was stable at acidic pH, the pigments were stable in the dark and it was found that chloroform was the best solvent for pigment extraction (399.2 CVU/gds) followed by methanol (398.32 CVU/gds).

Natural pigments obtained from *Aspergillus terreus* are of practical interest in many industries like the food, pharmaceutical and they have cholesterol lowering and a promising drug candidate for anti-influenza virus. Further work need to be carried out to characterize these pigments before they can be evaluated for industrial applications.

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LIST OF ABBREVIATIONS

PDA: Potato dextrose agar

SSF: Solid state fermentation

SF: Submerged fermentation

MSG: Monosodium glutamate

OD: Optical density

UV: Ultra violet

CVU/gds: Color value unit/ gram dry substrate