

**STUDIES ON A NEW GENERATION BIOCOLOURS FROM
Monascus spp. AND RELATED FUNGI FOR USE IN
FOOD INDUSTRY**

Thesis submitted in partial fulfillment of the requirements for the award of the
Degree of **DOCTOR OF PHILOSOPHY in AGRICULTURAL MICROBIOLOGY**
to the Tamil Nadu Agricultural University, Coimbatore.

By

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2001

CERTIFICATE

This is to certify that the thesis entitled "**STUDIES ON A NEW GENERATION BIOCOLOURS FROM *Monascus* spp. AND RELATED FUNGI FOR USE IN FOOD INDUSTRY**" submitted in partial fulfillment of the requirements for the award of the degree of **DOCTOR OF PHILOSOPHY in Agricultural Microbiology** to the Tamil Nadu Agricultural University, Coimbatore is a record of bonafide research work carried out by **Mr. B.TAMILSELVAM** under my supervision and guidance and that no part of this thesis has been submitted for the award of any other degree, diploma, fellowship or similar titles or prizes and that the work has not been published in part or full in any scientific or popular journal or magazine.

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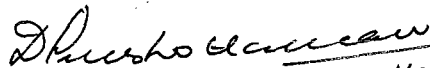
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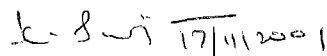
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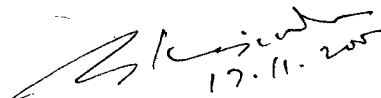
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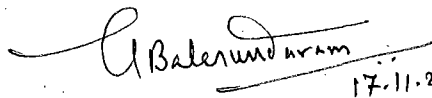


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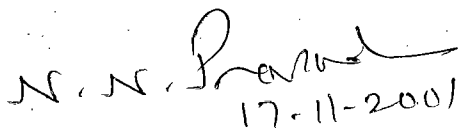
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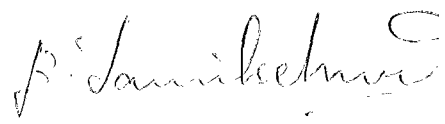
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(B.TAMIL SELVAM)

Abstract

ABSTRACT

STUDIES ON A NEW GENERATION BIOCOLOURS FROM *Monascus* spp. AND RELATED FUNGI FOR USE IN FOOD INDUSTRY

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(The search for newer sources for biocolourants is the need of the hour in the scenario of wide spread use of synthetic colours that has sufficiently spoiled our health. Though many natural colours are available, considerable interest has been generated on microorganisms as a source of biocolourants. Among various groups of microorganisms, fungi offer greater promises for the production of biocolourants. The fungus, *Monascus* sp. has been used as a colourant in the Far Eastern countries since long time. The present study adds a few more potential species of biocolour producing fungi and their utilization in food industry.

As soil is the “treasure house” of microorganisms, soil samples across the country were collected from forest ecosystems, cropped fields and rhizosphere soils of certain plants. Newer isolates of fungi were screened out that resulted in the recognition of *Penicillium purpurogenum* (PF 1), *Verticillium* sp. (PF 12) and *Humicola* sp. (PF 16). These fungi produced crimson red and yellow pigments.

Along with standard *Monascus purpureus* and *Monascus ruber*, the new isolates were studied in great detail. Generally, for maximum pigment production, glucose, sucrose and starch were preferred; biomass production was also high with these sources of carbon. Ammonium nitrate, yeast extract and monosodium glutamate served as preferred sources of nitrogen. An optimum pH of 5.0-6.0, with an agitation of 100-150 strokes per minute, an optimum temperature of 35°C and incubation in darkness were recognized as ideal for growth and pigment production.

All the five fungi selected for the study produced pigments both under solid state and liquid fermentation conditions. The use of polypropylene bags can replace conventional fermentors for pigment production. The study further revealed that the low cost growth media, rice kanji and raw rice could be used economically for pigment production.

The pigments of the fungi were harvested by air-drying or by solvent extraction procedures and concentrated by lyophilization. The crude pigments were quite stable at 100°C, varying pH levels and exposure to ultraviolet light, fluorescent light and unscreened sun light. The crude pigments were purified by thin layer chromatography and the components of pigments were analysed using HPLC.

For enhancing pigment production, genetic improvement of the fungi was attempted using the mutagen, nitrosoguanidine. A few of the mutants have registered significantly greater yield of pigments.

Toxicological study has been made on the pigments using a sensitive target organism, the silk worm. Mulberry leaves fortified with the pigments were preferred by the worms and there was no symptoms of toxicity as evidenced by hundred per cent survival of the worms.

Using *Monascus* spp. pigments as colourants a few food products like wheat halwa, plum squash and soft cakes were prepared and evaluated by a panel of judges. These products were readily accepted by the panel.

A potential cardio-vascular drug, Monacolin K was present in the pigments of *Monascus* spp. Monacolin K powder was prepared from the fungus and encapsulated in gelatin capsules.

In short, this study brings out the potentials of *Monascus* spp. and other species of fungi as excellent sources of biocolour production. Future food industry relies more on such non toxic natural biocolours.)

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ABBREVIATIONS

min.	-	minutes
atm.	-	atmosphere
tsp.	-	table spoon
l	-	litre
°C	-	Degree Celcius
conc.	-	concentrated
PDA	-	Potato Dextrose Agar
nm	-	nano metre
cm	-	centimetre
dia	-	diameter
µg	-	microgram
hrs	-	hours
rpm	-	revolutions per minute
ml	-	millilitre
× g	-	gravitational force
g	-	gram
kg	-	kilogram

Introduction

Chapter I

INTRODUCTION

("By recognizing the activities of microorganisms and learning to utilize them to his own advantage man has become masters of several over many natural processes thereby contributing greatly to his own economy and well being" so said the late Professor, Nobel Laureate Selman. A. Waksman (Waksman, 1963). Since the dawn of Microbial Biotechnology two decades back, world around there is increasing interest in the utilization of microorganisms. Newer and potential microorganisms are being discovered for wider application in agriculture, industrial fermentations, pharmaceutical/antibiotic industries, food fermentations, and food processing in combating dreadful diseases in man, animals and crop plants and also in environmental pollution abatement strategies. It is indeed hard to figure out any human endeavour where microorganisms are not associated with.

One of the upcoming areas of industrial utilization of microorganisms is the production of biocolourants usable in food and beverage industries. Attractive colours of foods and beverages are very important determinants for acceptability from the public. They fetch better price and consistent market too. For imparting pleasing and attractive colours to foods and beverages, many natural colours have been in use since ancient times (Francis, 1987). These colours were obtained from several vegetables and animals. Sooner a vast array of synthetic colours have flooded the food industry since five decades. Synthetic colours are found technically more suitable than natural colours because the former are known for their fastness, availability in a wide range of colours, low cost even at high concentration in low volumes and they are devoid of aroma and taste.

However, in the world market the number of permitted synthetic colours has declined in the past; colours like 'AZORUBIN' or 'TETRAZIN' caused allergies in human beings. Many are carcinogenic and teratogenic in nature. With the increasing awareness and concern for health, people avoid using synthetic colours and law also has abandoned them.

Search for harmless natural food colours intensified and plants, animals and microorganisms were rigorously screened. (Patnaik *et al.*, 1997). Among the natural sources of colourants, microorganisms offer great hope and scope. The ease of cultivation, extraction and more than anything else, the existing genetic diversity in microbes and sophistication of technology has made their choice more feasible (Juazlova *et al.* 1997).

Among the different groups of microorganisms like bacteria, yeast, algae, fungi and actinomycetes, filamentous fungi appear more efficient and attractive sources of bicolourant than others. From time immemorial the fungus, *Monascus* sp. has been used to impart desirable colour to food in the East. 'Benikoji', 'Anka' are traditional names for food colours (Dizon and Sanschez, 1984). *Monascus purpureus* and *Monascus anka* have already been extensively studied as potential sources of biocolourants (Hawkworth and Pitt, 1983). The yeast, *Phaffia rhodozyma* is yet another example of biocolour producing fungus enjoying industrial exploitation.

Bull *et al.* (1992) while reviewing the potentials of biodiversity among microorganisms indicated that fungi have great potentials for industrial exploitation and at present only 5 per cent of the estimated population of 1.5 million species alone is known. Fungi occurring in tropical ecosystem like ours display a much greater genetic diversity than are from temperate regions.

In India not much work has been accomplished in respect of biocolour production from fungi and bacteria. There appears wide scope in the industrial production of biocolours from fungi. It should be realized that not all biocolourants from microbial origin are harmless to human health. At present the pigments from *Monascus purpureus* are authorized for food use in Japan (Blanc *et al.* 1994). However, it is popular in Taiwan, Indonesia and Thailand. It is of interest to note that in USA there are over sixty *Monascus* pigment patents exist and this shows the trend of commercialization of microbial biocolourants.

With these background information, a detailed study on pigment producing fungi has been contemplated with the following objectives:

1. Isolation of pigment producing fungi from diverse ecosystems like agricultural soils, forest soils and rhizosphere of plants.
2. Target screening of the fungal isolates for maximum pigment yields and identification of the fungi.
3. Optimizing conditions for pigment production and characterization of the pigments.
4. Genetic improvement of the selected fungi for enhanced pigment production through mutagenesis.
5. Biological safety of fungal pigments and development of food products using fungal biocolours.

Review of Literature

Chapter II

REVIEW OF LITERATURE

1.1. Introduction

The use of colours as additives in food and drinks is a significant inevitable factor to food manufacturer and consumer alike in determining the acceptability of processed food. As processing of food sometimes may destroy its own colour, exogenous addition of desirable colours gives a “facelift” for the product. Artificial colourings, those commonly referred as 'coal tar dyes' or 'food dyes' have for many years been ideal for replacing natural colouring. However, in recent years, the number of dyes suitable for food colouring has been drastically reduced as a result of toxicological studies by Prevention of Food Adulteration Act of India, 1954. Besides Law enforcement, consumer has become aware of the health hazards due to synthetic food dyes. Naturally, the next alternative is 'natural colours' from sources like plants, animals and microorganisms.

This chapter, extensively reviews the synthetic dyes induced health hazards, natural colours and their sources. Importantly, it explores the microbial food colours and their suitability for food preparation. The review also considers the safety measures and regulatory aspects of natural and microbial pigments (Pattnaik *et al.*, 1997).

1.2. Synthetic food colours causing health hazards

From ancient times the colours of nature vegetable or animal origin such as saffron, turmeric, annato, chlorophyll, cochineal have been used by man. Poliny reported that in days of Roman Empire, both wine and bread were frequently coloured with such items as fruit berries and white earth.

With the advent of the modern dyestuff manufacturing industry after the discovery of Perkins Mauve in 1856 many new colours were synthesized and these were called as 'coal tar' colours (Santhanakrishnan, 1981). These colours were more suitable than the natural colours for the use in foods. Natural colours have: 1) fastness deficiencies, 2) restricted shade range, 3) high cost in use, 4) low effective agent concentration, 5) considerable batch to batch variation and 6) distinctive aroma or spicy taste.

Synthetic colours are complex compounds of carbon, hydrogen, oxygen, nitrogen and sulphur with metal ions (Anon, 1963). The manufacturers of dyes proceed from simple raw materials like aromatic hydrocarbons such as benzene, toluene, xylene, naphthalene, acenaphthalene, carbazole and anthracene with the introduction of other chemical groups such as nitro, amino, halogen and sulfonic acid. These intermediaries were further purified by diazotization, coupling, condensation and fusion to give the final dye stuff.

Synthetic dyes are divided into five classes:

- a. Monoazo, Diazo, Triazo compounds - Sunset yellow, FCF, Amaranth, Carmoisine, Ponceau.
- b. Triaryl Methane - Brilliant Blue, FCF
- c. Xanthene - Erythrosine
- d. Indigoid - Indigo carmine
- e. Azopyrazolone - Tartrazine

1.2.1. Health hazards of synthetic colours

Non permitted food colours are used alone or in combination or mixed with permitted food colours in various food products. Consumption of such food colours

causes irreversible degeneration of the seminiferous tubules, testicular lesions, formation of multinucleate giant cells and binding of the dye to serum tissue proteins. Similarly, there has been significant decrease in the weight of body organs like kidney, heart, lung, stomach, caecum and testes. Intake of synthetic colours may lead to alteration in physiological condition. Orange II dye causes local tumor formation in the lumen of the bladder of mouse intestinal gut microflora was affected due to the intake of synthetic colours in rats (Aries *et al.*, 1969). Metanil yellow, tartrazine reduced the levels of vitamins A and E in the body during continuous intake of such dyes in diets (Singh, 1997).

1.2.2. Present status of synthetic food colours

Of the eight permitted colours for food use under Prevention of Food Adulteration Act, there are red, two yellow, two blue and one green synthetic colours. However, nine colours listed as non permitted based on the toxicological studies. India produces about 135 tonnes of synthetic food colours every year representing 1.5 per cent of the world production of food colours. Among the permitted synthetics, sunset yellow, tartrazine account for 70 per cent net production. Indian population consumes 220 mg of food colours per year which is 50 and 35 times lesser than the per capita intake in USA and UK respectively (Singh, 1997).

Although the reasons for colouring foods have remained unchanged, food manufacturers have realised that the trend towards natural food colours has increased in recent years. Hence, many natural colours were explored from mother nature.

1.3. NATURAL FOOD PIGMENTS

The natural colours are now being favoured, as alternative to synthetic colours a few preparations have come to the user market (Lewis *et al.*, 1977).

Natural colourants are crude extracts of pigments, which are basically unstable. It is not true that all natural pigments are safe for human consumption. Pigments present in edible materials such as fruits and vegetables are generally considered safe. The trend towards natural ingredients in foodstuff is continuing and evidenced by consumer acceptance of "natural" foods and the various National Regulations which completely or selectively ban artificial colours from food (Timberlake and Bridle, 1980).

The source of natural colours and their choice are also limited in general. The bottle necks in using them are their unstable nature in pH, heat, light, oxidation, etc.

Natural colours available for food colouration fall into (1) carotenoids, (2) Anthocyanins (3) Betalains (4) Chlorophylls and (5) Miscellaneous (Britton, 1983).

1.3.1. Carotenoids

Among natural pigments, carotenoids are popular group consisting of about 300 natural compounds widely occurring in nature. For over 200 naturally occurring carotenoids the chemical structure have been elucidated (Baurenfield, 1975). They are responsible for yellow, orange and red pigments in a large variety of plants, animals, algae, fungi and bacteria.

Carotenoids are broadly divided into carotenes and xanthophylls.

Carotenoids occur in fat deposits as in solution form and in lipid media (or) combined with protein in the aqueous phase. Carotenoids are found in all plant parts

such on leaf, flower, bark, root and fruit, algae, fungi, bacteria, mammalian ovaries and muscles (Britton, 1983).

The role of carotenoids in biological system appears interesting.

1) Carotenoids take part in photo reception like vision in animals, 2) They harvest light during photosynthesis, 3) They offer protection against intense light or UV light 4) Carotenoids found involved in phototaxis 5) They stimulate sporangioophore formation in fungi and 6) They serve as integumental colour of fish, bird and marine invertebrates (Pattnaik *et al.*, 1997).

Carotenoid pigments are widely used in many spheres. In aquaculture, to increase the flesh colour of fish, carotenoids are mixed with fish feed. In food industry, to increase the egg yolk colour, *Spirulina* xanthophylls and Astaxanthin of *Phaffia rhodozyma* are mixed with poultry feed.

Paprika (*Capsicum annum*) is the oldest and most important natural carotenoid in food. Capsanthin and Capsorubrin are the main carotenoids in paprika (Kauli and Bavernfein, 1981).

Kauli and Bavernfein (1981) reported that saffron and its crocin, carotenoid is costliest pigment used to impart yellow colour. Tomato produces lycopene and β -carotene. Carrot produce more amount of α and β carotenes.

1.3.2. Anthocyanins

Anthocyanins are the water soluble pigments which are present in the sap of plant cells and highly responsible for the colour of the fruits, flowers and leaves (Timberlake and Bridle, 1975). Anthocyanins are widespread in plant kingdom and provide potential colours.

Anthocyanins are a part of flavonoid group of compounds and having flavylium nucleus. Among 4000 flavonoids, about 260 are anthocyanins (Francis, 1989).

All the fruits and flowers are rich in anthocyanin pigments. Shrikande (1983) described a process to extract pigments from plant sources.

Anthocyanins are legalized food colourants. An anthocyanin, called enocyanin from skins of wine grapes, red cabbage are good sources of food-grade pigments (Francis, 1989).

1.3.3. Betalains

Betalains are the water soluble colouring materials responsible for the red and yellow shades found in numerous varieties of beetroot and also found in Pokeberries (Spears, 1988) Betalains are classified into (1) the red pigment known as betacyanins and (2) the yellow ones known as betaxanthin (Mabry, 1966; Mabry and Dreiding 1968; Von Elbe, 1977). Unlike anthocyanins, they are less sensitive to changes in pH. But other variables like humidity, UV light, oxygen and temperature can be influencing factors (Von Elbe, 1977). Orange to red water soluble pigments found in beets, cactus fruits, bougainvillea flowers etc. have betalains (Hari *et al.*, 1994).

Several reports have illustrated the application of betalain pigments as colourants for food such as dairy products (Pasch *et al.*, 1975), confectionary products, meat products (Von Elbe and Maing, 1973 and Von Elbe *et al.*, 1974), poultry products, soft drinks and in dry mixes such as gelatin desserts (Driver and Francis, 1979).

1.3.4. Chlorophylls

Chlorophylls (mainly a and b) are widely distributed in nature as the pigments of green leaves. It is the universal natural pigment and acts as a catalyst in biochemical photosynthesis converting solar energy into food for plants. It is always accompanied by carotene and xanthophyll pigments (Coulson, 1980).

The heme pigments are found in meat and fish (Coulson and Humphrey, 1980). The oil soluble chlorophylls are not widely used as food colouring agents. The water soluble chlorophylls show good stability towards light and heat, and moderate stability to both acids and alkalies. So they are utilized in food preparations, canned products, confectionary, soups and dairy products (Coulson, 1980).

1.3.5. Miscellaneous

Apart from the major classes of pigments, there are several other naturally occurring pigments. Quinones (in lichens and fungi), xanthonenes (mangiferin in mango), curcuminoids (in turmeric), cochineal (in female cochineal insect *Coccus cacti*) and anhydro bases (in red sandal wood, bar wood and cam wood) are a few examples (Hari *et al.*, 1994).

1.3.5.1. Caramel

Caramel is the amorphous dark brown colouring material formed by heating food grade carbohydrates. It consists of volatile and non-volatile low molecular weight compounds. Caramel is mainly used in meat products, brewing, baking, confectionary, vinegar, pickle, soft drinks and spirit industries for both colour and flavour (Coulson, 1980).

1.3.5.2. Cochineal pigments (Pigment from insects)

Cochineal has been suggested as 'the best of all natural pigments' (Riboh, 1977). It has been used for many years in the form of extracts from the bodies of the coccid insects from the families *Coccidea* and *Aphidoidea*. A number of preparations are available under various names (Lloyd, 1980). Armenian red is obtained from the insect *Porphyrophera hameli*, Kermes is obtained from the old world insects *Kermes ilicis* or *Kermococcus vermilis* which grows on oak, particularly 'Kermis Oak'. Polish cochineal is obtained from *Margarodes polonicus* or *Porphyrophera ammonica* found on the roots of *Scleranthus perennis*. Lac is obtained from *Lacifera lacca* which is found on the trees of *Zizyphus mauretanica* and *Butea monosperma*. American cochineal obtained from *Dactylopus coccus* is found on the arial parts of *Opuntia* and *Nopalea*. There are 80,000 - 1,00,000 insects in a kilogram of raw, dried cochineal being exported from Central and South America.

Cochineal is extracted from the bodies of female insects just prior to egg-laying time, and the pigment may constitute as much as 22 per cent of their dry weight (Donkin, 1977).

Cochineal carmine can be used in powder form for colouring various instant foodstuffs. It can also be used in an alkaline solution in ammonia for colouring a wide range of foodstuffs. Usage of the colour is generally in the range of 0.04 to 0.2 per cent (Francis, 1987).

1.3.5.3. Alkannet

It is a related pigment extracted with alcohol from the roots of *Alkanna tinctoria* Tausch and *Anchusa tinctoria* Lom from Southern Europe. The red pigment is slightly soluble in water, but readily soluble in organic solvents. It has been used to colour confectionary, ice cream and wines (Coulson, 1980).

1.3.5.4. Turmeric (Curcumin)

Turmeric or curcuma is the fluorescent yellow coloured extract from the rhizome of various species of curcuma plant, *Curcuma longa* L. giving the best quality colouring matter. The colouring principle is curcumin (Govindarajan, 1981). Turmeric is insoluble in water, but soluble in alkali, alcohol and glacial acetic acid. The extracts have a strong characteristic odour and sharp taste and are utilized for both their taste and colour (Coulson, 1980). Stability of curcumin and oleoresins in the presence of light is low and stable in heat processing. Hence, they are utilized in canned products, soups, pickles, mustard, mixes and wrapped confectionary (Coulson, 1980).

1.3.6. Phycocyanin

The blue pigment phycocyanin can be extracted from cyanophyceae species by treating the biomass with a cold aqueous solution containing calcium ions. Phycocyanin is also obtained from *Spirulina*. The extract can be concentrated and used directly as a food colourant. Addition of antioxidants will increase its stability (Kawasaki and Kaneko, 1978).

1.3.7. Others

A number of colourants from unusual sources have been reported in the patent literature. For example, Iacobucci and Sweeney (1981) reported the isolation and properties of Rubrolone, a red pigment from *Streptomyces echinoruber*. Moll and Farr (1976) reported a series of red colourants by reacting *Monascus* with chitosan or monascorubin with the hydrogen chloride salts of glucosamine. Watanabe (1974) reported the isolation of a red pigment from *Penicillium purpurogenum*. The isolation of berberin, a isoquinoline - type pigment, was reported by a Japanese company,

Kakko (1980). Berberin is obtained from the bark of *Phellodendron amurense*, xylem of *Berberis thunbergii* and leaves of *Phellodendron amurense*. The yellow pigment, berberin is stable to heat and light.

1.3.7.1. Annato

Annato is a yellow carotenoid preparation obtained from the seeds of the plant, *Bixa orellana*. The pigment in annatto is a mixture of bixin, the mono methyl esters of a dicarboxylic carotenoids, norbixin also comes under this classification.

Annato extracts are sold in several physical forms, including dry powders, oil solutions and suspensions and alkaline aqueous solutions (Sampathu *et al.*, 1981). Annato has been used in foods, especially dairy products. It can be prepared as both water and oil soluble preparations.

1.3.7.2. Saffron

A dicarboxylic carotenoid, crocetin is found in saffron together with its crocin. Stigmas of the flowers of *Crocus sativus* yield saffron. Saffron provides both colour and flavour and is considered to be a gourmet item (Francis, 1987).

1.3.7.3. Carthamine

It is a yellow dye from the petals of safflower (*Carthamus tinctorius*), and known as a good source of yellow dye for food industry. CFTRI, Mysore optimized the production technology of carthamine (Sampathu *et al.*, 1981). It is stable in light, temperature and pH range normally encountered in food system.

1.4. MICROBIAL FOOD COLOURS

The search for naturally produced, stable and reliable colourants as substitutes or alternate to synthetic colourants has led to the recognition of colourants produced from microbial species. Microbes produce a variety of colourants such as chlorophyll and carotenoids as well as some unique pigments (Table 1).

The rapid growth of microbes cuts production time to a matter of days and the process lends itself to continuous operations (Taylor, 1984). Compared to plant or animal sources, the production is flexible and can easily be controlled.

1.4.1. FUNGAL PIGMENTS

Among the microbial sources of pigments, fungi have their unique advantage over the other organisms. Fungi can be scaled up in fermentor and its yield is comparatively high due to its faster growth rate. Extraction of pigments from fungi is easy whether it is intracellular or extracellular in nature.

Fungal pigments are broadly classified into two, 1) carotenoids and 2) other pigments.

1.4.2. Fungal carotenoids

Carotenoids are present in most of the organisms including fungi. In more recent years there has been an increasing demand for carotenoids from natural sources such as marine algae and fungi (Ninet and Renaut, 1997 and Lampila *et al.*, 1985). Detailed extraction procedure to extract carotenoids from fungi was reviewed by Britton (1985), Liaaen - Jensen and Andrewes (1985) and Taylor and Krinsky (1990).

Table 1. Pigments from microbes

Microorganism	Compound	Solubility	Colour	Use
Bacteria				
<i>Mycobacterium lacticola</i>	Astaxanthin	Fat	Orange	Fish feed
<i>Brevibacterium 103</i>	Canthaxanthin	Fat	Red	Dairy products
<i>Flavobacterium sp.</i>	Zeaxanthin	Fat	Orange	Poultry feed
Yeasts				
<i>Phaffia rhodozyma</i>	Astaxanthin	Fat	Orange	Fish feed
<i>Rhodotorula sp.</i>	β -Carotene	Fat	Orange	Poultry feed
<i>Sporobolomyces sp.</i>	γ -Carotene	Fat	Orange	Poultry feed
<i>Rhodospirillum sp.</i>	Torulene	Fat	Orange	Poultry feed
<i>Sporodiobolus sp.</i>	Torularhodin	Fat	Orange	Fish feed
Fungi				
<i>Monascus purpureus</i>	Polyketides	Water	Orange red, reddish purple	Food colourant
<i>Blakeslea trispora</i>	β -carotene	Fat	Yellow	Food colourant
<i>Gibberella fujikuroi</i>	Neurosporaxanthin	Water	Orange	---
<i>Phycomyces spp.</i>	δ -carotene, Lycopene	--	Orange	Food colourant
Algae				
<i>Dunaliella salina</i>	β -Carotene	Fat	Yellow	Food colourant
<i>Spirulina platense</i>	Phycobili protein	Fat	Bluish Green	Food
<i>Haematococcus pluvialis</i>	Astaxanthin	Fat	Orange	Fish feed

(Hari *et al.*, 1994)

Carotenoids have been detected in over 200 species of fungi, but examination of another 130 species has shown that they lack carotenoids and certain species produced carotenoids under light conditions (Goodwin, 1980). β and γ carotenes are abundantly present in fungi (Table 2).

1.4.3. Localization of fungal carotenoids

Carotenoids are found predominantly in lipid droplets in fungi, with only minor amounts associated with membranes (Ciegler *et al.*, 1959). In ascomycetes, synthesis and storage of carotenoid occur in different cell components, as the sphaerosomes function mainly for the storage of carotenoids (Anderson *et al.*, 1958).

1.4.4. Polyketide pigments

Polyketide pigments are produced by many fungal species. Among them, *Monascus* spp. have been well utilized in food industries.

1.4.4.1. *Monascus* spp.

The genus, *Monascus* produces several natural pigments, including primary colours red and yellow and the secondary colour, orange. These colours are widely used in Asia as food colourants (Han, 1990; Anonymous, 1999). *Monascus* pigments are produced commercially in China, Japan and Taiwan, primarily by a traditional method of food fermentation known as the Koji process. Mudgett (2000) extensively reviewed the organism and its applications.

1.4.4.1.1. Historical

History of *Monascus* dates back to two thousand years, hence it is called "Millenium Fungus". Far Eastern countries like, China, Thailand, Taiwan, Japan

Table 2 . Source of fungal pigments

Fungi	Carotenoid	References
Phycomyces	β -carotene	Alasodura and Visser, 1972.
<i>Blastocladales</i>	γ -carotene	
Ascomycetes	β and γ carotenes and apocarotenoids	
<i>Neurospora crassa</i>	Torulene	Riley and Bramley, 1982
	Neurosporoxanthin	Valadon <i>et al.</i> , 1980
<i>Ustilago sp</i>	Xanthophylls	Garber <i>et al.</i> , 1975
	β , γ carotenes	
<i>Poxizales</i>	phillipsiaxanthin, aleuriaxanthin, plechaniaxanthin	Haxo, 1950.
Hymenomycetes	Keto carotenoids	Alasodura and Visser, 1972
<i>Cantharellus</i>	Canthaxanthin	Riley and Bramley, 1982
<i>Cantharellus spp</i>	Neurosporene, lycopene	
<i>Sphaerobdus stellatus</i>	Echinenone	Feofilova and Pivovarova, 1976.
Deuteromycetes	Carotenoids, apocerolerooids	
<i>Phaffia rhodozyma</i>	Astaxanthin	Aragon <i>et al.</i> , 1976.
<i>Rhodotorula</i>	Rhodoxanthin	Feofilova and Pivovarova; 1976

consumed rice based foods fermented by *Monascus* spp. The name of the product differs based on the country, region, product preparations and usage.

In the literature, the first account of *Monascus* products appeared in 1818 (Hall, 1818). He referred on the fermented 'tofu' in Okinawa islands, Japan. The first scientific account on *Monascus* fungi was made by Went (Went, 1895). He described the fungi from 'Ang-Khak' on Java. Uyeda (1901) explored the flora of 'Beni-Koji' and he found that the fungus was *M. purpureus*, Oliver (1905) gave an account of the morphology of *Monascus*. The use of *Monascus* on soybean fermentation products was reviewed by Wai (1929, 1964). From the Western nations, the first systematic study on *Monascus* was done by Hasseltine (1965). Su and Huang (1977) reviewed *Monascus* in Taiwanese fermented foods. Wang and Hasseltine (1982) gave an account on oriental fermented foods including *Monascus* fermented foods. After 1970's the fungus, *Monascus*, its pigment production and medicinal values attracted the scientists.

1.4.4.1.2. Taxonomic considerations

Fungi are members of the division Thallophyta. They do not possess chlorophyll, nor are they differentiated into leaves, stem or true roots and they are widely distributed in nature, especially in the soil (Prescott and Dunn, 1959).

The genus, *Monascus*, a member of the class Ascomycetes, is descended from the Eumycetes, or true molds, the subclass Plectomycetes and the order Plactascales. The genus has also been considered as a member of the Fungi Imperfecti. *Monascus* spp. produce septate mycelia and produce sexual spores exogenously in sacs.

Van Tiegrhem (1884) first isolated *Monascus* spp.. Ainsworth *et al.* (1973) reported that the Monascaceae, one of the lower Ascomycetes consists of a single genus, *Monascus*. Hawksworth and Pitt (1983) reviewed the genus and recognized three spp. viz., *Monascus purpureus*, *M. pilosus* and *M. ruber*.

Earlier, the sexual cycle of this genus was critically viewed by different authors (Went, 1895; Barker 1903; Ikeno, 1903; Dangeard, 1907). Finally, Young (1931) proposed an interpretative that has virtually unchallenged. Carels and Shepherd (1975) studied sexual reproduction of *Monascus* in submerged culture and confirmed Young's findings.

1.4.4.1.3. *Monascus* Pigments

Monascus pigments receive increasing interest in the Western world as they are used as food colourants. It was not until early 1960's systematic studies of *Monascus* were undertaken. Later work helped in the isolation and characterisation of the pigments, 'monascin' and 'monascorubin' from *Monascus purpureus* Went (Fielding *et al.*, 1960, 1961; Haws and Holker, 1961 and Kumasaki *et al.*, 1962). The structure of six water-insoluble *Monascus* pigments are shown in Figure. (Lin and Demain, 1994). The physical and chemical properties of these compounds are also presented in Table 3.

Based on the colour, the pigments were classified into 1. Red pigments – Rubropunctamine ($C_{21} H_{23} NO_4$) and Monascorubramine ($C_{23} H_{27} NO_4$) 2. Yellow pigments – Monascin ($C_{21} H_{26} O_5$) and Ankaflavin ($C_{23} H_{30} O_5$), 3. Orange pigments – Rubrapunctatin ($C_{21} H_{22} O_5$) and Monascorubin ($C_{23} H_{26} O_5$) (Sweeny *et al.*, 1981).

Table 3. Selected pigments of *Monascus* spp. and their characteristics

Pigment	Molecular weight	Melting point (°C)	Absorption wavelength (λ nm)	Solvent system	References
Monascin	358	142-145	232, 238, 392	Ethanol	Chen <i>et al.</i> , 1971
			225, 288, 385	Methanol	Enouye <i>et al.</i> , 1962
			231, 287, 390	Ether	Fielding <i>et al.</i> , 1961
Ankaflavin	386	120-121	212, 228, 382	Dioxane	Marchand and Whalley, 1973
Rubrapunctatin	354	156-157	218, 246, 460	Ether	Haws <i>et al.</i> , 1959
Monascorubrin	382	141-143	286, 416, 470,	Ethanol	Hadfield <i>et al.</i> , 1967
			470, 500, 556,	Methanol	Kumasaki <i>et al.</i> , 1962
			246, 278, 410	Cyclohexane	Fielding <i>et al.</i> , 1960
Rubropunctamine	335	217-218	252, 305, 426	Ether	Haws <i>et al.</i> , 1959
Monascorubramine	381	207-208	303, 370, 480	Ethanol	Hadfield <i>et al.</i> , 1967

(Han, 1990)

Su and Huang (1980) reported that these *Monascus* pigments were readily soluble in ethanol and only slightly soluble in water. But monascorubrin from *Monascus purpureus* Went is soluble in ether, methanol, ethanol, benzene, chloroform, acetic acid, acetone and insoluble in water and petroleum ether (Kumasaki *et al.*, 1962). The ethanol solution of this compound becomes red in alkali and yellow in acid. Su and Huang (1980) showed that the pigment assumed different colours due to change in pH, reddish – orange at pH 3-4, red at pH 5-6 and purplish red at pH 7-9 with an absorption maximum 618-700nm. Little information is available regarding the stability of pigments. *Monascus anka* pigments faded in solution during prolonged exposure to strong light, like the carotenes, and were more photostable in 70 per cent ethanol than in water. The pigment was heat stable in 70 per cent ethanol upto 1000°C and was stable under natural and alkaline conditions.

Monascus pigments readily react with compounds containing aminogroups via a ring opening with a Schiff rearrangement to form water soluble compounds (Francis, 1987).

To increase solubility in water, pyronoid oxygen in the side chain of capryl (C₇) and caproyl (C₅) are replaced by nitrogen by adding glutamic acid in the growth medium. Blanc *et al.* (1994) and Hajjaj *et al.* (1997) found that monosodium glutamate served as a source of nitrogen that increased water soluble compounds.

1.4.4.1.4. Extraction and Analysis of Pigments

Hajjaj *et al.* (1997) reported that the n-butanol extraction stabilized the pigments against day light for several months. Watanabe *et al.* (1997) developed a capillary electrophoretic method to analyse pigments as food additives. Sato *et al.*

(1997) found eight major pigments in *Monascus* sp. Hajjaj *et al.* (1998) also developed sampling and extraction methods to obtain pigments. Teng and Feldhelm (1998) recovered purplish pigments from *M. anka*. Martinkova *et al.* (1999) isolated 8 pigments and characterized them using HPLC and NMR techniques.

1.4.4.1.5. Biosynthesis of Pigments

Biosynthesis of fungal secondary metabolites such as *Monascus* pigments, may be grouped in several metabolic classes according to the pathway from which they originate (Turner, 1971). Polyketides are formed by the condensation of one molecule of acetyl-CoA with at least three molecules of malonyl-CoA.

1.4.4.1.6. Factors influencing pigment production

Lin and Iizuka (1982) used rice meal, wheat meal, wheat bran, bread meal, lean meal and a number of other substrates for pigment production. Others (Toda *et al.*, 1982) have also studied the growth process to optimise the type and amount of pigments.

1.4.4.1.7. Physical factors

Although this fungus is amenable for commercial solid state fermentation, little information is available on the major factors influencing pigment production. Organism varies in its need for tolerance to oxygen. High oxygen partial pressure is toxic to *Monascus* spp. due to either inhibition of essential enzymes, production of radicals and reactive compounds hydrogen peroxide, superoxide, or hydroxyl radicals or a shifting of the redox equilibrium inside the cell and subsequent disturbing of the biosynthesis reactions (Onken and Liefke, 1989). For *Monascus* pigment production, one of three inoculation methods is generally used. These include spore suspensions

(Lin,1973), mycelial suspensions (Lin and Suen, 1973; Yoshimura *et al.*, 1975; Bau and Wong, 1979; Broder and Koehler, 1980; Su and Huang, 1980; Wong *et al.*, 1981 and Wong and Koehler, 1981a, 1981b) and mycelial homogenates (Carels and Shepherd, 1977,1978). The effect of inoculum source, size and preparation method on growth and pigment formation are not well documented.

The optimum temperatures for pigment production were reported at 30°C (Su and Huang, 1980), 35°C (Wong and Bau, 1977; Bau and Wong, 1979; Broder and Koehler,1980; Wong and Koehler, 1981 a,b and Wong *et al.*, 1981), 33°C (Miyake *et al.*, 1982), 32°C (Lin and Suen, 1973; Yoshimura *et al.*, 1975; Su and Huang, 1980 and Riken Vitamin Co., Ltd, 1982), 28°C (Broder and Koehler, 1980) and 25°C (Carels and Shepherd, 1977; 1978 and 1979).

Maximum pigment yields were generally obtained at the initial pH of inoculum at 5.5 to 6.5.

Evans and Wang (1984) demonstrated that by providing solid support for the mycelium as in immobilised cultures pigment production would be improved. Han (1990) optimized O₂ and CO₂ requirements for pigment production at 0.12 to 0.20 atm partial pressures of CO₂. The dissolved oxygen level would increase and might result in higher growth and pigmentation . The ratio of biomass to pigment yield decreased significantly as oxygen levels were increased or as carbon dioxide levels were decreased in the gas atmosphere (Ohantaek and Mudgett, 1992). Mak *et al.* (1990) used rotating vessel in the fermentative production of pigments using roller bottles, which reduced time for pigment production and 10 fold increase in yield due to better gas exchange, higher medium pH, efficient pigment secretion, solid support for mycelium and retarded conidiation.

Agitation rates significantly affect fungal morphology in submerged culture. Mycelia grow in pulp, pellet and intermediate forms (Takahashi *et al.*, 1965; Yoshimura *et al.*, 1975) that depended on media composition. Mycelia may grow into homogenous and filamentous suspensions ("pulp type"), globose colonies ("pellet type") or intermediate forms between pulp and pellet type (Takahashi *et al.*, 1965). Yoshimura *et al.* (1975) investigated relationships between morphology and pigment formation by a *Monascus* spp. At 300 rpm, mycelia were produced in the pulp form with low pigment formation. At 600-800 rpm, mycelial growth and pigment production were high and mycelial forms were intermediate between pulp and pellet forms. At 1200 rpm, pellets were formed and pigment production decreased significantly.

Porous resin spheres (Nagasawa *et al.*, 1987) and a polymeric resin (Evans and Wang, 1984) were used to absorb pigments. Adding a sterilized nonionic adsorbent resin directly to a growing submerged culture did not enhance pigment production. However, when resins were used to create an immobilized *Monascus* culture (Evans and Wang, 1984), pigment production was much higher, suggesting that mycelial support might offer an explanation for enhanced pigment production as in solid substrate fermentation.

Pigment production can also be negatively affected by the accumulation of pigments and by acidic pH. The two factors are inter-twining, since *Monascus* pigments are acid insoluble, and acidic pH inhibits pigment secretion. High pH was also associated with the chemical conversion of the orange pigment to the red pigment (Nakanishi *et al.*, 1959; Fielding *et al.*, 1961).

1.4.4.1.8. Chemical Factors

Pigment production is often influenced by different carbon and nitrogen sources. Lilly and Barnett (1962) reported that growth rate of *Monascus purpureus* on fructose, glucose and sucrose increased in that order. McHan and Johnson (1970) showed that *Monascus purpureus* grew better in glucose-peptone-yeast extract broth than in any other complex medium. Zinc and a special combination of glycine, leucine and tryptophan yielded more growth (Johnson and McHan, 1975). McHan and Johnson (1979) also studied the effects of zinc on the utilization of nitrogen sources in which growth studies indicated that ammonium compounds were preferred over inorganic nitrogen sources and nitrites were better than nitrates. Among several aminoacids, glycine, leucine, valine, aspartate, glutamate and urea were superior to ammonium salts. Wong *et al.* (1981) investigated the effect of various glucose and ammonium nitrate levels. Recently, Lin and Demain (1991) used *Monascus* strain TTWMB 6042 to investigate the regulation of pigment production on nutrient composition. Chen and Johns (1993) carried similar studies based on HPLC analysis. Further, Lin and Demain (1995) found that media with ammonium nitrate gave low level extracellular pigment and high level of intracellular pigment.

Recent studies have also shown that *Monascus purpureus* DSM 1379 oxidized fatty acids to methyl ketones (Peters *et al.*, 1993) and influenced the order of monosaccharide metabolism in two phase growth cycle. Although it is known that *Monascus* strains produce ethanol in both solid substrate and submerged culture fermentation (Johnson and McHan, 1975; Endo, 1985; Matsumoto *et al.*, 1987; Han and Mudgett, 1991), little information is available on nutritional effects on production of ethanol by *Monascus* and its role in pigment synthesis. Juzlova *et al* (1994) showed that pigment synthesis at 2 per cent ethanol by *Monascus purpureus* CCM 1852 was much higher than on maltose.

Table 4. Pigments yield from *Monascus* through submerged and SSF (1973 - 1990)

Fungal strains	Pigment yield ^a	Productivity ^b	References
A. Submerged fermentation			
<i>Monascus anka</i>	5	55	Lin and Iizuka, 1982
<i>Monascus</i> sp. No.2 AJ 7744	36	215	Yoshimura <i>et al.</i> , 1975
<i>Monascus</i> sp. 2	24	250	Lin, 1973
<i>Monascus</i> sp. NRRL 1993	60	389	Evans and Wang, 1984
<i>M. kaoliang</i> S-11	92	1282	Lin and Suen, 1973
<i>M. anka</i> V-204	156	1080	Su and Huang, 1980
<i>M. purpureus</i> ATCC 16365	14	282	Han 1990
B. Solid state fermentation			
<i>M. anka</i> Namazawa et sato	174	1040	Lin and Iizuka, 1982
<i>M. anka</i>	240	-	Lin, 1973
<i>M. anka</i>	1690	8800	Lin and Iizuka, 1982
<i>M. anka</i> UN 202-13'	666	3470	Hiroi <i>et al.</i> , 1979
<i>M. kaoliang</i> R-1087	5433	37730	Lin and Iizuka, 1982
<i>M. purpureus</i> ATTC-16365	6016	41780	Han, 1990

^a OD at 500 nm expressed as units ml⁻¹

^b OD at 500 nm expressed as units l⁻¹ h⁻¹ or units kg⁻¹ dry fermented solids h⁻¹

1.4.4.1.9. Genetic improvement of *Monascus*

The yield of pigment can be improved by genetic manipulation of the organisms. Mutation appears the simple tool to get genetically improved strains. Lin and Suen (1973) produced mutants in *Monascus* by using UV irradiation and NTG mutagenesis and the mutants produced higher quantities of pigments in submerged culture. Wong and Koehler (1981) developed a mutant, *Monascus purpureus* G.1 through NTG mutagenesis. Lin and Iizuka (1982) developed a hyper pigment producing mutant, R-10847 from *M. kaoliang* - F2 through a series of NTG mutagenesis and UV irradiation. Youngsmith *et al.* (1994) developed a yellow pigment producing mutant of *M. kaoling*.

1.4.4.1.10. Antimicrobial activity

Wang and Hesseltine (1979) commented on the absence of mycotoxin in the *Monascus* fermented foods. Parenthetically, *Monascus* is not reported to cause any disease in human by Dubos and Hirsch (1965). The antibacterial activity of *M. purpureus* was first reported by Wong and Bau (1977). Wild and mutants of *M. purpureus* were active against *Bacillus*, *Streptococcus* and *Pseudomonas*.

Wong and Koehler (1981) suggested that addition of acetate to the medium enhanced transformation of yellow pigments to red or orange pigment and inhibited antibiotic compounds. Nozaki *et al.* (1991) studied secondary metabolites of *M. anka*, ankalactone, which they reported as bacteriostatic. Similarly Blanc *et al.* (1995a) have reported the presence of monascidin A in submerged cultures of *M. purpureus* and *M. anka* which they identified as citrinin. Blanc *et al.* (1995 b) also found that addition of cerulenine and ethionine as specific inhibitors of citrinin and pigment synthesis. Martinkova *et al.* (1995) found antibiotic activity in *M. purpureus* extracts containing

the orange pigments against various bacteria, fungi and yeast. Blanc *et al.* (1995b) investigated the production of citrinin using various media and culture conditions. Pastrana *et al.* (1996) found a metabolic pattern in which citrinin was produced during the lag phase of growth. Sabet-vilar *et al.* (1999) estimated that the level of citrinin from 0.2 to 17.1 $\mu\text{g g}^{-1}$ of commercial products. Hajjaj *et al.* (1999) explored the biosynthetic pathway of citrinin production in *M. ruber* using ^{13}C Nuclear Magnetic Resonance. Hajjaj *et al.* (2000 a) also explored that fatty acids in the medium affected the citrinin production in *M. ruber*. Organic acids accumulation during the culture decreased red pigment production and increase citrinin accumulation (Hajjaj *et al.*, 2000 b). Even though citrinin was investigated in *Monascus* fermented foods, there was hardly any report related to the toxicity as of yet. These foods are consumed past 2000 years by people in the Far East.

Methyl ketones are important flavour constituents of blue cheese (Morgan and Anderson, 1956). Kranz *et al.* (1992) reported that the free fatty acids were converted into methyl ketones by *M. purpureus*, which are responsible for the flavour. The influence of carbohydrate dissimilation on the fatty acid metabolism leading to methyl ketones synthesis has been reported in *M. purpureus* (Peters *et al.*, 1993).

1.4.4.1.11. Productivity of *Monascus* pigment

Monascus pigment are produced both by submerged and solid state fermentation. Both methods are followed based on their own merits and demerits. The East follows the traditional solid state fermentation on rice, wheat, sorghum, maize, cassava etc. while the West follows the submerged fermentation because of their deep commitment to fermentors and sophisticated control devices. Though many elaborate studies have been made on submerged fermentation the yields of pigments have been much lower than in solid state fermentation. The following table illustrates this.

1.4.4.1.12. Food Applications of *Monascus* spp.

Monascus pigments have been used in the oriental foods since thousand years. The traditional foods prepared using *Monascus* are red Chinese rice (Ang-khak), red soybean cheese, kaoliang brandy, red wine and red rice wine (Lin and Iizuka, 1982). The traditional techniques for making red rice were recorded by Li Shizhen in a monograph of Chinese medicine and in Song Yinxing's "Tian Aong Kai Wu". Steinkraus (1995) published the preparation of red rice in Taiwan. *Monascus* has been used to prepare wine in China (Xu and Bau 1999). In food-related products *Monascus* pigments are used as a substitute for nitrate and nitrites as colorants for sausages and other cured meat products, with obvious health implications (Cheng and Ockerman, 1998). Other applications including the production of α -glucosidase, malic acid and *Tofu* were extensively studied by Yasuda *et al.* (1993, 1995a, 1996). Fabre *et al.* (1993) listed some uses of red pigment in meat products.

Many patents are available in Japan and USA to utilize *Monascus* pigments in food industries. Sea foods are mixed with *Ang-kak* red rice in Philippines (Steinkraus, 1995). In Germany, batches of sausages prepared with 1000-4000 ppm of *M. purpureus* extracts compared with 0-72 ppm of nitrite salts (Fink-Gremmels *et al.*, 1991).

1.4.4.1.12. Medicinal applications of *Monascus* spp.

The reports citing *Monascus* pigments being used on herbal medicines and dietary supplements are numerous, as seen from internet websites through *Google* and other search engines. These include the suppression of tumour promotion, regulation of immunoglobulin of tumour promotion, lowering of serum lipids in hyper lipidemia and reduction of aminocetaphen induced liver toxicity by antioxidase action.

Table 5. Recommended dosages for use of red rice powders as colourants of various food products

Food Product	Quantity (per cent w/w)
Sausages, Ham	0.005 - 0.025
Ice cream	0.02 - 0.10
Tomato paste	0.5 - 2.0
Curry	0.005
Fruit juices	0.002 - 0.005
Meat	0.10 - 0.20
Sauce	0.02 - 0.10
Jam	0.02 - 3.00
Sweet	0.001 - 0.005
Wine	0.2 - 1.0

(Anonymous, 1999 c)

Cholestin or *Monacolin K*, obtained from red rice imported from China as a nutraceutical, contained a natural inhibitor of the rate-limiting step in cholesterol synthesis mediated by HMG-CoA (3-hydroxy, 3-methyl, glutaryl coenzyme) reductase (Hardness, 1998). Heber *et al.* (1999) conducted detailed research on the Monacolin-K activity from red rice on human beings and concluded that effect of red rice had cholesterol lowering effect.

Oral administration of *Monascus* pigments suppressed tumor promotion in rice and rat (Yasukawa *et al.*, 1994, 1996). *Monascus* pigments have also been shown to inhibit the immunoglobulin, IgG or IgM production at concentration as low as 1 mM, suggesting that natural pigments may have a role in regulating immunoglobulin activity (Kuramoto *et al.*, 1996).

1.4.4.1. 13. Nontraditional products from *Monascus* spp.

Monascus anka IFO 4478 produced coenzyme Q₁₀ in the solid state (Dainippon Ink and Chemicals Inc., 1982a), *Monascus purpureus* M-023 also produced coenzyme Q₁₀ in submerged culture (Dainippon Ink and chemicals Inc. 1982b). A nuclease inhibitor was also obtained from *M. purpureus* Went 908 in bran culture (Saruno *et al.*, 1980). *M. anka* was also found to produce substances that flocculated *Saccharomyces cerevesiae* (Nakamura *et al.*, 1976). More recently, an albino mutants of *M. araneosus* did not produce any pigments, but did produce L-malic acid at 28 g l⁻¹ (Lumyong and Tomita, 1993). A ribonuclease was obtained from *Monascus* spp. NO.3403 and purified (Yasuda *et al.*, 1995b). Eighty volatile compounds were identified from samples of rice fermented by *M. purpureus* and in cultivation media following fermentation (Patakova-Juzlova *et al.*, 1998).

1.4.4.1.14. Present and Future trends of *Monascus* pigments

The attitude of regulatory authorities towards microbiologically produced colourants is impossible to anticipate, but no doubt, the strains would have to be proved 'pathogen free' and free of any toxic component. The precedent established by FDA which limits the production of enzymes for food use to just three organisms viz. *Aspergillus niger*, *A. oryzae* and *Bacillus subtilis*, might count against *Monascus* produced pigment (Coulson, 1980). Only pigments of *M. purpureus* are authorized for food use in Japan (Blanc *et al.*, 1994). In Germany, the *M. purpureus* pigments are advised to use as dietary supplement. Investigation of the practical use of *Monascus* as a source of food colourant is popular in Taiwan and Japan. More than 60 patents are available on *Monascus* in the United States of America and this shows the trend of commercialization of the pigments.)

1.4.4.2. *Blakeslea trispora*

The filamentous fungus, *Blakeslea trispora*, is regarded as an industrial carotene source, containing β -carotene and precursors of its synthesis, phytoene, phytofluene, lycopene and γ -carotene (Mehta and Cerda - Olmeda, 1995).

Accumulation of β -carotene in *Blakeslea* is strongly linked to their sexual interaction. Mating of the two sexual forms results in a spectacular increase in mycelial β -carotene (Ciegler, 1965 and Lampila *et al.*, 1985). Mixed cultures of strains of opposite sex usually contain 5-15 times more pigment than separate cultures. Trisporic acids, substances with hormonal activity formed upon mating have been shown to mediate the stimulation of β -catotene biosynthesis (Lampila *et al.*, 1985).

In *Blakeslea trispora*, the cyclisation of lycopene to β -carotene can be prevented by changing the growth condition or by adding inhibitors to the medium. (Ceigler, 1965). Mutation of this fungus is difficult because all the stages of the fungus are multinucleate. Mutants produced 3-6 times more carotene than the wild type but have defective sporulation (Mehta and Cerda-olmeda, 1995).

In the original process of Ceigler, about 1 g of β -carotene l^{-1} (17-20 mg g^{-1} dry mycelium) was produced (Ceigler *et al.*, 1959). Ninet and Renaut (1979) subsequently improved the yield to 3 - 3.5 l^{-1} by the addition of carotenogenic chemicals and antioxidants. Despite these higher production, this β -carotene price is rather high as compared to the synthetic β -carotene (Ceigler, 1965 and Ninet and Renaut, 1979).

In the Netherlands, β -carotene project started based on *Blakeslea trispora* is underway. The product is recovered and purified from the biomass by high-pressure extraction, yielding crystals of β -carotene with a purity of more than 96 per cent.

1.4.5. FOOD PIGMENTS FROM YEAST

Yeast has been used by mankind for the manufacture of beverages and leavened products for many thousands of years. Yeasts can also be used to generate colouring substances for use in food systems. These colours are predominantly carotenoid pigments. *Phaffia*, *Rhodotorula*, *Cryptococcus* and *Sporobolomyces* are species of yeast used industrially.

1.4.5.1. *Phaffia rhodozyma*

This is a red astaxanthin (3, 3'-dihydroxy β - β -carotene 4, 4'-diene) producing yeast. It was first isolated during 1970 by Herman Phaff and his associates from

exudates of deciduous trees in Japan, Alaska and Russia. Phaff *et al.*, (1972) proposed the name *Rhodozyma montanae* which was later changed to *Phaffia* in honour of many years of research in yeast biology by Dr. Herman Phaff.

Phaffia yeast produces astaxanthin, as its major pigment (85%) (Andrews *et al.*, 1976; Haard, 1988 and Johnson *et al.*, 1991). Interest towards natural pigments is gaining momentum in aquaculture (Johnson *et al.*, 1977 and Johnson *et al.*, 1980). This could become an attractive alternative to the synthetic carotenoids (Ravinder *et al.*, 1992).

The genus, *Phaffia* is characterized by the synthesis of carotenoid pigments, production of cell surface - associated amyloid pigments, a coenzyme Q10 system, and the ability to ferment sugars. They have ability to ferment sugars like D-glucose, maltose, sucrose, cellobiose, trehalose, raffinose, soluble starch (latent or negative), ethanol, mannitol, lactate, succinate and glycerol. They do not utilize galactose, lactose, glucosamine, arabinose and do not utilize nitrate but hydrolyse urea. It grows from 0 to 27°C.

Andrews *et al.* (1976) determined the configuration of yeast astaxanthin and suggested that the *cis* isomer represented a genuine biosynthetic product of yeast and not an artifact of isolation. Wild strains produced 500 µg g⁻¹ of dry weight of yeast, of which 40 to 95 per cent is astaxanthin. Pigmentation of lobsters and eggs of laying hens was also observed in laboratory (Johnson *et al.*, 1980).

Due to high cost of growth media, commercialization of *Phaffia rhodozyma* is still in its infancy (Nelis and De Leenheer 1991). Extraction of pigments from cells is

also a bottle neck. The growing market for astaxanthin, together with the trends towards using natural sources of feed nutrients, make the production of astaxanthin by *P. rhodozyma* attractive (Meyer and Du Preez, 1993).

1.4.5.2. *Rhodotorula* spp.

Rhodotorula, is an oleagenous yeast belongs to the group of carotenoid forming imperfect yeasts. β -carotene is an example of a carotenoid found in *Rhodotorula* showing commercial importance as precursor of vitamin-A and yellow pigment for foods (Costa *et al.*, 1984).

Rhodotorula also finds use as protein rich feed in aquaculture. The major pigments produced by *Rhodotorula* are torulene and torulahordin (Simpson *et al.*, 1964).

In *R. rubra* carotenoid synthesis occurs in three main phases (Goodwin, 1959) (1) a period of active synthesis leading to maximal concentration and (2) a period of persistence during which the concentration stays relatively constant during which the pigments gradually disappear from the yeast. Carotenoid biosynthesis in the yeast *R. rubra* was shown to occur mainly after growth had stopped (Goodwin, 1971).

Rhodotorula rubra has been reported for its ability to utilize glycerol, galactase, maltose, sucrose etc. but failed to utilize complex carbohydrates like 2-keto gluconate and methyl glucoside (Hari *et al.*, 1992).

The high biomass concentration and pigment yield were obtained at a pH of 5.5 (Martin *et al.*, 1993).

According to Goodwin (1980) *Rhodotorula* spp. can be cultivated at a wide range of temperatures. Simpson *et al.* (1964) have reported that *Rhodotorula* accumulated carotenes at low temperatures and torulahordine at 25°C.

Costa *et al.* (1987) suggested that the uncoloured or slightly coloured cells were transferred and agitated for 24 hours in distilled water to produce orange yellow colour and the concentration of pigment increased by 4.2 to 4.8 times.

Extraction of pigments from yeast cells was hindered by the rigid cell wall and due to its glucan or polysaccharide constituents (Phaff *et al.*, 1966). Cell disruption by French press method is not a viable solution for commercialization (Okagbue and Lewis, 1985). Martin *et al.* (1993) found a new method by which the cell wall could be disrupted by using organic solvent dimethyl sulfoxide.

R. rubra, because of its versatility with respect to nutrient requirements, may be grown at commercial scale on industrial wastes (Hari *et al.*, 1992). β -carotene found in such yeasts has attracted commercial interest as a precursor of vitamin A and as a yellow pigment for foods (Nelis and De-Leenheer, 1991 and Johnson *et al.*, 1977). Also, cultured shellfish and salmonids gained red colouration and better price (Okagbue and Lewis, 1984 a and Hari *et al.*, 1992).

1.4.6. FOOD COLOURS FROM ALGAE

Micro algal biotechnology is rapidly moving from laboratory R & D and pilot plant operations to commercial reality. The major development in micro algal biotechnology was the discovery during the late 1970s of the *Dunaliella* strains which accumulated upto 10 per cent of its dry weight as β -carotene.

Apart from yielding anti-cancer drugs it is used for food colouring in margarine and also as a vitamin supplement. Algal products have the advantage of being "all natural".

1.4.6.1. *Dunaliella* spp.

This unicellular bi-flagellated green alga belongs to the class Chlorophyta and occurs in marine environments (Schlipalius, 1989). *Dunaliella* spp. are of interest for the production of protein-enriched biomass and speciality chemicals such as beta-carotene.

Ben-Amotz (1986) extensively reviewed the culture production and biosynthesis of *Dunaliella* spp. *Dunaliella* requires an arid area with high proportion of sunny, cloudless days conducive for its growth and β -carotene production. In highly saline waters, it dominates over other algae and produces high concentration of β -carotene (Schlipalius, 1989).

After the removal of the aqueous phase, the biomass is treated with sodium or potassium carbonate or bicarbonate to dissolve the protein-bound pigment (Francis, 1987).

Tanaka (1990) reported the applications of *Dunaliella* in many fields. *D. salina* finds its use in pigmentation of marron (Sommer *et al.*, 1990). So far three finished products have been developed from *D. salina* (1) four per cent soluble β -carotene in soybean oil, (2) two per cent spray dried β -carotene for use in animal feeds, (3) thirty per cent crystalline β -carotene in soybean oil for use as a food colorant (Curtain *et al.*, 1987).

1.4.6.2. *Haematococcus* sp.

The alga, *Haematococcus* sp. grows in potable water and accumulates astaxanthin to a level of 0.2 to 2 per cent of dry weight. *Haematococcus* produces astaxanthin, which varies significantly during its life cycle. Astaxanthin is formed during encystment of algae and accumulates in the cytoplasm (Czygan and Kessler, 1967; Lang, 1968).

The pigment extract can be concentrated and used directly as a food colorant. The pigment is soluble in either water or alcohol, which can be used in chewing gum, frozen confectionaries and ice creams (Francis, 1987). Due to higher antioxidant properties, it can be applied as a food colorant and also as a medicine (Tjahjono *et al.*, 1994).

1.4.7. FOOD COLOURS FROM BACTERIA

Bacteria also serve as excellent source of biocolours. Basically bacteria are concerned with the production of carotenoids. β -carotene, lutein and/or other xanthophylls are present in cyanobacteria and photosynthetic bacteria.

1.4.7.1. *Brevibacterium* KY - 4313

This bacterium was originally isolated from a petroleum field in Japan. It is a Gram positive, strict aerobic organism exhibiting a rod-coccus growth cycle. Many of

the cells arranged at an angle to give 'V' shape. It contains canthaxanthin together with smaller amounts of echinenone and β -carotene (Sakurai *et al.*, 1971).

Brevibacterium is primarily found in dairy products. It contributes to the aroma and colour of surface ripened cheese (Clancy *et al.*, 1993).

1.4.7.2. *Rhodococcus maris*

Rhodococcus is a Gram positive, aerobic organism having an oxidative type of metabolism. Koronelli *et al.* (1987) reported the other name of this organism as *Mycobacterium brevicale*. The pigment was extracted and used for food applications. In addition to the enormous economic potentials of microbial pigments, their gradual accepted application in foods and feeds and the current strong interest in "natural" pigments have opened the flood gates of microbial biotechnology for better exploitation of these microorganisms.

1.5. Toxicology of Food Colours

The colour is associated with flavour, texture, nutritive value and thus, the wholesomeness of the food. Unfortunately, because of this added colour can also deceive by implying richness, flavour, or maturity in foods where these traits do not exist. Due to widespread practice of adulteration of food stuffs which goes unchecked, it is of interest to know the toxicological aspects of these colours and regulations governing their use.

1.5.1. Toxicity and biocolours

The most important aspect of food colour usage is its toxicity. Microbial food colours were not allowed in the Western countries due to their suspected toxicity. But *Monascus* pigments were allowed as a food colour in Japan.

1.5.2. Natural Colours

All the substances, natural or synthetic can be injurious to life when administered in large quantities. Although most permitted natural colours have been present in human diets from time immemorial, the specification of commercial products may differ substantially from that found in food (Table 6).

1.5.2.1. Carotenoid pigments

Hyper carotenaemia is not thought harmful, and does not produce hyper vitaminosis on ingesting β - carotene over prolonged periods (Drake, 1975). Formal toxicological testing has not been conducted on the natural dietary constituents α -and γ -carotene or on capsanthin.

The only xanthophylls to be examined individually from a toxicological stand point is canthaxanthin, which has been isolated from certain edible mushroom, but can also be prepared synthetically . It produced no untoward effects when fed to rats at dietary levels up to 5 per cent for 2 years and to dogs at upto 15 per cent for 15 weeks (Drake, 1975).

1.5.2.2. *Monascus* pigments

Some synthetic colorants have been implicated as carcinogenic and teratogenic. This has invited interest in the natural pigments of *Monascus*. It is interesting to note that the toxicity studies on *Monascus* pigments have yielded no adverse effects. Acute oral and intraperitoneal toxicity on mice showed LD₅₀ values of 33.3g and 8.7g/kg body weight respectively (Kaio *et al.*, 1978) Commercial preparative of *Monascus* pigment had increased effects on fetal rat hepatocyte enzymes important in detoxification processes (Sako *et al.*, 1983). These results need to be confirmed (according to European Union legal procedures) before use as food additives. (Blanc *et al.*, 1994).

Table 6. Toxicological status of certain natural pigments

Common Name	Typical uses	Estimated ADI (mg kg ⁻¹ body wt)	Toxicological studies required
β-carotene	General	5	Adequate
α and γ carotene	General	5	Adequate
Annato	Bulter, Margarine	1.25	Adequate
Capsanthin	Soups, sauces	Not possible	Adequate
Chlorophylls	Soups, sauces	Nil	Adequate
Anthocyanin	Wine	Grape skin specification	Adequate
Cochineal red	Yoghurt	Not specified	Further studies required
Curcumin	Food products meat preparations	2.5	Acceptable
<i>Monascus</i>	Red rice, wine, as spice	-	Under study

Santhanakrishnan, 1981

Effects of *Monascus pilosus* extracts on blood pressure of rats were experimented by Tsuji *et al* (1992). It has been shown that preparation suppressed rises in blood pressure.

Samples of a methanol extract of rice fermented by *M. purpureus* were used in genotoxicity tests and it was concluded that *M. purpureus* did not show significant genotoxicity (John, 1991). Fink-Gremmels *et al.* (1991 b) also tested cytotoxicity and genotoxicity of *Monascus* extract. Ames test done on the extracts showed mutagenic effects. The observed genotoxicity and mutagenity level of *Monascus* is much lower than those of nitroso compounds formed in cured meat.

Mycelial extracts from *Monascus* were shown to exhibit antibiotic (Wong and Bau 1977; Wong and Koehler, 1981; Nozaki *et al.*, 1991 and Martinkova *et al.*, 1995) embryotoxic and teratogenic effects (Martinkova *et al.*, 1995, 1999) Sabater vilar *et al.* (1999) conducted hepatocyte test and Ames mutagenity test of *Monascus* extracts. She found that low level of response to mutagenesis and concluded that *Monascus* extracts failed to induce mutagenicity, neither with nor without metabolic activation by sa-mix. No adverse effects have been reported as of yet, which might be attributable to the low concentrations applied in food technology. However contamination of *Monascus* products with citrinin should be avoided as citrinin can exert mutagenic effects after metabolic activation.

1.5.2.2.1. Citrinin toxicity of *Monascus*

The occurrence of antibacterial activities among crude pigments has been shown by several authors (Wong and Koehler, 1981; Lee *et al.*, 1995 and Martinkova *et al.*, 1995). In 1995, Blanc *et al.* isolated and identified monascidin A out of various species of *Monascus*. Mass spectroscopy analysis indicated that it is identical in structure to citrinin (Blanc *et al.*, 1995 and Pastrana *et al.*, 1995).

Citrinin (IUPAC (3R, 4S) -4,6-dihydro-8- hydroxy 3,4,5-trimethyl-6-oxo-3H-2 benzopyran-7-carboxylic acid) has been identified and characterized as a potential causative agent in human endemica Balkan nephropathy from *Aspergillus* and *Penicillium* (Frank, 1992). Despite a common biosynthetic pathway for the red pigment and citrinin, their production is differently affected by nutritional and environmental factors. In conditions of high oxygen supply, citrinin production was favoured and moreover, a linear relationship was observed between the oxygen supply and the citrinin yield (Hajjaj *et al.*, 1999). Hajjaj *et al.* (1999, 2000) elucidated the biosynthetic pathway of citrinin. Sabater-Vilar *et al.* (1999) conducted embryotoxicity test and hepatocytes test and concluded that low levels of citrinin in food application did not affect human beings.

1.5.3. Other natural pigments and their toxicity

In recent times, a rare congenital error of metabolism involving 'phytol intolerance' has been identified, but this cannot be regarded as significant from the view point of the general populations. When chlorophyllin copper complex (0.25 per cent ionic Cu) was fed to rats for their entire life span at dietary levels of upto 3 per cent, no untowards effects were noted. Mokady *et al.* (1989) assessed the safety of alga *Dunaliella bardawil* for food use and evaluated in a multigeneration study with rats. Imada *et al.* (1983) found that the Neopurpuratin, a purplish red pigment from a mixed cultures of *Streptomyces propurpuraties* and *Bacillus* sp. No 751 were found to be safe in biological tests.

Hence, before accommodating any new food colours from natural sources, toxicity analysis should be carried out to avoid future problems.

1.6. Regulation of Natural food colours

Consumer consciousness towards many natural products leads many of the researchers to explore new and viable alternatives to the synthetics. All natural colours are not suitable for consumption for human beings. Many of them are certified as unsuitable for consumption. The use of colouring in food in the European Commission is controlled by EC Directive and in United States of America by the Colour Additives Amendments of 1960 to the Food Drug and Cosmetic Act of 1938 . In our country, under the Prevention of Food Adulteration Act 1954, some 8 food colours were permitted and today 3 more added. Simultaneously, specifications were laid by ISI for all the permitted synthetic or natural food colours and their preparations. Thus there are 24 for Indian Standards, 11 for Synthetic food colours, 10 for natural foods colours are effected (Santhanakrishnan, 1981)

1.6.1. Toxicological Aspects

Food Additive Toxicology began to emerge as a scientific discipline in the early 1960s and the following toxicological information on colours was sought by the Joint Expert Committee on Food Additives in 1963. Acute toxicity studies, short-term and long term toxicity studies (in at least two species), carcinogenicity studies (in two species) and metabolic studies should be conducted before an approval of any synthetic or natural colours. Recently, teratological and mutagenic tests are also required. With a view to stream line the costly and time consuming testing programme now required for food additives, the US Food safety council has advised to follow their procedure strictly.

Public concern over the use of food additives and colours in particular may be due to a question of need and of safety. Some major International authorities would

need to permit the new source of colour whether it is synthetic or natural. They are U.K. Food Additives and Contaminants Committee (FACC), EEC Scientific Committee for Food (SCF), the USA Food and Drug Administration (FDA), the FAO / WHO. Joint Expert Committee on Food Additives (JECFA) and The Bureau of Indian Standards (BIS).

In the case of microbial food colours, the only permitted colour is from *Monascus* in Japan. However in United States, FDA opposed the use of *Monascus* pigments as a food colour. The Administrative Court of Baden, Wuer Hermberg (VGH) has argued that the evaluation on *Monascus* as an additive *i.e.* *Monascus*, red fermented rice, is a characteristic ingredient of food. Its overwhelming usage for colouring purposes does not destroy its nature as food. Aside the colouring effects, medicinal benefits enhanced the consumption of *Monascus* in the Western world too.)

Materials and Methods

Chapter III

MATERIALS AND METHODS

3.1. General

3.1.1. Cleaning of glassware

The glassware of 'Borosil' and 'Corning' qualities were used in all the experiments. They were cleaned by soaking in chromic acid solution (100 g Potassium dichromate dissolved in 1 lit water with 500 ml of conc. sulfuric acid) for two hours and washing in tap water.

3.1.2. Chemicals used in this study

All the chemicals used in this study were of high purity Analar, BDH, Qualigens, Hi Media and E Merck specifications. Whenever necessary, 'Sigma' grade chemicals were also used.

3.1.3. Sterilization techniques

All glassware were sterilized in a hot air oven at 180°C for three hours or autoclaved at 1 atm for 15 min according to the requirement. All growth media, broth and water blanks were steam sterilized in an autoclave at 1 atm pressure for 15 min.

Vitamin solutions and growth factors were filter sterilized using sintered glass filter or membrane filter. Isolation, purification, inoculation and other microbiological procedures were carried out in the Laminar flow chamber (Airflow, India).

3.1.4. Sources of authentic cultures of *Monascus* spp.

Two authentic isolates of *Monascus* spp. viz., *Monascus purpureus* # 3139 and *Monascus ruber* # 4958 were obtained from the culture collections of Indian Type

Culture Collection, Division of Plant Pathology, Indian Agricultural Research Institute, New Delhi.

3.2.1. Collection of soil samples

Soil samples for the isolation of pigment producing fungi were collected across the Western Ghats, North Indian Plains, rhizosphere samples of selected crops and compost yard soils of Tamil Nadu Agricultural University, Coimbatore. Soil samples were collected in polyethylene bags and stored at 5°C in a refrigerator until use.

3.2.2. Isolation of pigment producing fungi from soil samples (Warcup, 1950)

As pointed as above, the soil samples were used for the isolation of pigment producing fungi. Two grams of soil sample was taken for serial dilution upto 10^{-4} using sterile water blanks. After thorough shaking, one ml aliquot from the 10^{-4} dilution was drawn and pour plated in Potato Dextrose Agar Medium (PDA) (Appendix - I) amended with 50 ppm of streptomycin to control bacteria. The plates were incubated at 35°C in an incubator for five days.

Colonies of fungi which produced extracellular pigments that changed the colour of the medium were observed. Fungi displaying intense attractive bright colours were selected and transferred to fresh PDA medium.

3.2.3. Purification of pigment producing fungal isolates

Promising fungal isolates which produced attractive colours were purified by single hyphal tip method.

3.2.3.1. Single hyphal tip method of purification (Mundkur, 1959)

Plain agar medium containing 2 per cent agar-agar in tap water, after sterilization was poured into a sterile petri plate. After solidification, carefully lifted

peripheral mycelial bits were inoculated. After incubation for two days at 35°C, the colonies were observed for hyphal developments. Peripheral tip of the mycelial growth was taken from the plates and reinoculated to PDA medium. After proper documentation and coding, these purified colonies were transferred to PDA slants for further study.

3.2.4. Rapid screening of fungi for pigment production

A screening procedure that employed growth (biomass production) and pigment production (optical density units) was adopted.

3.2.4.1. Preparation of inoculum of fungal isolates

A basal mineral salt medium, 'Czapek- Dox' medium (Appendix - I) with 20 g of glucose per litre with 15 g l⁻¹ of agar and pH adjusted to 5.5 was dispensed and autoclaved at 1 atm pressure for 15 min.. About 20 ml of this medium was poured to petri dish. The purified fungal isolates were inoculated and incubated for 5 days at 35°C and then transferred to a fresh dish carrying this medium for another 5 days. Mycelial discs, 0.6 cm dia were cut from the margin of the mycelial mat with the of a flame sterilized cork-borer and used as inoculum.

3.2.4.2. Biomass production of the fungal isolates

One hundred ml of Czapek-Dox mineral medium was dispensed in 250 ml Erlenmeyer flasks and sterilized. After cooling, the flasks were inoculated with a 0.6 cm fungal mycelial disc and incubated at 35°C for seven days. At the end, the mycelia were filtered, washed twice with distilled water over a preweighed Whatman filter paper circle. The filter paper with fresh mycelium was dried in hot air oven at 80°C for four hours and weighed.

3.2.4.3. Extracellular pigment production of fungal isolates (Lin and Iizuka, 1982)

From the experimental set up, the mycelial mat served to determine biomass production and the culture filtrate was used for the quantification of pigments. Twenty ml of culture filtrate was taken in a centrifuge tube and spun down at 5000 x g for 10 min to remove debris. One ml of the clear culture filtrate was diluted with 9 ml of distilled water. The absorbance of the solution at 300 nm and 500 nm was read in a Beckman DU 64 Spectrophotometer. The absorbance values were converted to colour units.

3.2.4.4. Intracellular pigments from fungal isolates

From the above experimental set up, a portion of the mycelial mat was used for the biomass estimation. One gram of fresh mycelial mat was taken in a test tube. Five ml of 90 per cent hot ethanol was added and extracted for 30 min over a boiling water bath. The mycelium was well ground in a clean pestle with a mortar with a pinch of acid washed sand. The slurry was centrifuged for 10 min at 3000 x g. The clear supernatant was read in a Beckman DU 64 spectrophotometer at 300 nm and 500 nm respectively. The absorbance values were converted to colour units.

3.2.4.5. pH shifts in the culture broth during pigment production

From the above experimental set up, 20 ml of the culture medium was withdrawn and the pH was read in a pH meter (Elico, Hyderabad).

3.2.5. Selection of promising pigment producing fungal isolates

After thorough screening of pigment producing isolates, promising isolates were selected based on biomass production and amount of extra and intracellular pigments.

3.3. Taxonomic identification of pigment producing fungi

Among the pigment producing fungal isolates, PF1, PF13 and PF16 were obtained from soils. These isolates were identified.

3.3.1. Moist chamber technique (Cappucino and Sherman, 1996)

The isolates of fungi were grown on glass slides placed in petri dish moist chambers. The petri plates were provided with adequate humidity. PDA medium was pour plated in a sterile petri plate as a thin film. The medium was sectorized into squares (0.5cm x 0.5cm) using sterile scalpel. Each agar block was transferred to the moist chamber aseptically. The four sides of the agar piece were inoculated with the fungus. A clean cover slip was placed on the agar block. The assembly was kept for incubation for five days at 30°C and the characters of each fungi were observed under a light microscope. The coverslip was carefully removed with fungal mycelium and a drop of lactophenol cotton blue stain (Jensen, 1931) was added and this coverslip was placed on a new clean glass slide. The agar block was carefully removed and a drop of lactophenol cotton blue was added on the mycelial growth on the glass slide. A new clean cover slip was placed over the glass slide without airlock. These slides were examined under microscope and the different features of the fungi were noted. Manuals carrying the description of fungi were consulted for identification (Barnett, 1958; Dennis, 1968; Ainsworth, 1973; Gilman, 1975 and Cannon, 1985).

3.3.2. Growth characters

The selected five fungi were grown on different media, like Potato Dextrose agar, Czapek- Dox agar, Mycological agar, Yeast Morphology agar, Sabouraud agar, Martin's Rose Bengal agar, Malt extract agar and *Monascus* fermentation medium (Appendix- I). Sterile media were poured into petri dishes. After solidification, 0.6 cm

diameter mycelial disc of the selected fungi were introduced into the media. The plates were incubated for seven days at 35°C. The plates were examined for growth and pigmentation on different media.

3.4. Characterization of pigment producing fungal isolates

The following three cultures viz., *Penicillium purpurogenum*, *Verticillium* sp. and *Humicola* sp. were selected for further studies. Besides these isolates the standard cultures, *Monascus purpureus* #3139 and *M. ruber* # 4958 were also used. Factors which might influence pigment production like carbon and nitrogen sources, temperature, pH of the medium, aeration and light were studied.

3.4.1. Effects of different carbon sources on pigment production

The effect of different carbon sources on the growth and pigment production of the fungi was studied. In the Czapek-Dox mineral medium, various sources of carbon were added at one per cent level. The carbon sources included glucose, fructose, xylose, sucrose, maltose, galactose, lactose, arabinose, glycerol, sorbitol, mannitol, starch, and ethyl alcohol. One hundred ml of the medium was dispensed in 250 ml Erlenmeyer flasks and steam sterilized. pH was adjusted to 5.5 before sterilization. Mycelial disc of 0.6 cm diameter was inoculated aseptically.

The flasks were incubated at 35°C for seven days under static conditions. During their growth, pigment excretion was also observed. After seven days of incubation the following parameters were estimated.

3.4.1.1. Biomass yield

The mycelial growth was separated by filtration, dried at 105°C for 3 hrs. and estimated as fungal biomass.



3.4.1.2. Pigment excretion

The effect of different 'C' sources on initiation of pigment excretion was recorded by visual observation.

3.4.1.3. Extracellular pigments

The influence of different 'C' sources on pigment production was studied. The pigment was extracted from the culture filtrate and quantified in units as detailed earlier (3.2.4.3.)

3.4.1.4. Intracellular pigments

The effect of various 'C' sources on the intracellular pigment content of the fungi was studied by following the method described earlier (3.2.4.4.).

3.4.1.5. Changes in pH

The changes in the pH of the culture medium in the course of fungal growth and pigment production were determined using a pH meter.

3.4.2. Effect of different nitrogen sources on the pigment production

The effect of different nitrogen sources on the growth and pigment production of the selected fungi were studied. The nitrogen source in Czapek - Dox medium was replaced with various organic and inorganic nitrogen sources at 0.3 per cent and 0.1 per cent levels respectively. The inorganic nitrogen sources were: ammonium chloride (NH_4Cl), sodium nitrate (NaNO_3), diammonium hydrogen phosphate ($(\text{NH}_4)_2\text{HPO}_4$), potassium nitrate (KNO_3), ammonium ferrous sulphate ($(\text{NH}_4)_3\text{SO}_4 \cdot \text{FeSO}_4 \cdot 6\text{H}_2\text{O}$), ammonium nitrate (NH_4NO_3), ammonium sulphate ($(\text{NH}_4)_2\text{SO}_4$) and ammonia (NH_3).

Organic nitrogen sources were: yeast extract, glycine, gelatin, peptone, urea and mono sodium glutamate. One hundred ml of culture medium amended with different nitrogen sources was taken in 250 ml Erlenmeyer flasks and steam sterilized. pH of the medium was adjusted to 5.5. Mycelial discs of 0.6 cm dia were inoculated and the flasks were incubated at 35°C for seven days under static condition. Biomass yield, pigment production time, intra and extracellular pigment production and change in pH shift of the medium were observed. Required number of replications was maintained with appropriate controls.

3.4.3. Effect of temperature on pigment production

Czapek - Dox broth with one per cent glucose as carbon source was prepared. The pH of the medium was adjusted to 5.5. One hundred ml of the medium was dispensed in 250 ml Erlenmeyer flasks and steam sterilized. The flasks were inoculated with 0.6 cm dia of mycelial discs of the selected fungi and incubated at different temperatures, 15°C, 35°C and 45°C for seven days under static condition. Adequate number of replications were maintained. After seven days, the flasks were withdrawn and the parameters were studied as in the earlier experiments.

3.4.4. Effect of pH on pigment production

Czapek - Dox broth with one per cent glucose was prepared and the pH was adjusted to 4.0, 5.0, 6.0, 7.0, 8.0 and 9.0 by adding calculated quantities of 0.5 N HCl or 0.5 N KOH. One hundred ml of medium was dispensed in 250 ml Erlenmeyer flasks and steam sterilized. The broth was inoculated with 0.6 cm dia mycelial disc of selected fungi. The flasks were incubated for seven days at 35°C. Required replications were maintained. After the incubation period, all the parameters listed in earlier experiments were observed.

3.4.5. Effect of aeration / agitation of culture on pigment production

Czapek - Dox medium was prepared with one per cent glucose as carbon source. One hundred ml of the broth was dispensed in 250 ml Erlenmeyer flasks, the pH adjusted to 5.5 and autoclaved at 1 atm for 30 min. The flasks were inoculated with 0.6 cm dia mycelial discs of selected fungi. They were kept over a shaker whose frequency of shaking was adjusted to 100, 150, 200 and 250 strokes per min. After seven days of continuous shaking, all the parameters were observed.

3.4.6. Effect of light on pigment production

The influence of light on the growth and pigment production of the selected fungi was investigated. One hundred ml of Czapek - Dox medium with one per cent glucose was taken in 250 ml conical flask and sterilized at 1 atm for 15 min.. The flasks were inoculated with 0.6 cm dia mycelial discs of selected fungi. Each set of flasks was incubated under different wave length of light like red (640-740 nm), yellow (570-600 nm), green (520-570 nm), blue (450-520 nm), darkness and unscreened white light (350-740 nm) for seven days. The flasks were drawn and the parameters like biomass production, pigment production time, intracellular and extracellular pigments and pH shift in the medium were studied by following the methods described earlier. Required number of replications was maintained.

3.5. Pigment production through fermentation

The selected fungi viz., *Monascus purpureus* *M. ruber*, and the isolates *Penicillium purpurogenum*, *Verticillium* sp. and *Humicola* sp. were grown under solid state fermentation and liquid fermentation conditions. The aim was to standardise optimum conditions for maximum pigment production.

3.5.1. Solid state Fermentation

For solid state fermentation, the following substrates were used:

- | | |
|------------------|----------------------|
| 1. Rice grain | 5. Rice bran |
| 2. Wheat grain | 6. Wheat bran |
| 3. Sorghum grain | 7. Pulses waste |
| 4. Maize grain | 8. Sugarcane bagasse |

A quantity of 50g of cereal grains was taken in a 250 ml conical flask and soaked for 10 hrs in water. After draining the excess water, flasks were steam sterilized. Two 0.6 cm dia discs of selected fungal mat were inoculated to the flask. The content of the flask was stirred with sterile scalpel. After incubation for 15 days, at room temperature (28° C) the content was dried at 40° C in an incubator. After thorough drying, the material was ground to fine powder in a porcelain pestle and mortar. The fine powder was used to estimate the pigment production and fungal biomass.

3.5.1.1. Quantification of pigment production

One gram of the powdered solid state fermented product was taken in a test tube and 10 ml of 70 per cent ethyl alcohol was added and the colour was extracted by boiling over a water bath for 10 min. The content was centrifuged at 5000 x g for 10 min. The clear supernatant was read in a spectrophotometer at 300 nm and 500 nm with appropriate control. The absorbance values were converted to pigment units.

3.5.2. Liquid fermentation

The selected five fungi were grown in Potato Dextrose Agar plates. After sufficient growth and sporulation, 0.6 cm dia mycelial discs were cut out and

inoculated into 100 ml of Czapek - Dox broth in 250 ml Erlenmeyer flasks. The flasks were placed over a wrist action shaking machine (Orbitek, India) set at 150 strokes per min. This served as inoculum for 2 litre Czapek - Dox broth taken in a 5 lit Haffkin's flask. The flasks were incubated at room temperature (27°C) for 10 days, under static conditions.

The mycelial biomass was filtered through cheesecloth and the clear filtrate was saved. The mycelial mat was used for the extraction of intracellular pigments. Yield of pigments, mycelial biomass and changes in pH of the broth were observed.

3. 6. Mass production of fungal pigments

The fungal pigments were mass produced following the techniques like solid state fermentation and liquid fermentations. To get higher yields and to reduce the cost of production, some modifications were introduced in the procedure.

3.6.1. Low cost polybag fermentors for pigment production

Both solid state and liquid fermentations were performed in polypropylene bags. Polypropylene bags (32 x 16 cm) were used for this study. PVC rings of 2 cm dia and 3 cm length were used as bottle neck and plugged with cotton wool. The polybags were filled with the fermentation medium and was sterilized in an autoclave at 1 atm pressure for 30 min.)

3.6.2. Comparison of low cost media for pigment production

Potato Dextrose broth, Czapek-Dox broth and Rice kanji broth were used in polybags for pigment production.

Rice kanji obtained during rice cooking is available in plenty in hotels, hostels and hospitals. Usually it is drained-off as a waste. As rice kanji is rich in soluble carbohydrates it can substitute the conventional formulation media.

Rice kanji was diluted with water and supplemented with 0.1 per cent glucose/sucrose and micronutrient solution (0.1%). The pH of the medium was adjusted to 6.0. The whole polybag assembly with medium was sterilized in an autoclave.

One ml of the spore suspension of the fungus was inoculated and mixed well in polybags. The bags were placed over tabletop in such a position that it offered maximum surface area and also the top portion with air interface for good growth. The bags were shaken after 3 days to mix the mycelia evenly. After 15 days, the content of the bag was collected and pigments harvested.

3.6.3. Solid state fermentation in polybags

Polybags were also used for solid state fermentation. Different form of rice varieties were studied for their suitability to produce pigments. The selected forms of rice were, 1) parboiled rice, 2) raw rice, 3) broken rice, 4) "Cheeraga Samba" rice and 5) Basmathi rice. For preparation and solid-state fermentation, the method suggested by Steinkraus (1995) was followed. Rice was soaked in water for 12 hrs. After decanting the excess water, it was steam sterilized in polybags. The fungal inoculum was added and the bags were incubated for fifteen days at room temperature (28°C). Samples from each treatment were analysed for pigment yield. The fermented rice was air dried at 40°C in an incubator and made into powder using a blender.

3.6.4. Large scale pigment production using rice substrate

Based on the above comparative studies, parboiled rice was used for mass production in polybags following the method of Steinkraus (1995). After harvest, the rice was air dried at 40°C, powdered and stored in polybags.

3.7. Genetic improvement of pigment producing fungi

Mutation / Mutagenesis appears to be a tangible method for developing strains with improved traits. For enhancing pigment production, this strategy could be applied. It was hoped to develop mutants of fungi showing greater efficiency in pigment production.

3.7.1. Collection of spores

The wild strains of fungi were grown on PDA medium for 5 - 7 days at 30°C in petri dishes. Spores were collected by flooding the plates with sterile distilled water and gentle ransacking with sterile inoculation needle. The spore suspension was gathered in Eppendorf tubes containing one ml of sterile saline water (0.89 per cent sodium chloride). After 5 min, 0.1 ml of one per cent Triton-X was added to reduce the surface tension of spores to facilitate settling. The Eppendorf tubes were centrifuged for 10 min at 5000x g and the supernatant was discarded. Spores were washed twice with sterile saline to get rid off any inhibitor on the spores. The spores were finally suspended in sterile distilled water.

3.7.2. Mutagen (N-methyl, N nitro, n- nitrosoguanidine)

NTG was used as the mutagen. Calculated amount of NTG was added to worm water to prepare stock solution carrying 10000 $\mu\text{g ml}^{-1}$ of NTG. Calculated quantity of mutagen was transferred to get final concentration of 200 $\mu\text{g ml}^{-1}$.

3.7.3. Spore germination and competency for mutagenesis

Harvested spores were transferred to 100 ml sterile distilled water and further diluted to obtain 10^8 to 10^9 spores per ml. The population of spores was calculated by pour plate technique using PDA medium. The spores in the flask were allowed to germinate by keeping over a shaker for 3 hours (100 strokes per min).

3.7.4. Mutagenesis (Clowes and Hayes, 1968)

A quantity of 4 ml of the spore suspension was added to 95 ml sterile distilled water in a 250 ml conical flask. Calculated quantities of NTG were added to make up the volume to 100 ml. The flasks were incubated under room temperature (28°C) for one hour with light wabbling (75 - 100 strokes per min). After incubation, 100 ml of prechilled ($5 - 6^{\circ}\text{C}$) sterile distilled water was added to the flask to arrest the action of mutagen.

3.7.5. Mutant cloning

One ml aliquot was pipetted out from the reaction mixture and diluted sufficiently. One ml of the diluted sample was plated on PDA medium. The plates were incubated for 4 days and observed for the growth of colonies.

Besides spore dilution and plating, one ml of aliquot of the spore suspension was aseptically pipetted out into 100 ml of PDA broth and kept over a shaker for 120 hrs to permit as much recombination as possible to take place. After incubation, one ml of aliquot was diluted suitably in sterile water blanks and plated in PDA medium. The plates were incubated at 28°C for 4-5 days. From the developing fungal clones the mutants were scored.

3.7.6. Scoring of mutants

The clones were observed for the appearance of changes in mutants were observed (i) growth (slow or fast), (ii) pigmentation (pigment intensity) and (iii) morphological characters (mycelial colour, colony morphology and sporulation). The mutants were spotted and colonies were restreaked on PDA medium. Discarding the revertants and leaky mutants the stable ones were streaked continuously on PDA medium for 6 to 7 successive generations to stabilise characters.

3.7.7. Characterization of mutants

The mutants were grown in PDA medium for 5 - 7 days at 35°C. After good growth and sporulation, the mutants and the wild strains were inoculated into 100 ml of Czapek - Dox broth in 250 ml flasks. The flasks were incubated for 7 days at 35°C under static condition. The flasks were drawn after incubation and the parameters like biomass production, pigment initiation time, intracellular and extracellular pigments and changes in pH of the medium were estimated by following the methods already described.

3.8. Pigment extraction and characterization

Pigments were extracted following two methods.

- 1) Extraction using solvents
- 2) Pigment recovery by sundrying /air drying.

3.8.1. Extraction with solvents

The pigments of fungi were extracted by solvents of polar and non-polar nature. As many as 20 different solvents were used and they included : 1) methyl alcohol 2) ethyl alcohol 3) acetone, 4) ethyl acetate, 5) ethyl acetoacetate, 6) diethyl

ether, 7) petroleum ether 8) benzene, 9) acetonitrile, 10) n-butanol 11) propan-2-ol, 12) n-propanol, 13) chloroform, 14) cyclohexane, 15) n-heptane, 16) isobutyl alcohol, 17) hexane, 18) lactic acid, 19) carbon tetra chloride 20) acetic acid, 21) nitrobenzene and 22) water.

The pigments were initially immobilised onto activated silicagel (100 - 600 μ , E-Merck). A quantity of 0.1 gm of adsorbed silicagel was transferred to a boiling tube and 10 ml of each solvents was added separately. The test tubes were incubated for 30 min with intermittent shaking. The solvent that extracted the maximum quantity of pigment was selected.

In a 2000 ml capacity separating funnel, 500 ml of the broth was taken and 500 ml of the solvent was added. The mixture was thoroughly shaken and left aside for 12 hrs. Ensuring complete extraction, the solvent phase was collected and air dried to one third of the original volume. The pigment was freeze dried in the lyophilizer (Christ Alpha 401) and preserved.

3.8.2. Air drying process

A quantity of 500 ml of the broth having the pigment was taken in a clean enamel tray and placed under sunlight in a dust free chamber. The trays were covered with a thin muslin cloth to avoid dust contamination. After 8 hrs of drying, the volume of the broth was reduced to one third.

The condensed broth was lyophilised using the Lyophilizer (Christ Alpha 401 Lyophilizer). The powdered pigment was stored at 10°C.

3.8.3. Characterization of the pigments

Chemical and physical characteristics of the pigments were studied (Lee and Chen, 1998).

3.8.3.1. Colour content

A quantity of 0.1 g of pigment was dissolved in 70 per cent methanol and read at 500 nm using spectrophotometer.

3.8.3.2. pH stability

A quantity of 0.1 g pigment was taken in a test tube and 10 ml of distilled water was added. The mixture was subjected to pH change in the range of 3, 5, 7 and 9. Visible solubility of the pigment was noted.

3.8.3.3. Hygroscopy

A quantity of 0.1 g of pigment was taken in a clean glass dish and added with distilled water dropwise to know the hygroscopic nature.

3.8.3.4. Sunlight exposure

A quantity of 0.1 g of pigment was taken in a clean dish and kept under sunlight for 4 hrs during noon (11.00 a.m. to 3.00 p.m). After exposure the pigment was dissolved in 70 per cent methanol. As a control unexposed 0.1 g of pigment mixed in 10 ml of 70 per cent methanol was taken. Both were read at 500 nm in a spectrophotometer.

3.8.3. 5. Exposure to fluorescent light

A quantity of 0.1g pigment taken in a clean dish was kept under a fluorescent tube light (58 W) 30 cm away. After 4 hrs exposure, the pigment in the dish was

extracted in 10 ml of 70 per cent methanol. Unexposed pigment was taken as control. The samples were read in spectrophotometer at 500 nm and compared for pigment destruction.

3.8.3.6. UV light exposure

As described earlier, the pigment was exposed to 4 hrs to UV irradiation (2200°A; Philips, Holland). The pigment destruction if any was observed using a control.

3.8.3.8. Temperature stability

A quantity of 0.1 g of pigment was taken in a series of test tubes and 10 ml of distilled water was added. The test tubes were placed over a hot water bath for 20 min at different temperatures like, 70°C, 100°C and 120°C. For 120°C temperature, the test tubes were kept in an autoclave at 1 atm pressure for 15 min. Unexposed control was also maintained. The content of the tubes was read at 500 nm in spectrophotometer. Temperature stability was determined.

3.8. 3. 8. Stability of pigment to dry heat

A quantity of pigment was taken in a clean petri dish and treated in a microwave oven to make heat emission (1FB Microwave Oven, Hyderabad, India) for one minute to know the dry heat stability of pigments.

3.8.3.9. Preservatives treatments

To know the pigment stability in the presence of food preservatives, 0.1 g of pigment was taken in 10 ml of distilled water and a quantity of 10 mg of food preservatives like citric acid, sodium benzoate and potassium meta bisulphite (KMS)

was added. The test tubes were kept in a water bath set at 80°C for one hour. Untreated pigment in water was also kept over the water bath. They were compared for the pigment stability in different preservatives by reading at 500 nm in spectrophotometer.

3.8.3.10. Absorbance of pigments

A quantity of 10 ml of pigment fermented broth was taken in a centrifuge tube and spun down at 10000 x g for 15 min.. Clear supernatant was collected and diluted adequately with distilled water. Similarly, solid state fermented fungi with solid substrates were ground with 90 per cent ethyl alcohol. The slurry was spun down at 10000xg for 15 min. Clear supernatant was collected and adequately diluted with distilled water.

The samples were scanned for their maximum absorbance at different wavelengths in a Varian Cary Scan - 50 (USA) Spectrophotometer from 200 nm to 700 nm. The maximum absorbance at different wavelengths was recorded.

3.8. 3. Isolation and purification of pigments (Blanc *et al.*, 1994).

Mycelium of the fungi harvested from liquid fermentation was lyophilized and the pigment obtained from the broth were collected. Crude pigments were extracted from the lyophilized powder with chloroform : methanol (50:50 v/v) until exhaustion (total colorant extract). This extract was made into minimum volume of 10-20 ml. The pigments were resolved into different fractions by Thin Layer Chromatography (TLC).

3.8.4.1. Thin layer chromatography of the pigments (Blanc *et al.*, 1994)

TLC plates (30 x 15 cm glass plates) were coated with silicagel - G (200 mesh, E-Merck) to 1 mm thickness using a spreader. After air drying, the plates were

activated by exposure at 100°C for 15 min in a hot air oven. A quantity of 200 µl of the crude pigment was spotted. The plates were developed in a solvent system of chloroform: methanol: acetic acid (65:24:4 v/v). Individual spots on the TLC plates were marked and Rf values calculated. Each spot was collected from the plates and stored in a clean glass vial.

3.8.4.2. High Performance Liquid Chromatography (HPLC) (Blanc *et al.*, 1994)

The free and complexed pigments thus isolated were further purified using HPLC. The bands recovered from the plates were used to re extract pigments in 10 ml of methanol. The extract was centrifuged and the supernatant was collected and used for the study.

Free and complex pigments were analysed by using HPLC on a C₁₈ column by using the following linear separation solvents of 80:20 (methyl alcohol : water v/v). The detector used was Varian Pro Star UV Detector at 480 nm. Retention time of the pigments was 20 min. A quantity of 0.5 µl of the sample was injected for the experiment. A flow rate of 0.8 ml min⁻¹ was maintained.

3. 9. Toxicological study of fungal pigments

The pigments extracted from the fungi were examined for their toxicity if any. Second instar larvae of silkworm (*Bombyx mori*) were selected for the study as silkworms are highly sensitive even to lower concentrations of toxins in the feed.

The experiment was conducted at the Laboratories of Sericulture, Dept. of Sericulture, TNAU, Coimbatore.

One hundred number of second instar larvae of silkworm were taken in a clean, formalin sprayed bamboo basket and labelled. A quantity of 100 g of clean, tender and chopped leaves of mulberry was taken in a clear tray. One gram of pigment from fungi was dissolved in minimum quantity of water and thoroughly mixed with the feed. The leaves were allowed to air dry. After starvation for 3 hrs, the larvae were allowed to feed the treated leaves. Untreated leaves were also fed to another set of larvae which served as control. Observations were made after 24 hrs of feeding on (i) the worm movement (ii) feeding rate, (iii) symptoms of ill-health, (iv) abnormal excretion and (v) death of worms.

3.10. Developing food products using biocolours

Biocolourants from *Monascus ruber* and *M. purpureus* have been recommended for food colouring at present in other countries. The biocolour of these fungi obtained on rice grain was used in the preparation of a few food recipes. The products developed were, Halwa, Plum squash and Cake.

3.10.1. Wheat Halwa

A delicious sweet product that requires additional colouring. Halwa is one of the common sweet preparations in India.

Recipe

<i>Monascus</i> pigment	-	15 g (Rice based)
Maida flour	-	1 kg (flour of wheat)
Sugar	-	1.5 kg
Water	-	500 ml
Ghee	-	500 g
Dried cashew nuts and raisins	-	50 g
Cardamom powder	-	5 g

Kitchenware and a LPG gas stove.

Procedure

A quantity of one kg of maida flour (wheat flour) was mixed with 500 ml water and made into dough. The dough was immersed in water for 30 min. The starch solution was recovered. Again water was added and starch solution was recovered until the starch in the dough was almost removed. The collected solution was kept for settling and the clear solution was drained off. Starch that settled at bottom ('Wheat milk') was taken in a frying pan over medium flame. A quantity of 1.5 kg of sugar was added and continuously stirred. A quantity of 500 g of ghee was added. 15 g of *Monascus* powder was added into the mix for colouring. The stirring was continued till the consistency of halwa reached by excreting ghee out. Fried cashew nuts and raisins were added and the whole content while hot was poured into a ghee greased plate. After the product solidified, it was made into slices. A control preparation without pigment was also prepared for evaluation.

3.10.2. Plum Squash

Squashing of seasonal fruits makes the fruits available at off seasons too. Generally, squash contains 30 per cent of suspension solids. Plum squash was prepared and coloured using *Monascus* pigments.

Recipe

Plum fruits	-	1 kg
Sugar	-	1.9 kg
Water	-	1.0 lit
Citric acid	-	10 g
Sodium benzoate	-	4 g
<i>Monascus</i> pigment	-	15 g

Clean kitchenware and a LPG gas stove.

Procedure

A quantity of one kg of clean, fresh plum fruits were made into pulp after removing nuts. The juice was extracted from the fruit. About 2 kg of cane sugar was made into a syrup solution using one litre water. The solution was kept on flame until it became syrup. Filtered fruit juice was added and heating was continued. A quantity of 5 g of *Monascus* powder mixed in small quantity of ethyl alcohol was added to the mixture. Stirring continued until the colour become homogenous. Citric acid (10 g) was added and mixed thoroughly, the mixture was added with preservative (sodium benzoate - 4.0 g) before bottling. The squash was bottled in a clean sterilized bottle. A control preparation without the addition of *Monascus* pigment was also prepared for evaluation purpose.

3.10.3. Soft Cake

Cake is a common food item used as a snack. It is synthetically coloured to decorate grand functions. *Monascus* pigments were tried to impart colour to cake.

Recipe

Maida flour	-	100 g
Vanaspathi	-	100 g
Powdered sugar	-	100 g
Egg	-	1 no
Baking powder	-	1 g
Salt	-	a pinch
Vanilla essence	-	1 tsp
<i>Monascus</i> pigments	-	10 g

Procedure

The flour was sifted and souted twice. Cream fat and sugar were added and slowly mixed to get fluffy consistency. Egg was added to the cream mix and slowly mixed. The *Monascus* pigment was added following the addition of eggs. The flour was folded well and milk was added if necessary. The cake mix was poured in a greased and dusted tray. The tray was kept in a microwave oven (IFB microwave oven) for 20-30 min.

3.10.4. Evaluation of the products

The products were given code names as A, C and E and their respective control products were named on B, D and F. The products were displayed and given for organoleptic evaluation to a 10 member panel of judges. They were requested to evaluate the products based on, colour and appearance, flavour, texture, taste and over all acceptability of the products. Their remarks to improve the products were also sought. Based on the scores obtained, the acceptance of *Monascus* pigments as a colouring agent was assessed.

3.11. Production of medicinal compound from *Monascus* spp. Monacolin-K

Besides producing the food pigments, *Monascus* spp. produce a valuable medicinal compound to reduce blood cholesterol level. The compound has been identified as Monacolin - K (3-hydroxyl-3-methyl glutaryl Coenzyme-A) reductase inhibitor).

3.11.1. Determination of Monacolin-K (Ganrong *et al.*, 1998)

Monacolin K was determined by spectrophotometrically. The commercial grade 'Lovastatin' was used as control.

3.11.1.1. Sample preparations

Monascus fermented red rice was powdered and a quantity of one gram was taken with 10 ml of methanol and the tubes were kept on a water bath at 40° - 50°C for 15 min. The quantity was collected in a centrifuge tube and spun down at 10000 x g for 15 min. The clear supernatant was collected for analysis. As a control, Lovastatin tablet (20 mg, was dissolved in 10 ml of methanol and was taken in a centrifuge tube. The content was spun down at 10000 x g for 15 min. Both the samples were adequately diluted.

3.11.1.2. Spectrophotometry

The samples were scanned through UV spectrum from 200 nm to 300 nm to locate the presence of Monacolin-K at 246 nm and 254 nm,. The peaks recorded at 246 nm and 254 nm were taken for the presence of Monacolin-K.

3.11.1.3. HPLC analysis

The prepared samples of *Monascus* spp. and a commercial 'Lovastatin' tablets were qualitatively analysed for the presence of Monacolin-K in the fungal extracts. Varian Pro Star HPLC system was used. Methanol: water (80:20 v/v) solvent system was used as a mobile phase at a flow rate of 0.8 ml min⁻¹. A quantity of 0.5 µl of the samples was injected and detected at 237 nm. The identity of the compound was confirmed with the commercial sample of Lovastatin.

3.11. 2. Product development - capsules of *Monacolin-K*

Monascus spp. were used for the production of Monacolin-K tablets. As described earlier, through solid state fermentation, non-glutinous, parboiled rice was used for the fermentation. *Monascus* spp. inoculum was added and fermentation was

carried out for 15 days. After good fermentation, the fermented rice ('Red rice') was harvested and air dried at 50°C in an oven for 6-8 hours. The dried 'red rice' was finely powdered. Calculated quantity (0.1 g) of finely powdered, sieved 'Red rice' was encapsulated in gelatin capsules and stored.

3.12. Statistical analysis

Wherever needed the data were subjected to statistical scrutiny by the standard procedure of Panse and Sukhathme (1976). In the analysis a few computing packages were used like, AGDATA, AGRES, *Microsoft's* EXCEL and SPAR1.

Results

Chapter IV

EXPERIMENTAL RESULTS

4.1 Isolation and purification of pigments producing fungi from soils

The data on the fungal flora of different soil samples and pigment producing fungi are presented in Table 1 and Plate 1.

From thirty one soil samples collected across the country and also from the rhizosphere soils of different crops, the fungal flora was isolated. Pigment yielding fungi were located, reisolated and purified. The data indicated that the preparation of pigment yielding fungi was indeed very low. Soil samples from forest ecosystem recorded maximum per cent (21%) of pigment yields. In other samples, the population of pigment producing fungi was meagre. The spectrum of colours ranged from deep red to yellow and fluorescent yellow. Most of the pigment producing fungi displayed red and different shades of red.

4.2. *In vitro* screening of fungal isolates for pigment production

The data on the *in vitro* screening of pigment producing fungi are presented in Table 2 and Plates 2 to 5.

Using a rapid method of screening the pigment producing fungal isolate, the ones excreting maximum pigments are recognized. Generally, pigment excretion in the medium started three days after inoculation. As many as twenty isolates of fungi were selected and they excreted pigments with brown to red and yellow shades of colours. The yellow pigments were absorbed maximally at 300 nm and the red pigments at 500 nm.

Table 1. Distribution of pigment yielding fungi in certain soil samples.

No	Soil samples collected from	Fungal colonies (CFU $\times 10^4$)	No. of pigment producing fungi (CFU $\times 10^4$)	Per cent of pigment producing fungi	Nature of pigment
1.	Silent valley forest soil	7.5	1	13.3	Red.
2.	Aliyar nagar field soil	14	-	-	-
3.	Parambikulam dam soil	18.5	2	10.7	Yellow, Fl. Yellow
4.	Ooty Teak forest soil	18.5	-	-	-
5.	Gudalore forest soil	16.5	2	12.1	Yellow, Pink
6.	Thotabetta soil	20.5	-	-	-
7.	Pollachi red soil	7.0	-	-	-
8.	Top slip peat soil	17.5	-	-	-
9.	Silent valley river soil	9.5	2	21.0	Orange, Red
10	Assam tea estate soil	3.0	-	-	-
11	Idukki soil	7.5	-	-	-
12	Coir compost	26.0	1	3.8	Yellow orange
13	Farm yard manure	16.5	-	-	-
14.	Thirupathi forest soil	19.5	-	-	-
15.	Dehra Dun forest soil	15.0	2.0	13.3	Yellow, Red
16.	Saharanpur river soil	5.0	-	-	-
17.	Badrinath field soil	10.0	-	-	-
18.	Canal pirivu pasture meadow soil	22.0	-	-	-

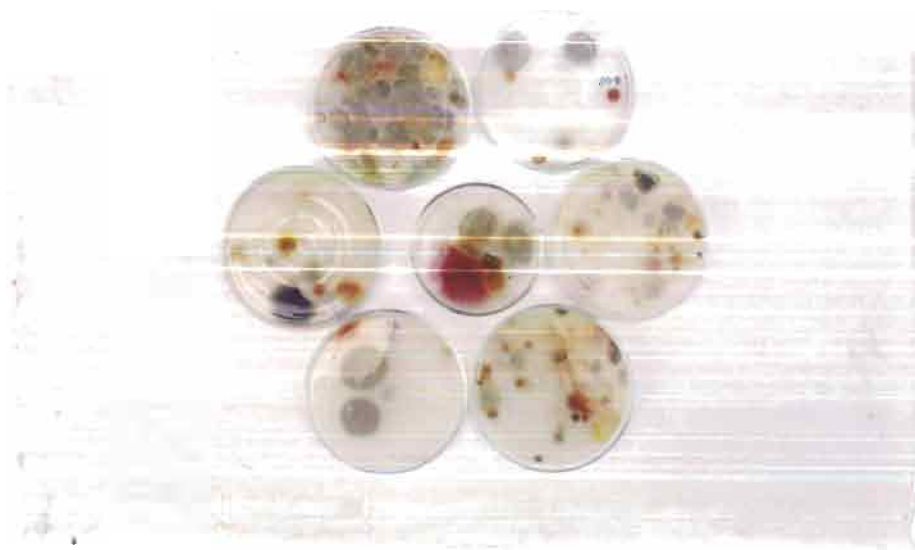
19.	Chullimedu Teak soil	24.0	3.0	12.5	Yellow, Red
20.	Walayar Dam site soil	16.5	1	6.06	Orange
21.	Chinnampathi forest soil	19.0	1	5.26	Brownish red
	Rhizosphere soils from				
22	Cotton , TNAU	32.5	-	-	-
23	Mulberry, TNAU	11.5	1	8.7	Brown
24	Carrot, Ooty	16.0	-	-	-
25	Cabbage, Ooty	25.0	-	-	-
26	Cauliflower, Ooty	27.0	3	11.1	Yellow, Red, Orange
27	Potato, Ooty	14.5	-	-	-
28	Tea, Valparai	24.0	-	-	-
29	Grass land soil, Ooty	24.0	-	-	-
30	Rice (Low land), TNAU	15.5	-	-	-
31	Green gram , TNAU	13.0	-	-	-

Population expressed per g sample on dry weight basis.

CFU: colony forming unit.

- Not detected.

ISOLATION OF PIGMENT PRODUCING FUNGI
FROM SOILS



Plates 1. Isolation of pigment producing fungi from soil.

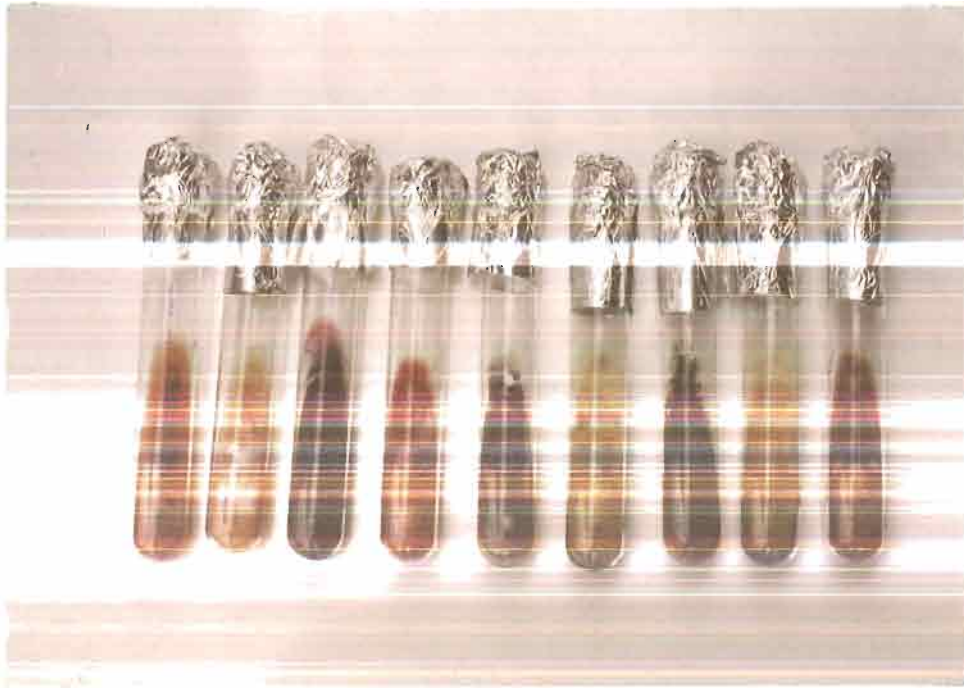


Plate 2. Promising pigment producing fungi on agar slops (Front view).



Plate 3. Certain promising pigment producing fungi (Rear view).

Table 2 . *In vitro* screening of fungal isolates for pigment production

No	Fungal isolate	Biomass (g l ⁻¹ dry weight)	Pigments in units l ⁻¹ *		Colour of the pigment	Time of pigment excretion (Days)
			300 nm	500 nm		
1.	PF1	4.37	131.6	967.3	Red	3
2.	PF2	4.16	31.4	61.4	Red, very low	4
3.	PF6	3.17	431.8	316.8	Yellow	4
4.	PF7	6.71	314.9	474.8	Violet	6
5.	PF8	5.34	613.4	113.4	Yellow	8
6.	PF9	2.14	416.5	136.3	Yellow	10
7.	PF10	3.73	536.8	173.4	Orange	3
8.	PF12	5.17	643.6	376.4	Orange	3
9.	PF13	6.18	714.8	568.9	Orange	4
10.	PF14	3.16	461.5	146.3	Fluorescent yellow	5
11.	PF15	7.14	314.9	615.3	Pink	6
12.	PF16	4.16	117.3	867.3	Dark reddish brown	7
13.	PF23	7.61	113.8	613.9	Pink	5
14.	PF26	2.46	114.7	67.5	Bright orange	9
15.	PF27	4.37	576.8	176.5	Yellow	3
16.	PF28	7.61	263.8	715.6	Red mycelium	3
17.	PF35	7.61	186.6	671.9	Red	4
18.	PF41	4.36	116.4	516.4	Wine red	5
19.	PF53	5.16	176.3	673.2	Violet	6
20.	PF55	6.63	416.9	132.3	Yellow	3

* One unit pigment is defined as equivalent to an absorption of 0.1 OD either at 300 or 500 nm



Plate 4. Extracellular pigment secretion by fungi in liquid medium.

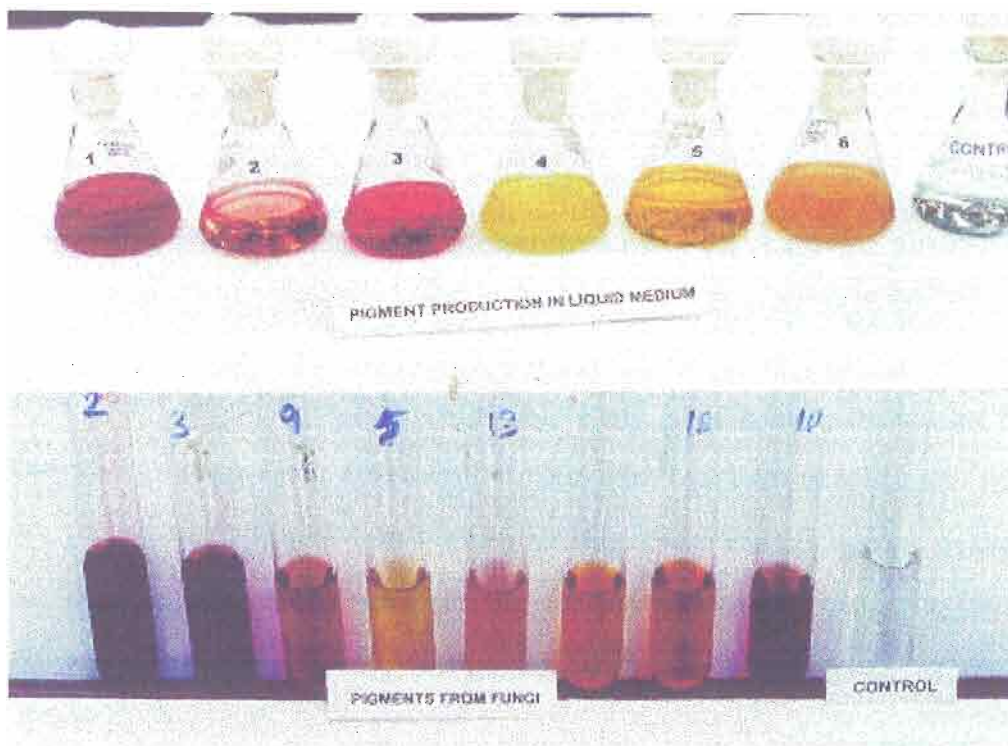


Plate 5. Variety of pigments from fungi

4.3. Taxonomic characterization and identification of pigment producing fungal isolates

As detailed under materials and methods, morphology and cultural characters of the pigment yielding fungi were determined. Colony morphology, mycelial structure, conidiation and pigment excretion pattern were studied and tabulated (Table 3). The taxonomic positions of the three promising unknown fungi were studied using 'moisture chamber technique' (Plate 6). Based on the instruction of Gilman (1975), the taxonomic position of the fungi was determined based on the conidial morphology, size, shape and colour of ascospores (Plates 7 to 31). The three fungal isolates were identified as follows.

PF1 - *Penicillium purpurogenum*

PF 12 - *Verticillium* sp.

PF 16 - *Humicola* sp.

4.3.1. *In vitro* growth and pigment production by the fungi

The data on the growth and pigment production of fungi in different growth media are presented in Table 4.

As potato dextrose medium supported fungal growth and pigment production over than any other media, invariably, PDA medium has been used. In Czapek-Dox medium, growth and pigmentation were low. Other growth medium like mycological agar had its own limitation of restricted growth and colour production. Malt extract medium supported growth and pigmentation in *Monascus purpureus*, *M.ruber* and *Verticillium* sp. Yeast morphology agar was not good as it failed to encourage fungal growth and pigmentation. The special medium developed for *Monascus* supported all fungi for their good growth and more pigment excretion.

Table 3. Morphological characteristics of the selected fungi on potato dextrose agar medium.

Characters	<i>M. purpureus</i>	<i>M. ruber</i>	<i>P. purpurogenum</i>	<i>Verticillium</i> sp.	<i>Humicola</i> sp.
Colony Morphology	Flat, leathery, circular, entire margin	Raised, fluffy with irregular margin	Leathery, Circular, colonies with entire margin	Leathery, cottony colonies with regular margin	Small, circular heaped colonies with regular margin. At maturity, produces reddish excretion
Mycelium	Reddish subsurface mycelium and arial colourless mycelium with conidiophores; septate; and grows faster.	Reddish subsurface mycelium with woolly arial mycelium bearing meager conidiophores.	Subsurface mycelium produces red pigment. Thickly oven arial mycelium.	Subsurface mycelium is orange and it has cream coloured arial mycelium.	Short, thick mycelium with septate cells. Scattered conidiophores dark brown colour and verrucose.
Conidiation	Sexual: Numerous Ascospores in a sac like structure produced in arial and sub merged mycelium. Asexual: 1-3 conidia present in arial conidiophores. Chlamydospores present.	Ascospores in asci. 2 to 3 profuse conidia in conidiophores. Chlamydospores present.	Ascospores rarely produced profuse conidiation on sterigmata. Velvet green colour colonies. No chlamydospores.	Conidia are typical clavate conidiophores, bear hyaline single spores. No sexual spores	Conidia born terminally, pyriform and dark in colour. Chlamydospores common.
Pigment production	Red pigment secretion in the medium.	Intense red pigment secretion in the medium	Intense red pigment secretion in the medium	Orange pigment secretion	Reddish brown pigment secreted.

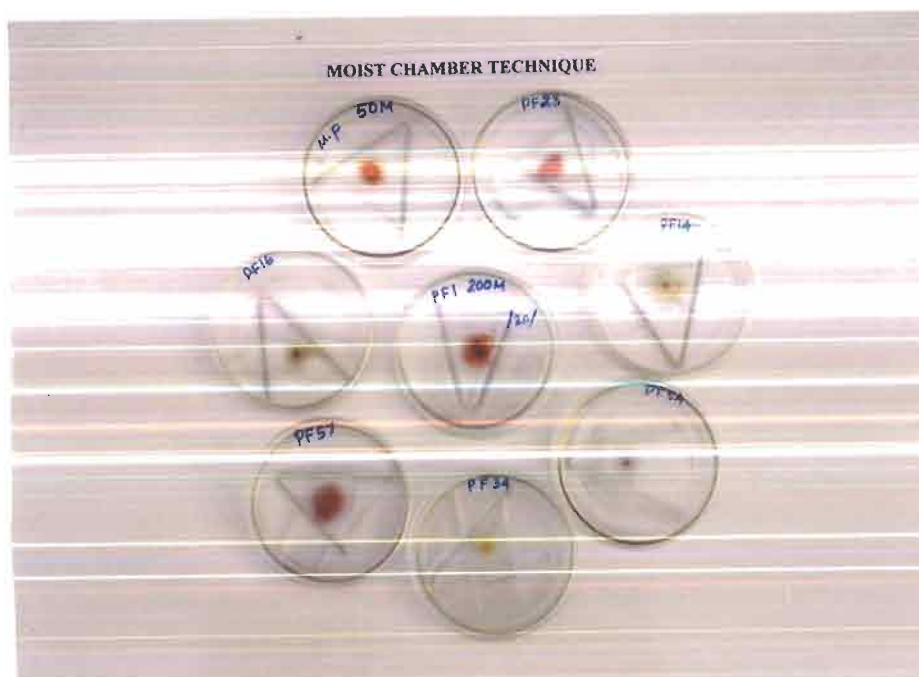


Plate 6. Moist chamber technique for the identification of fungi.

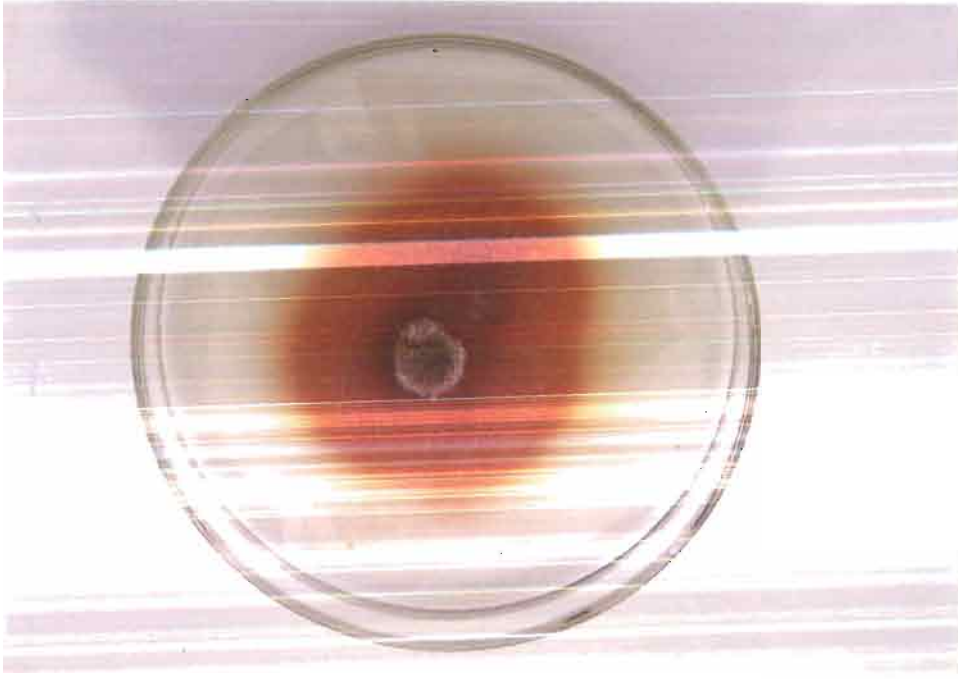


Plate 7. Growth of *Monascus purpureus* on potato dextrose agar medium.

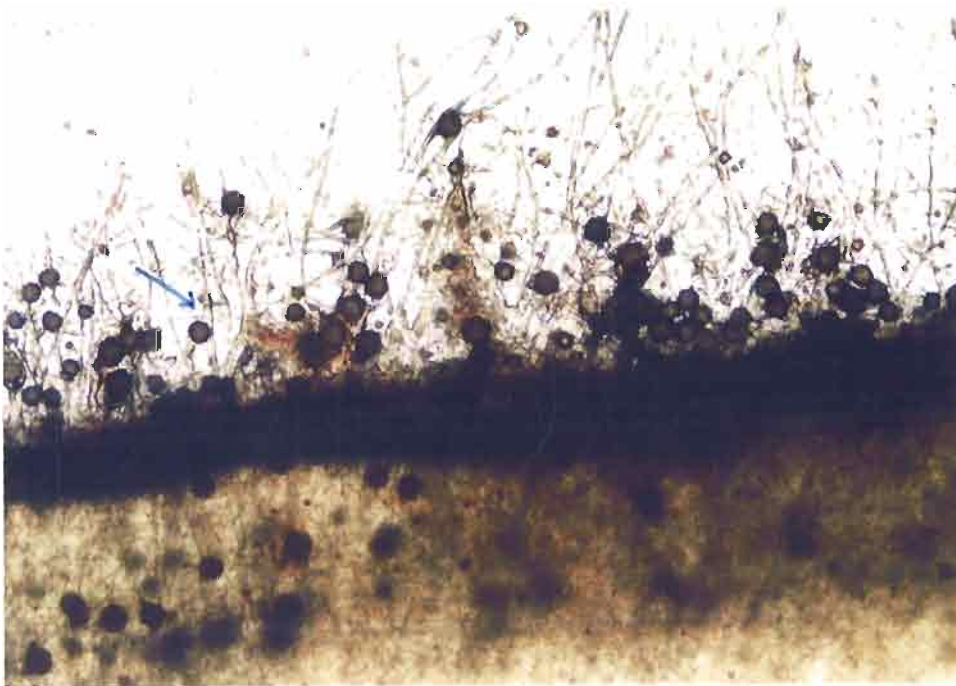


Plate 8. Ascospore production in *Monascus purpureus*. (10x)

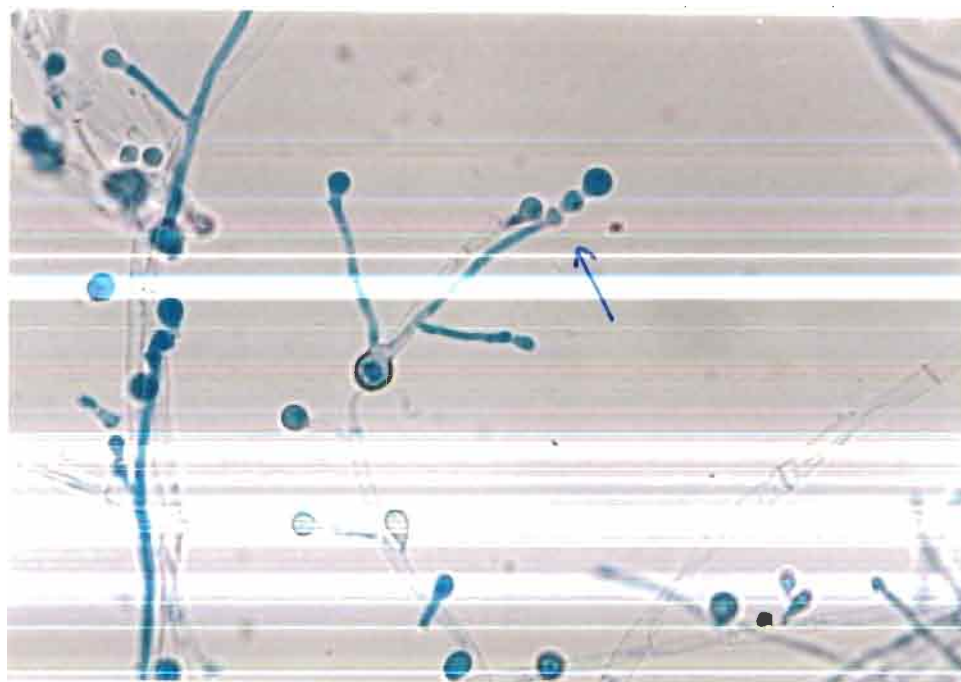


Plate 9. Conidiospores of *Monascus purpureus*. (300x)



Plate 10. Closer view of chlamydospores of *M. purpureus*. (300 x)



Plate 11. Ascospores of *M. purpureus*. (600x)

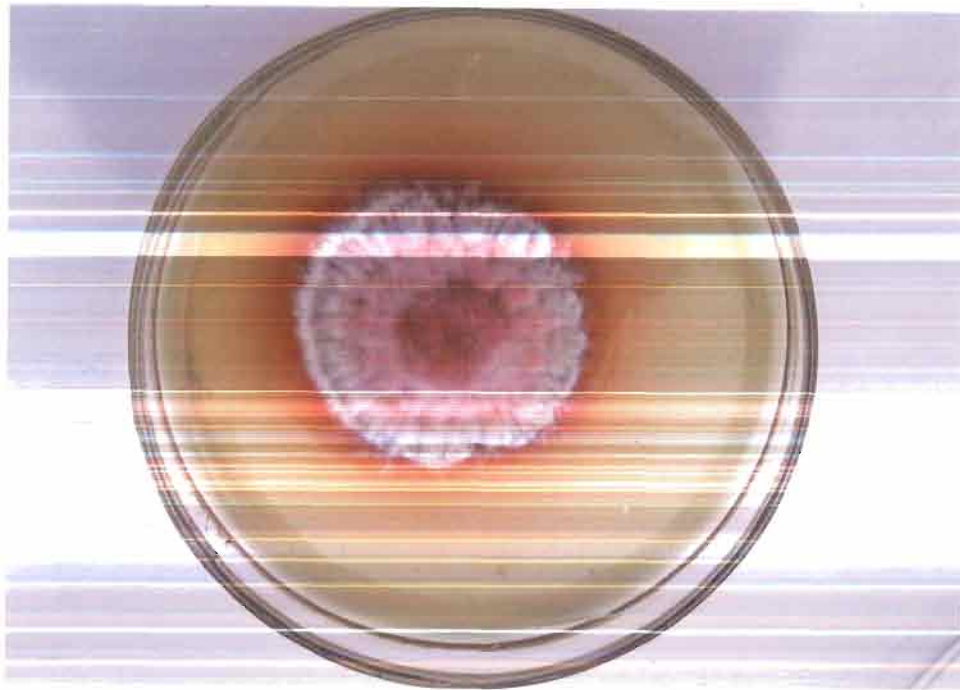


Plate 12 . Colony morphology of *Monascus ruber* on potato dextrose agar medium.



Plate 13. Conidiospores of *Monascus ruber*.(600 x)

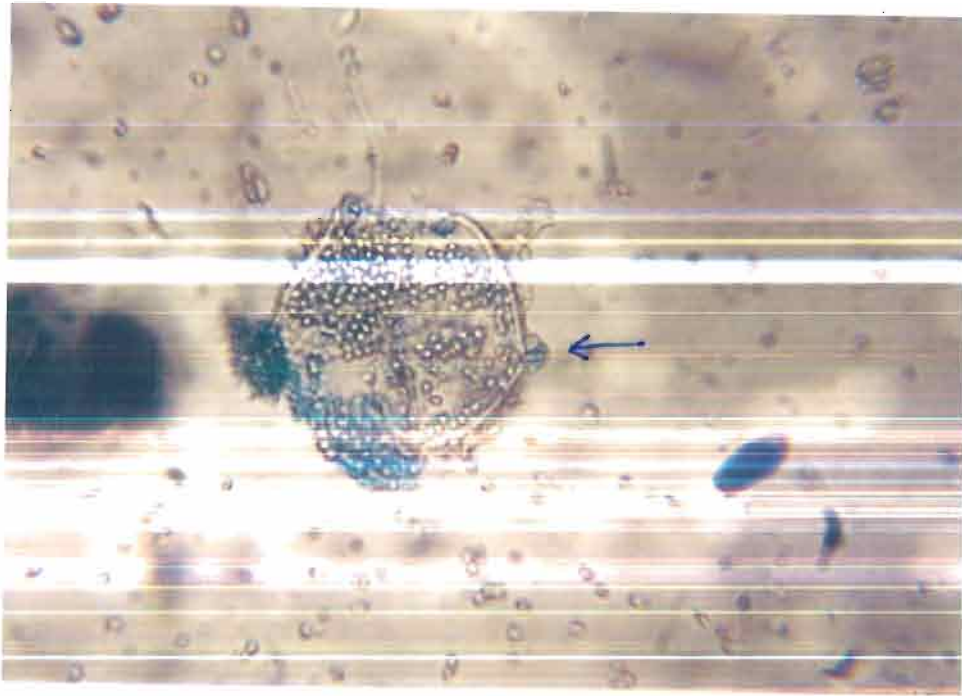


Plate 14. Ascus carrying ascospores of *Monascus ruber*.(600 x)

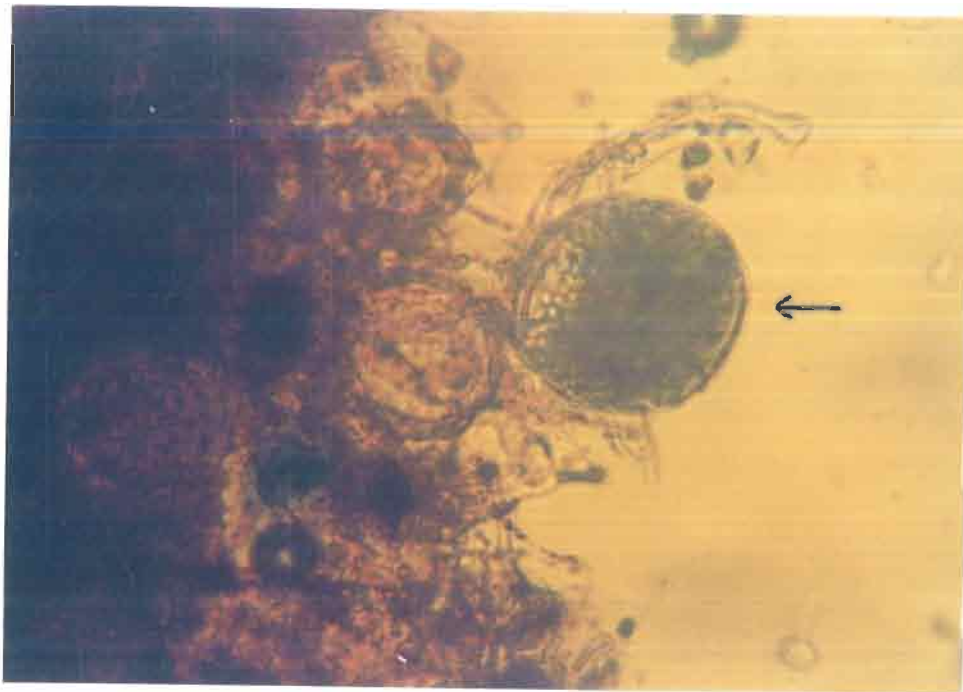


Plate 15. Ascospores of *Monascus ruber*.(600x)

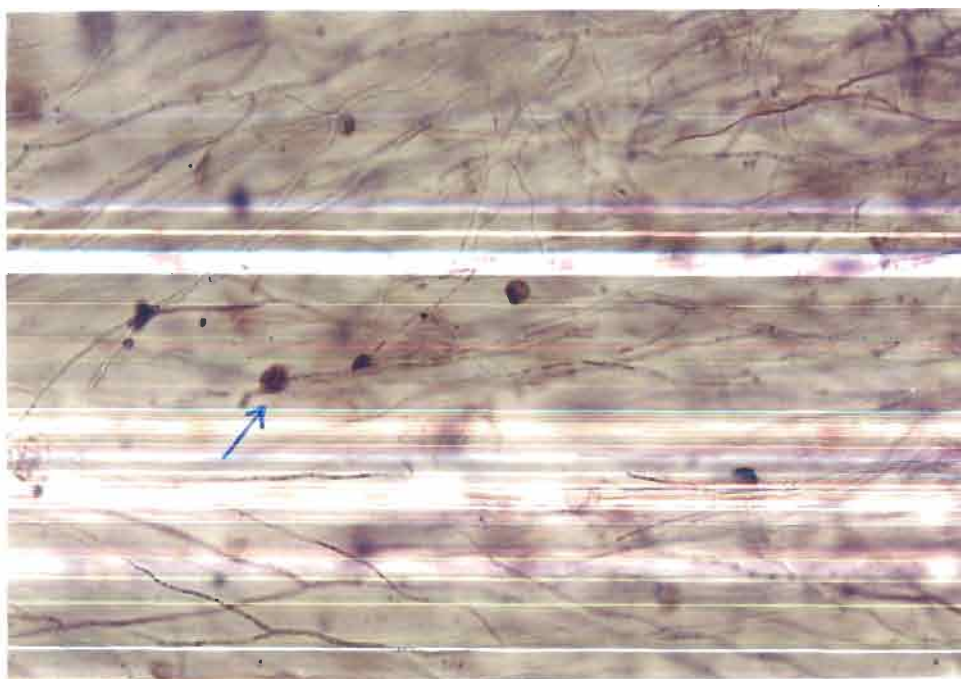


Plate 16. Hyphal tips showing pigment excretion in *M. purpureus* .(300 x)



Plate 17. Hypha displaying pigment accumulation of *M. purpureus*.(600x)



Plate 18. Pigment excretion from the hyphae of *M. ruber*. (600 x)

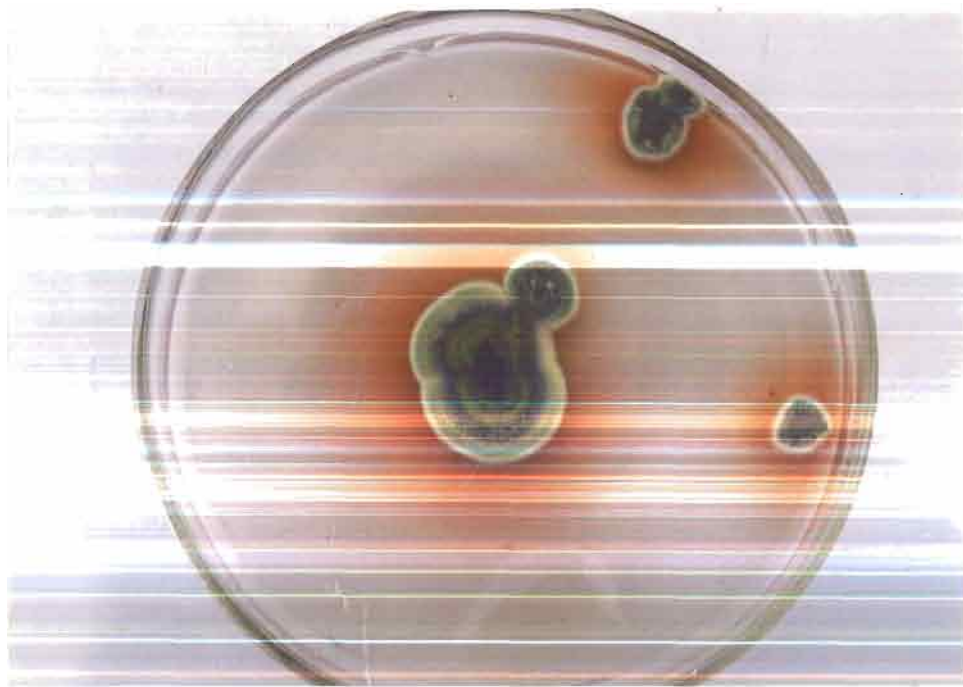


Plate 19 . A close up view of *Penicillium purpurogenum*.



Plate 20. Conidiophores (60 x) and Conidiospores (300 x) of *P. purpurogenum*.

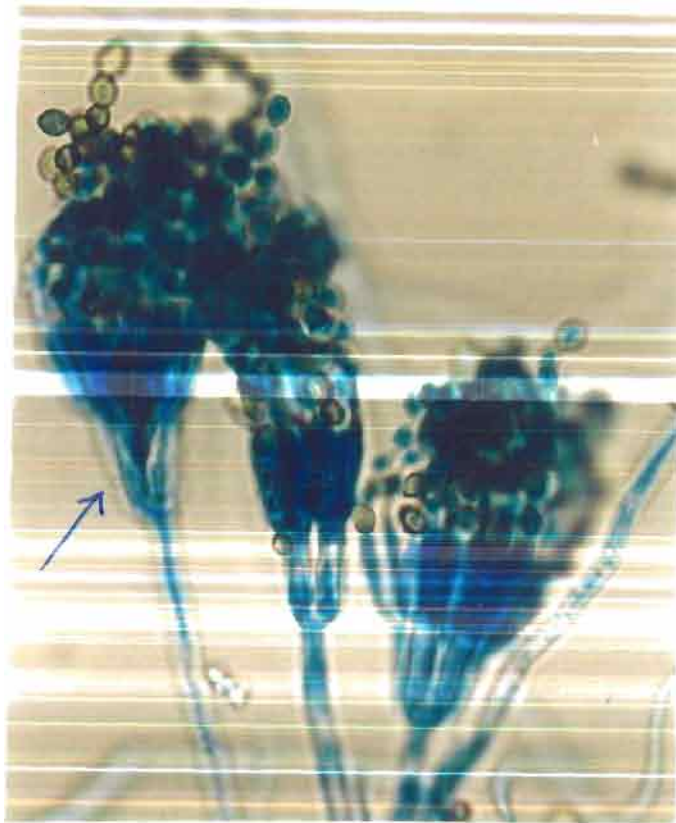


Plate 21. Conidial structure of *Penicillium purpurogenum*. (600 x)



Plate 22. Red pigment production by *P. purpurogenum* in agar medium. (60 x)

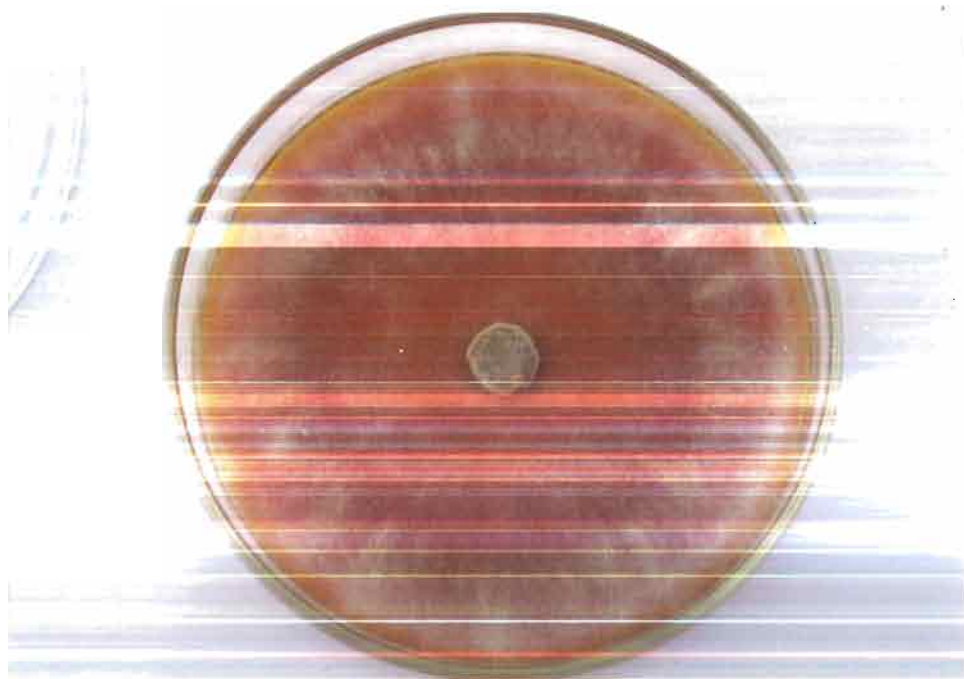


Plate 23. Growth of *Verticillium* sp. on agar medium (Abaxial view).

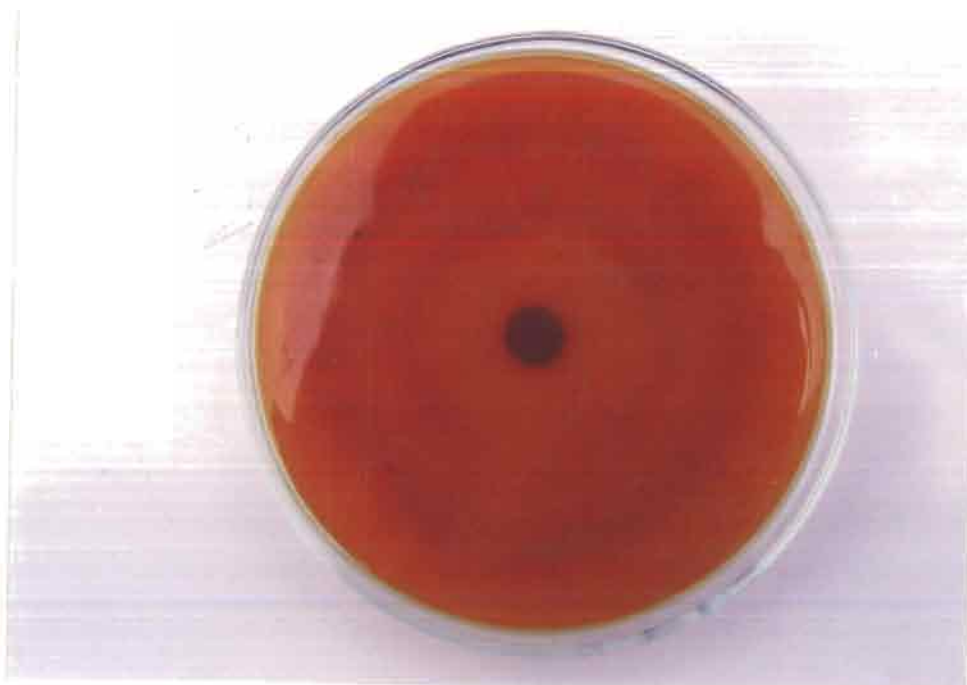


Plate 24. Pigment production by *Verticillium* sp on agar medium (Adaxial view).

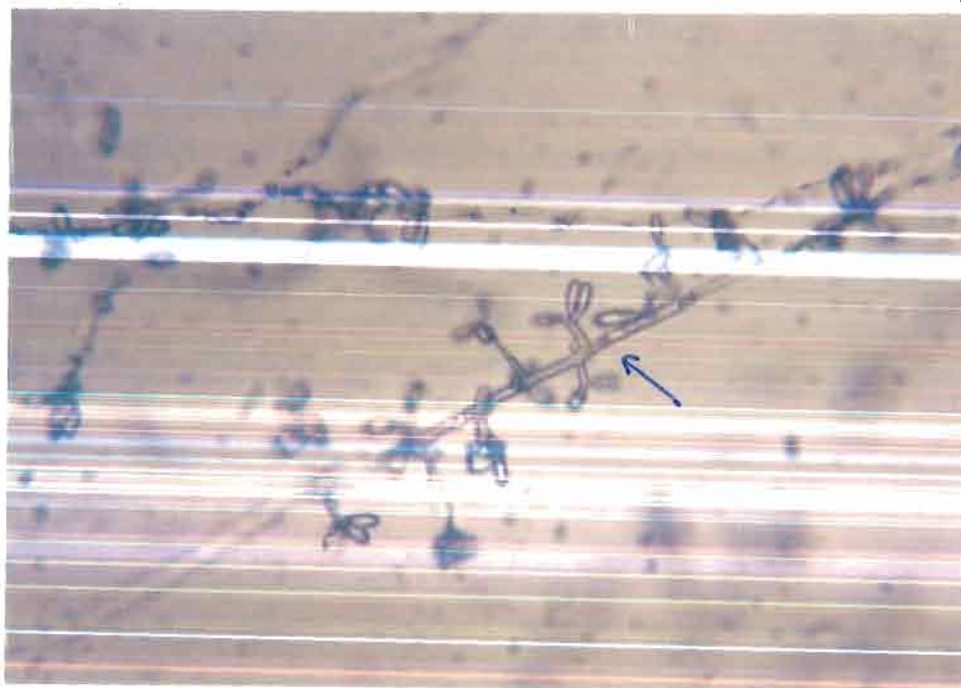


Plate 25. Hyphae of *Verticillium* sp. bearing conidia. (300 x)

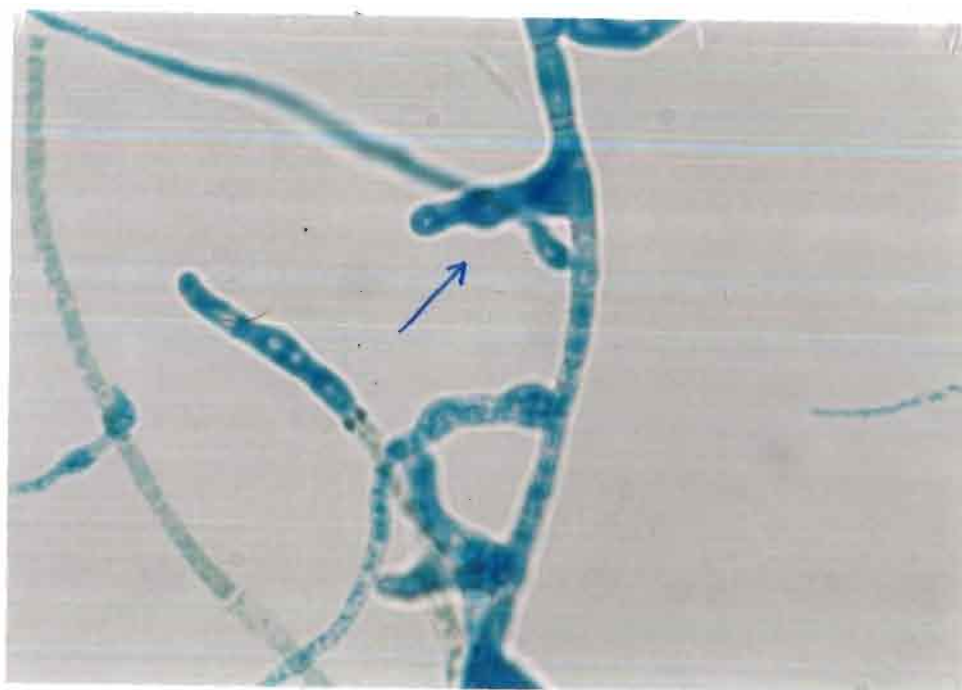


Plate 26. Formation of ascospores in *Verticillium* sp. (600 x)

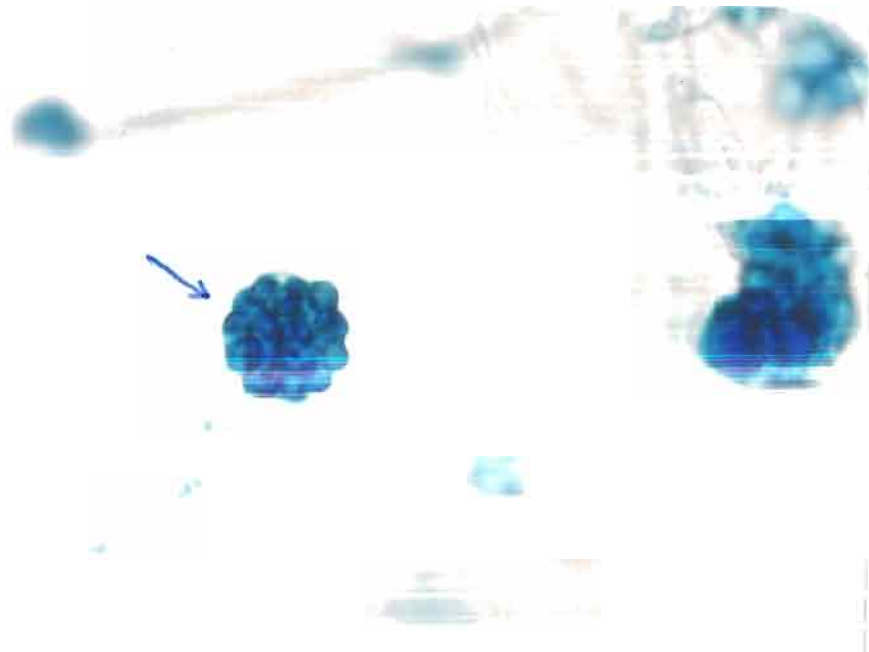


Plate 27. Ascospores of *Verticillium* sp. (600 x)

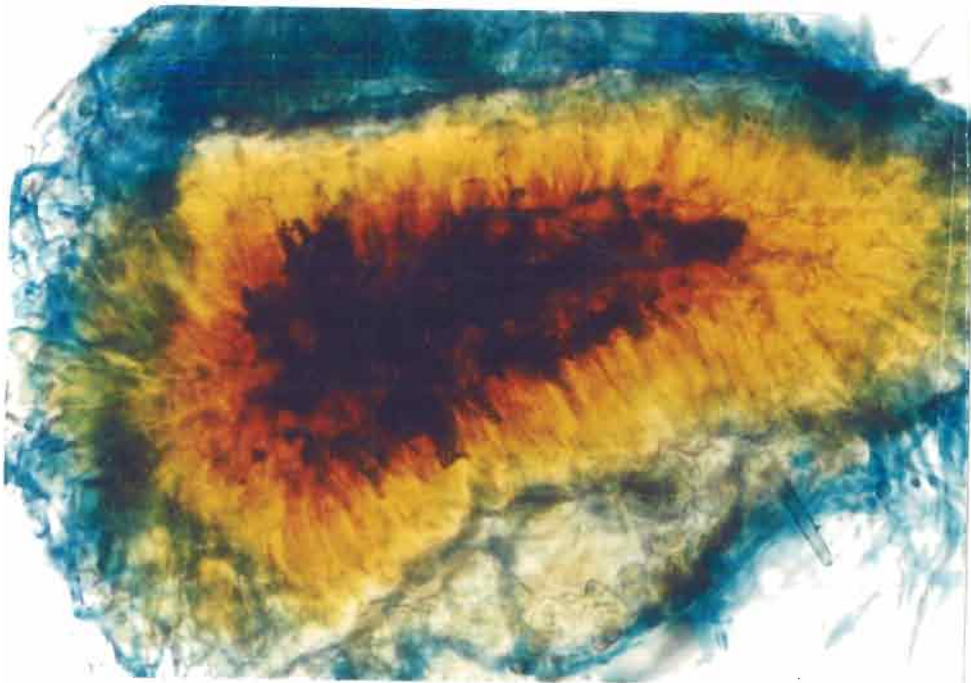


Plate 28. Reo pigment production by *Verticillium* sp. in agar medium. (300 x)

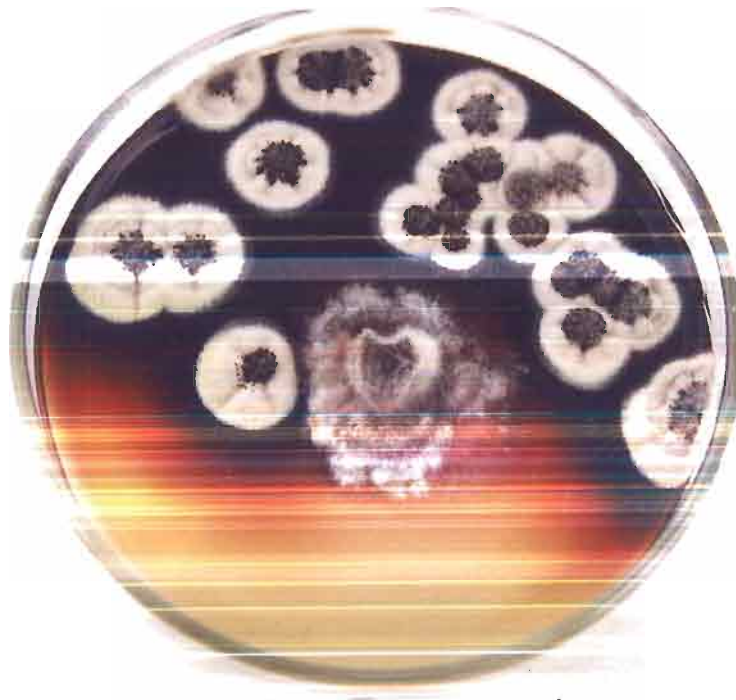


Plate 29. Pigment producing *Humicola* sp on agar medium.

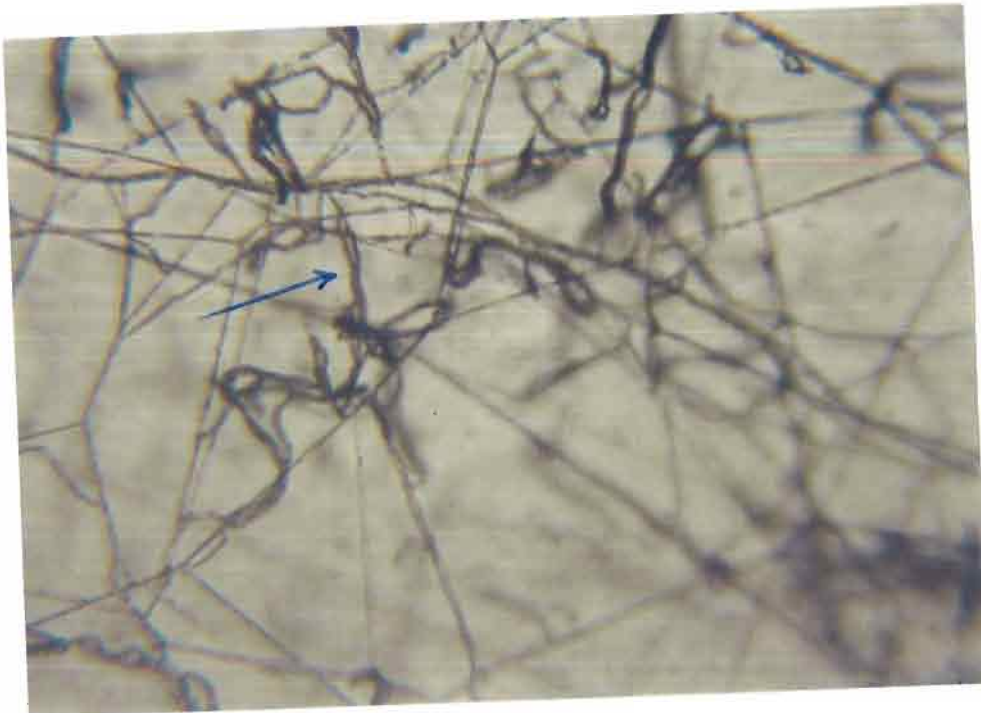


Plate 30. Spore bearing structures of *Humicola* sp. (300 x)

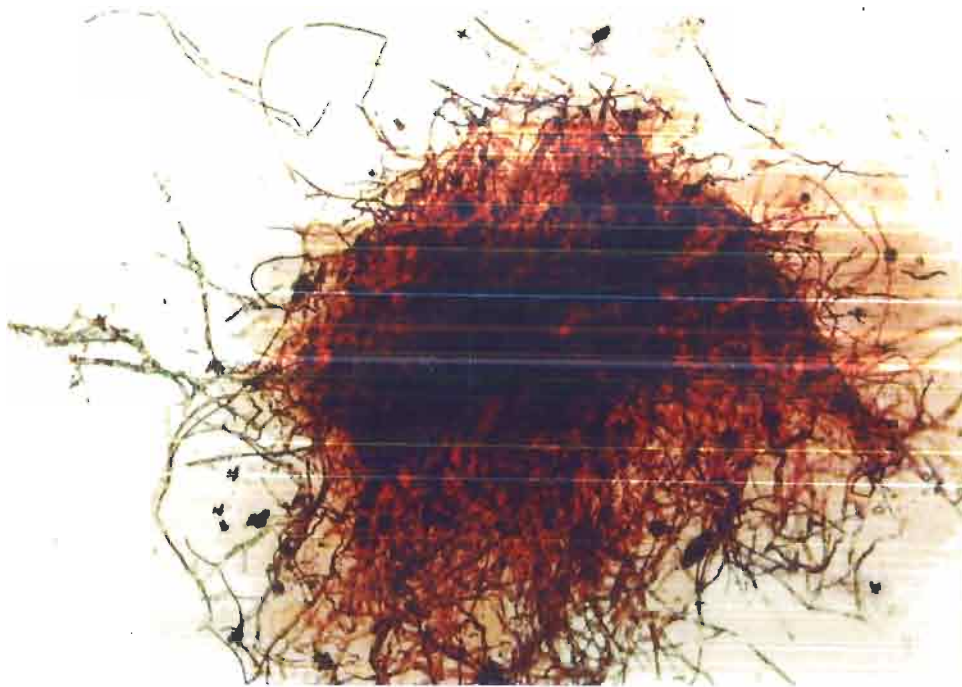


Plate 31. Brownish Red pigment production by *Humicola* sp.in agar medium.

Table 4. *Invitro* growth characteristics of pigment producing fungi on different media.

No	Growth media	<i>M. purpureus</i>		<i>M. ruber</i>		<i>P. purpureogenum</i>		<i>Verticillium</i> sp.		<i>Humicola</i> sp.	
		Growth	Colour production	Growth	Colour production	Growth	Colour production	Growth	Colour production	Growth	Colour production
1	Potato Dextorse Agar	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
2	Czapek-Dox Agar	++	++	++	++	+++	+++	++	++	+++	+++
3	Mycological Agar	+	+	+	+	+	+++	+	+	+	+
4	Malt Extract Agar	+++	+++	+++	+++	+	+	+	+	++	++
5	Yeast Morphology Agar	+	+	+	+	+	+	+	+	+	+
6	Sabouraud Agar	+++	+++	+++	+++	++	+++	+	+	+	+
7	<i>Monascus</i> Medium	+++	+++	+++	++	+++	+++	+++	+++	+++	+++
8	Martin's Rose Bengal Agar	++	+	++	+	++	-	-	-	+	+

- No growth / pigmentation

+ Poor growth / pigmentation

++ Medium growth / pigmentation

+++ Good growth / pigmentation

4.4. Certain physiological characters of the selected pigment yielding fungi

The five selected fungi were studied in a detailed manner. The factors influencing growth and pigmentation like effect of different carbon and nitrogen sources, temperature, pH, agitation (aeration) and light conditions were studied. The results are presented in the Tables 5 to 25 and Figures 1 to 14.

4.4.1. Effect of different carbon sources on the growth and pigmentation of selected fungi

The influence of different carbon sources on the growth and pigment production of fungi is set out in Tables 5 to 8 and Figures 1 to 3.

4.4.1.1. Effect of different sources of carbon on the biomass production

The data on the influence of different carbon sources on the biomass production of the selected fungi are presented in the Table 5 and Figure1.

In general, all the carbon sources used in the study encouraged fungal growth. Glucose, starch and mannitol recorded increased biomass production as compared to other sources of carbon. Among the five fungi, *Monascus* spp. registered higher biomass yield.

Correlation analysis of the data revealed that biomass production bore a positive relationship with pigment production and pH shifts in the medium (Appendix IIa).

4.4.1.2. Effect of different carbon sources on the pH of growth media

The data on the changes of pH of growth media as influenced by fungal growth are presented in Table 6.

Table 5. Effect of different sources of carbon on biomass production of fungi.

No	Carbon Sources	Biomass production (g l ⁻¹ dry weight)				
		<i>M. purpureus</i>	<i>M. ruber</i>	<i>P. purpurogenum</i>	<i>Verticillium</i> sp.	<i>Humicola</i> sp.
1	Glucose	8.79	8.86	2.71	2.15	3.33
2	Fructose	6.10	5.50	1.91	2.23	1.46
3	Xylose	2.83	1.43	2.21	1.76	1.67
4	Sucrose	7.20	6.36	2.51	2.67	1.25
5	Maltose	4.88	5.61	2.51	0.87	2.14
6	Galactose	2.30	1.30	2.07	0.76	2.36
7	Lactose	3.17	1.40	1.91	0.64	1.39
8	Arabinose	2.56	1.31	0.67	0.46	2.16
9	Glycerol	1.50	3.65	2.01	3.01	2.37
10	Sorbitol	1.43	1.03	2.41	2.74	2.20
11	Mannitol	6.20	4.31	2.61	5.05	2.49
12	Starch	3.17	6.03	2.81	2.81	2.71
13	Ethanol	4.14	4.64	2.62	1.06	2.16
SE		0.069	0.033	3.051	0.211	0.021
CD(<i>P</i> ₀ =0.05)		0.142	0.068	6.272	0.434	0.043

Fig.1. Effect of different sources of carbon on the biomass production of fungi

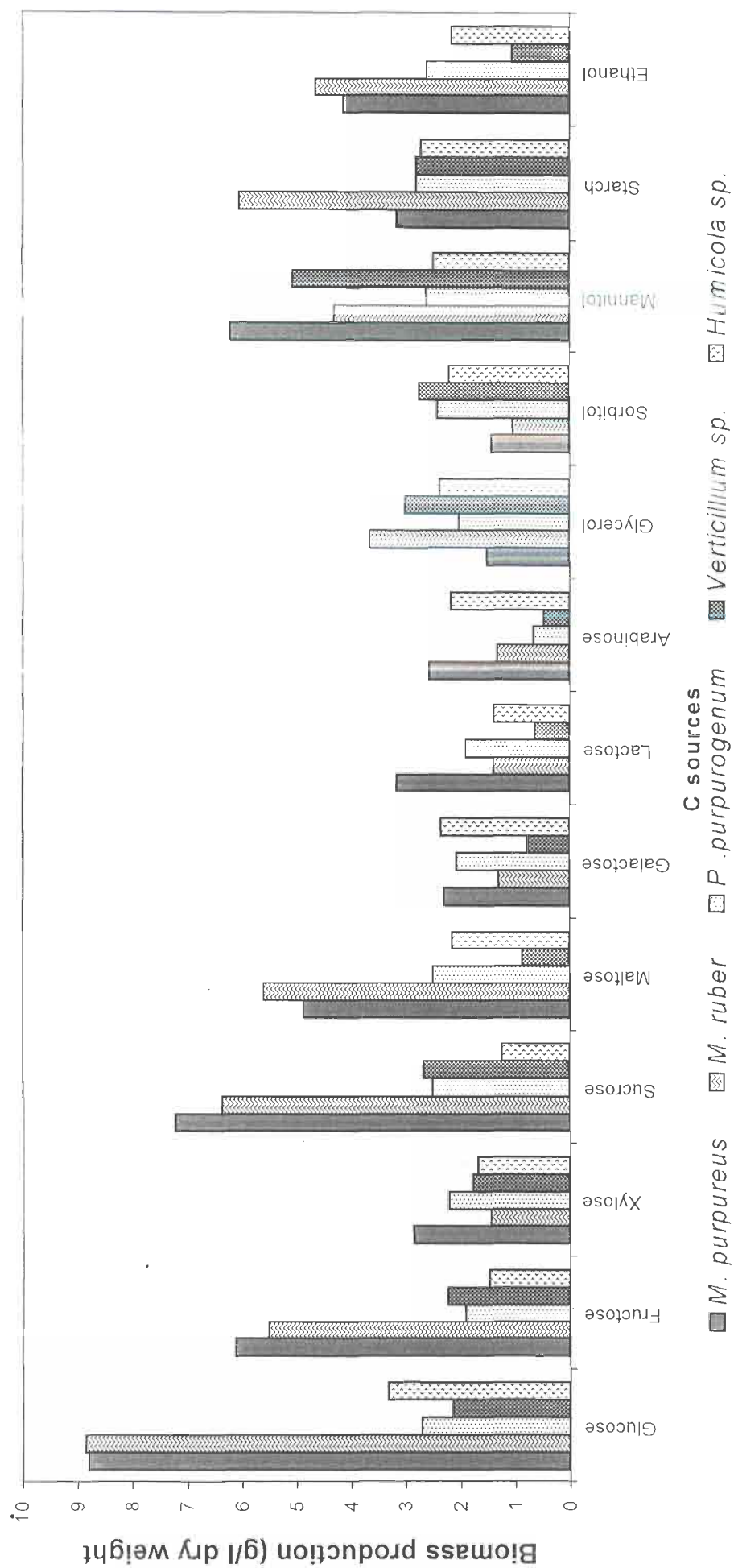


Table 6. Effect of different sources of carbon on the pH shift of the medium by the fungi.

No	Carbon sources	pH shift *				
		<i>M. purpureus</i>	<i>M. ruber</i>	<i>P. purpurogenum</i>	<i>Verticillium</i> sp.	<i>Humicola</i> sp.
1	Glucose	8.18	8.13	8.50	8.86	8.73
2	Fructose	8.45	8.14	8.18	8.76	8.54
3	Xylose	6.13	6.75	7.35	5.14	5.41
4	Sucrose	7.14	7.11	6.96	7.43	6.45
5	Maltose	8.47	8.01	8.12	8.75	7.16
6	Galactose	6.41	5.74	5.70	6.14	6.15
7	Lactose	6.31	5.76	5.81	6.68	5.76
8	Arabinose	6.13	5.61	5.96	5.73	5.81
9	Glycerol	7.12	6.14	6.93	8.77	7.13
10	Sorbitol	5.11	5.19	6.02	6.13	7.14
11	Mannitol	8.18	8.16	8.18	8.14	8.63
12	Starch	7.13	7.17	7.67	8.15	8.18
13	Ethanol	6.14	7.43	7.44	7.47	6.71

* Initial pH of broth : 5.5

Due to the growth of fungi, it is very common to observe changes in the pH. The different carbon sources also influence the pH changes. Carbon sources like glucose, fructose, maltose and mannitol shifted the pH from 5.5 to pH 8.0. The other carbon sources tended to decrease the pH of the medium to acidity. The pH of the medium with sorbitol recorded an acid pH.

pH shifts of the growth medium bear a positive correlation with fungal biomass of the selected fungi (Appendix IIa).

4.4.1.3. Effect of different carbon sources on intracellular pigment production

The results on the influence of different sources of carbon on the intracellular pigment production have been set out in Table 7 and Figure 2.

In general, all the carbon sources encouraged fungal growth and pigment production. All the five fungi tested produced both yellow and red pigments; red pigments predominated yellow pigments in quantity except in *Verticillium* sp. The data clearly show that glucose was found to support the highest pigment yield in most of the fungi. Fructose, maltose and mannitol do support pigment production. *Monascus ruber* has been found to synthesize more quantity of pigments than the rest of the fungi.

Analysis of the data revealed that the fungal biomass has a positive correlation with pigment production in all the fungi examined (Appendix IIa).

4.4.1.4. Effect of different sources of carbon on the production of extracellular pigments

The data on the effect of different carbon sources on the extracellular pigment production have been presented in Table 8 and Figure 3.

Table 7. Effect of different sources of carbon on the production of intracellular pigments in fungi.

No	Carbon source	Intracellular pigments in units *									
		<i>M. purpureus</i>		<i>M. ruber</i>		<i>P. purpurogenum</i>		<i>Verticillium</i> sp		<i>Humicola</i> sp.	
		300 nm	500 nm	300 nm	500 nm	300 nm	500 nm	300 nm	500 nm	300 nm	500 nm
1	Glucose	41.7	58.6	57.3	83.8	21.3	94.6	68.4	23.3	31.6	57.6
2	Fructose	36.4	48.4	48.4	74.6	16.7	83.7	73.6	24.7	36.4	56.8
3	Xylose	23.7	23.6	24.6	21.3	6.8	51.3	31.6	16.1	13.2	21.7
4	Sucrose	41.0	63.1	54.7	76.5	20.5	107.3	70.5	31.4	31.6	67.3
5	Maltose	12.2	63.4	26.8	56.4	18.3	76.4	31.6	21.3	16.3	26.3
6	Galactose	26.8	23.7	31.8	52.3	11.6	34.9	44.2	17.1	11.4	17.3
7	Lactose	18.4	20.6	31.6	26.7	5.2	38.7	40.1	15.9	10.8	14.6
8	Arabinose	23.5	21.3	27.5	22.6	6.7	17.3	31.9	7.6	11.8	6.1
9	Glycerol	24.2	31.8	36.8	61.3	17.6	57.1	42.4	25.3	16.7	27.6
10	Sorbitol	27.3	31.6	30.7	21.6	14.5	31.6	43.6	19.8	15.6	28.4
11	Mannitol	31.6	68.7	45.4	73.7	23.9	73.6	58.4	26.1	31.6	50.6
12	Starch	36.7	71.6	43.6	81.3	19.5	51.7	68.9	21.4	33.7	46.7
13	Ethanol	28.4	56.3	36.7	57.3	4.6	73.1	14.6	7.8	16.3	12.7
SE		0.280	0.693	0.080	0.593	3.903	0.928	0.113	0.795	0.126	1.160
CD($P=0.05$)		0.574	1.426	0.163	1.220	8.024	1.909	0.232	1.634	0.259	2.384

* One unit of pigment is defined as equivalent to 0.1 OD of an absorbance either at 300 nm and 500 nm.

Fig.2. Effect of carbon sources on the intracellular pigment production in fungi

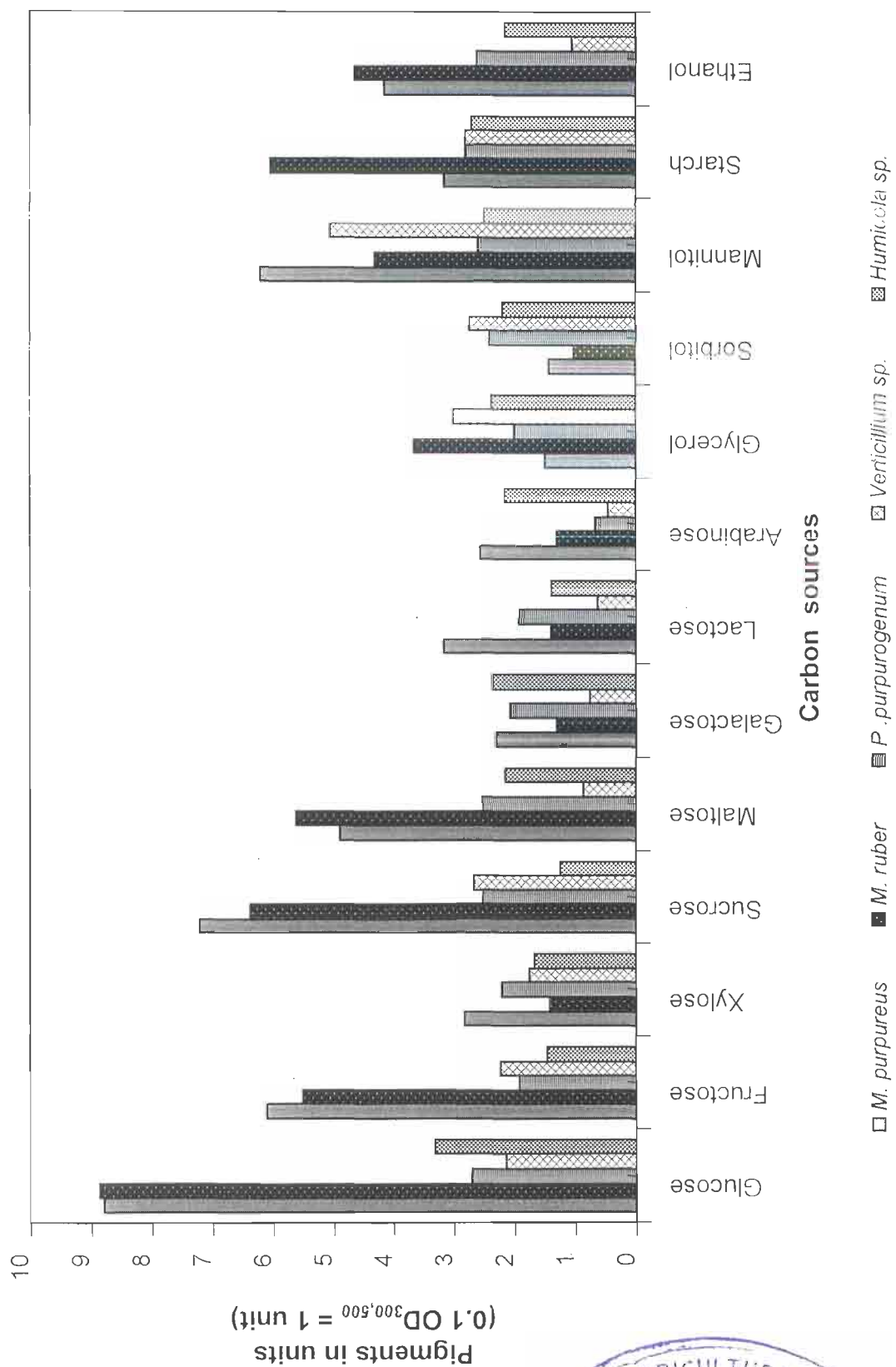
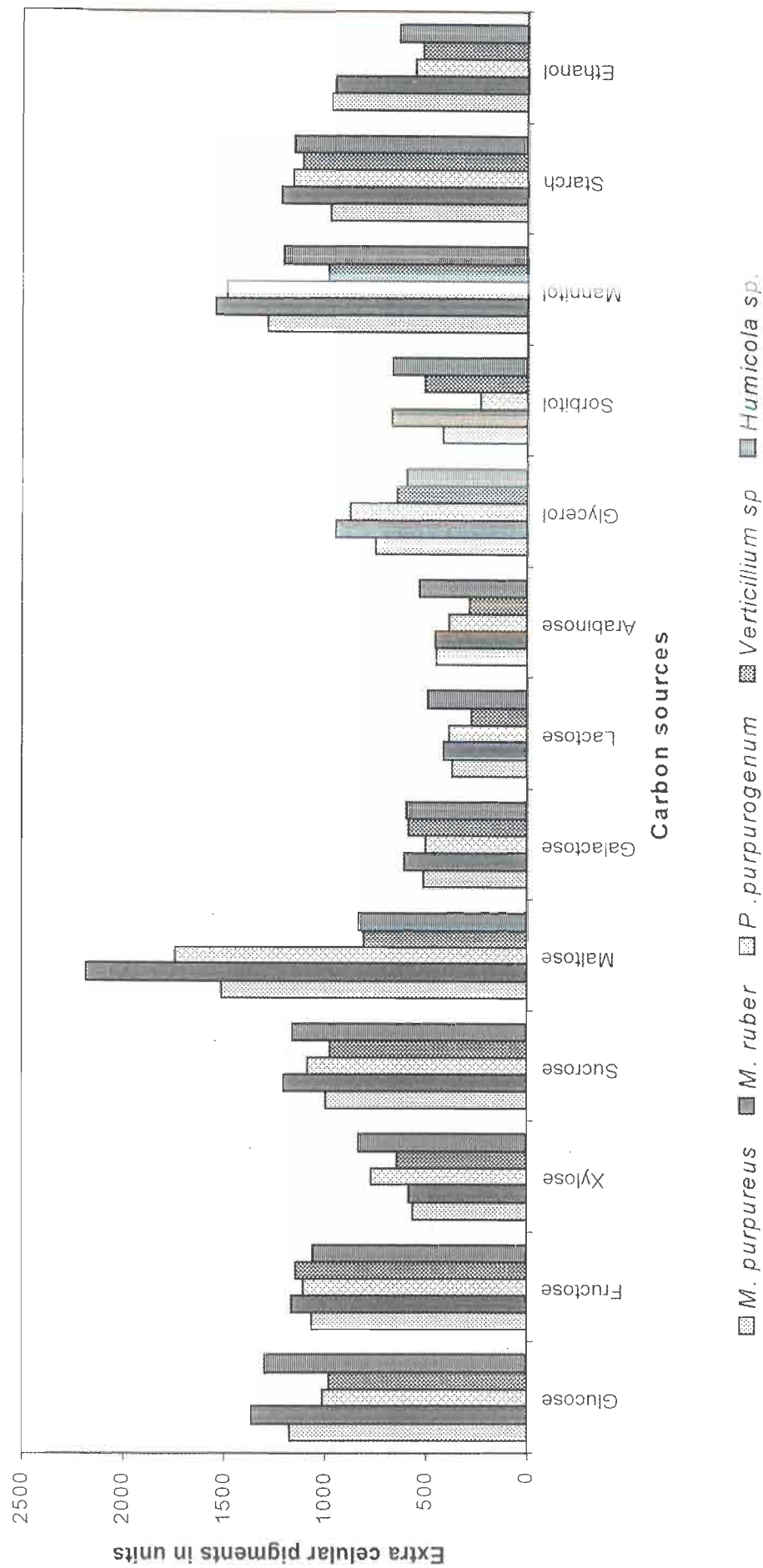


Table 8. Effect of different carbon sources on the production of extracellular pigments in fungi.

No	Carbon source	Extracellular pigments in units*									
		<i>M. purpureus</i>		<i>M. ruber</i>		<i>P. purpurogenum</i>		<i>Verticillium</i> sp.		<i>Humicola</i> sp.	
		300 nm	500 nm	300 nm	500 nm	300 nm	500 nm	300 nm	500 nm	300 nm	500 nm
1	Glucose	531	643	631	733	167	844	634	346	468	831
2	Fructose	483	584	517	647	138	967	767	376	393	667
3	Xylose	237	327	214	368	117	653	376	267	367	468
4	Sucrose	397	596	541	667	146	936	513	461	376	782
5	Maltose	314	1201	627	1556	108	1631	466	338	437	396
6	Galactose	206	304	136	468	67	432	367	216	227	367
7	Lactose	167	203	147	261	48	335	186	87	106	385
8	Arabinose	178	267	128	324	56	325	147	136	117	417
9	Glycerol	281	467	319	626	137	738	376	267	232	363
10	Sorbitol	137	276	187	479	68	163	336	168	277	386
11	Mannitol	564	723	576	967	176	1312	567	417	434	769
12	Starch	415	556	585	631	139	1021	646	466	474	678
13	Ethanol	336	631	416	532	87	463	387	129	213	417
SE		19.29	22.41	57.66	25.48	34.52	24.87	61.71	16.44	25.25	15.81
CD($P_o=0.05$)		39.52	46.07	118.1	52.39	70.72	51.12	126.4	33.80	51.74	32.49

* One unit of pigment is defined as equivalent to 0.1 O.D. of light absorption either at 300 or 500 nm

Fig. 3 . Effect of different sources of carbon on the production of extracellular pigments in fungi



All the carbon sources used in the study helped producing extracellular pigments. However, with glucose, mannitol, starch and maltose as carbon sources the pigment production was higher than with other sources of carbon. The data further revealed that the quantity of yellow pigments (at 300 nm) was always lower than the red pigments (at 500 nm) with the exception of the fungus *Verticillium* sp. Maltose appears to be the preferred carbon source for high yields of pigments in *Monascus* spp.

Both biomass production and the extracellular pigments in the medium were positively correlated (Appendix IIa).

As a conclusion of this experiment, all the carbon sources are supporting the growth of the selected fungi with varying growth rate and pigment production. Glucose sucrose, starch, mannitol and maltose were commonly increasing the pigment production and biomass.

4.4.2. Effect of different sources of nitrogen on biomass and pigment production

As many as 14 sources of nitrogen were used in the study. These includes nitrate nitrogen, ammoniacal nitrogen and organic source of nitrogen. As detailed earlier, the data on the biomass production and pigment synthesis are set out in the Tables 9 to 12 and Figures 4 to 6.

4.4.2.1. Effect of different nitrogen sources on the biomass production

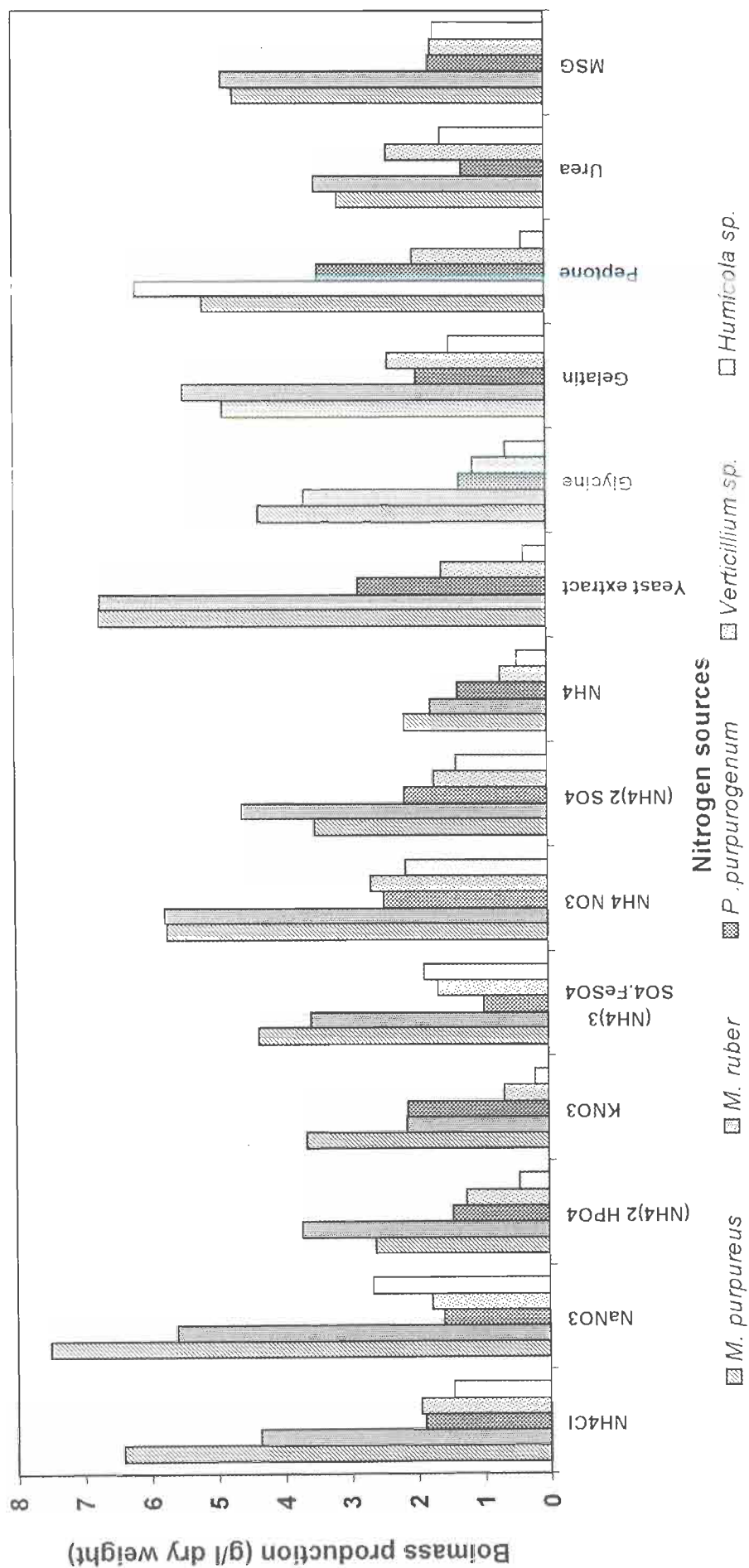
The data on the influence of different sources of nitrogen on the biomass production of the selected fungi are presented on the Table 9 and Figure 4.

Table 9. Effect of different sources of nitrogen on the biomass production.

No	Sources of nitrogen	Biomass production (g l ⁻¹ dry weight)				
		<i>M. purpureus</i>	<i>M. ruber</i>	<i>P. purpureogenum</i>	<i>Verticillium</i> sp.	<i>Humicola</i> sp.
1	Ammonium chloride	6.41	4.36	1.89	1.95	1.46
2	Sodium nitrate	7.50	5.61	1.60	1.77	2.67
3	Di ammonium phosphate	2.61	3.73	1.46	1.25	0.46
4	Potassium nitrate	3.64	2.14	2.13	0.68	0.21
5	Ammonium ferrous sulphate	4.37	3.56	0.98	1.67	1.87
6	Ammonium nitrate	5.73	5.76	2.48	2.67	2.14
7	Ammonium sulphate	3.50	4.61	2.15	1.71	1.37
8	Ammonia	2.16	1.76	1.35	0.71	0.46
9	Yeast extract	6.73	6.71	2.83	1.58	0.36
10	Glycine	4.33	3.64	1.32	1.11	0.61
11	Gelatin	4.86	5.46	1.95	2.37	1.46
12	Peptone	5.16	6.17	3.43	1.99	0.36
13	Urea	3.13	3.47	1.25	2.38	1.57
14	Monosodium glutamate	4.69	4.86	1.74	1.71	1.67
SE		0.0132	0.0130	0.3918	0.0377	0.0514
CD(<i>Po</i> = 0.05)		0.0269	0.0251	0.7981	0.0769	0.0148

Growth period five days at static conditions

Fig. 4. Effect of different sources of nitrogen on the biomass production



In general, organic sources of nitrogen supported better growth of the fungi than inorganic nitrogen sources. Yeast extract and peptone recorded more biomass yield. Among the inorganic sources of nitrogen, ammonium nitrate was found to be better suited for higher biomass production for all the fungi.

Analysis of the data reveals that in general fungal biomass is positively correlated with the pigment production in all the five fungi (Appendix II b).

4.4.2.2 Effect of different nitrogen sources on the pH of growth media

The data on the changes of pH of growth media as influenced by fungal growth are presented in Table 10.

Due to the growth of fungi, it is very common to observe changes in the pH. The different nitrogen sources also influence the pH changes. Sodium nitrate shifted the pH 5.5 to pH 8.0.

pH shifts of the growth medium bear a positive correlation with biomass of the selected fungi (Appendix IIb).

4.4.2.3. Effect of different nitrogen sources on the intracellular pigment production

The data on the influence of different nitrogen sources on intracellular pigment production are presented in Table 11 and Figure 5.

In general, all the nitrogen sources supported intracellular pigmentation production. The organic source of nitrogen was better than the inorganic sources. Monosodium glutamate strikingly increased the internal pigments of all the fungi.

Table 10 . Effect of different sources of nitrogen on the pH shift of the medium.

No	Sources of nitrogen	pH shifts *				
		<i>M. purpureus</i>	<i>M. ruber</i>	<i>P. purpureogenum</i>	<i>Verticillium</i> sp	<i>Humicola</i> sp.
1	Ammonium chloride	4.41	3.52	4.26	3.73	4.32
2	Sodium nitrate	7.57	8.48	6.92	7.79	8.14
3	Di ammonium phosphate	6.23	5.82	6.47	3.71	6.14
4	Potassium nitrate	7.83	7.26	6.78	6.87	7.63
5	Ammonium ferrous sulphate	5.81	5.87	6.39	7.50	6.78
6	Ammonium sulphate	4.41	5.30	2.77	3.55	4.38
7	Ammonium nitrate	5.92	6.50	6.32	7.07	6.63
8	Ammonia solution	3.32	3.24	5.42	5.89	3.36
9	Yeast extract	7.53	7.56	4.30	7.41	7.47
10	Glycine	5.51	5.15	5.15	5.14	5.03
11	Gelatin	7.25	7.38	6.56	6.91	7.17
12	Peptone	7.15	7.44	6.25	6.21	6.15
13	Urea	6.80	5.94	7.42	6.83	7.14
14	Monosodium glutamate	6.13	7.59	7.30	3.25	6.79

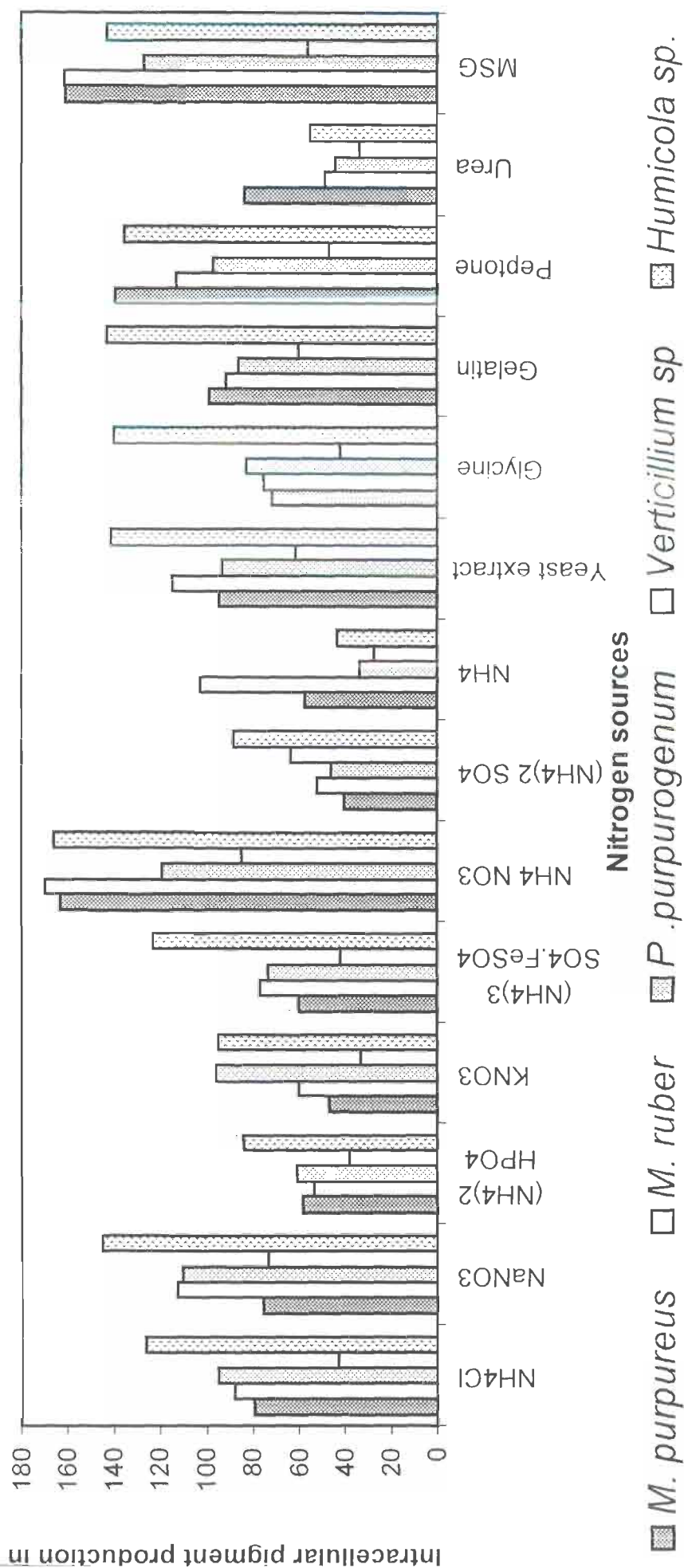
* Initial pH : 5.5

Table 11. Effect of different sources of nitrogen on the intracellular pigments production in fungi

No	Sources of nitrogen	Intracellular pigments units*									
		<i>M. purpureus</i>		<i>M. ruber</i>		<i>P. purpurogenum</i>		<i>Verticillium</i> sp		<i>Humicola</i> sp.	
		300 nm	500 nm	300 nm	500 nm	300 nm	500 nm	300 nm	500 nm	300 nm	500 nm
1	Ammonium chloride	37.9	41.6	47.6	40.3	17.1	77.8	16.5	26.4	57.7	68.7
2	Sodium nitrate	43.6	32.1	51.3	61.3	26.6	83.6	27.0	46.5	68.9	76.2
3	Di ammonium phosphate	16.4	41.8	21.6	31.6	16.8	44.1	14.8	23.1	52.1	31.8
4	Potassium nitrate	24.7	22.0	16.7	43.1	22.6	73.2	16.8	16.3	46.8	48.4
5	Ammonium ferrous sulphate	16.5	43.4	20.4	56.7	28.6	44.6	13.4	28.4	73.5	49.3
6	Ammonium nitrate	72.0	91.3	86.4	83.6	31.3	87.6	37.6	47.5	86.8	79.3
7	Ammonium sulphate	17.2	23.3	14.6	37.7	14.6	31.3	14.7	48.7	44.9	43.6
8	Ammonia solution	12.0	45.3	15.2	87.6	17.3	16.4	9.6	17.8	26.3	16.7
9	Yeast extract	24.9	69.5	46.8	67.7	16.3	76.7	24.6	36.7	59.7	81.7
10	Glycine	14.6	57.2	16.6	58.3	13.7	68.9	25.7	16.4	66.8	73.3
11	Gelatin	34.1	64.9	23.8	67.3	12.7	73.2	28.6	31.8	71.3	71.4
12	Peptone	35.0	104.2	41.8	71.3	15.8	81.4	25.5	21.3	73.6	61.8
13	Urea	18.5	64.8	16.7	31.9	7.8	36.4	16.1	17.2	37.8	17.3
14	Monosodium glutamate	68.3	93.0	63.8	97.6	17.7	109.3	36.8	19.4	71.2	71.6
SE		4.084	4.437	1.386	1.189	0.964	2.153	0.771	0.815	1.371	0.967
CD ($P_0 = 0.05$)		8.329	9.039	2.823	2.423	1.964	4.386	1.570	1.660	2.794	1.970

* One unit of pigment is defined as equivalent to 0.1 OD light absorption either at 300nm or 500 nm.

Fig.5. Effect of different nitrogen sources on intracellular pigments production in fungi.



Among the inorganic sources, ammonium nitrate yielded higher quantity of pigments in all the fungi. Interestingly, monosodium glutamate yielded the highest amount of pigments among the different sources of nitrogen.

The accumulation of both yellow and red pigments was positively correlated with fungal biomass yield in the presence of different nitrogen sources (Appendix IIb)

4.4.2.4. Effect of different sources of nitrogen on the extracellular pigment production

The data on the effect of different sources of nitrogen on the excretion of extracellular pigments are furnished in the Table 12 and Figure 6.

The data show that all nitrogen sources tested either organic or inorganic supported pigment secretion. Among the organic nitrogen sources, monosodium glutamate was found to yield the highest amount of pigment. Peptone and gelatin were also good enough. Among the inorganic sources of nitrogen, nitrate ranked first in the excretion of pigments.

Correlation studies indicated that the yield of extracellular pigments and fungal biomass production were positively correlated but at lower per cent (Appendix IIb)

4.4.3. Effect of pH of growth medium on pigment production

The data on the influence of pH of the growth medium on pigment production and biomass yield of selected fungi are set out in Tables 13 to 15 and Figures 7 and 8.

4.4.3.1. Effect of pH on the biomass production

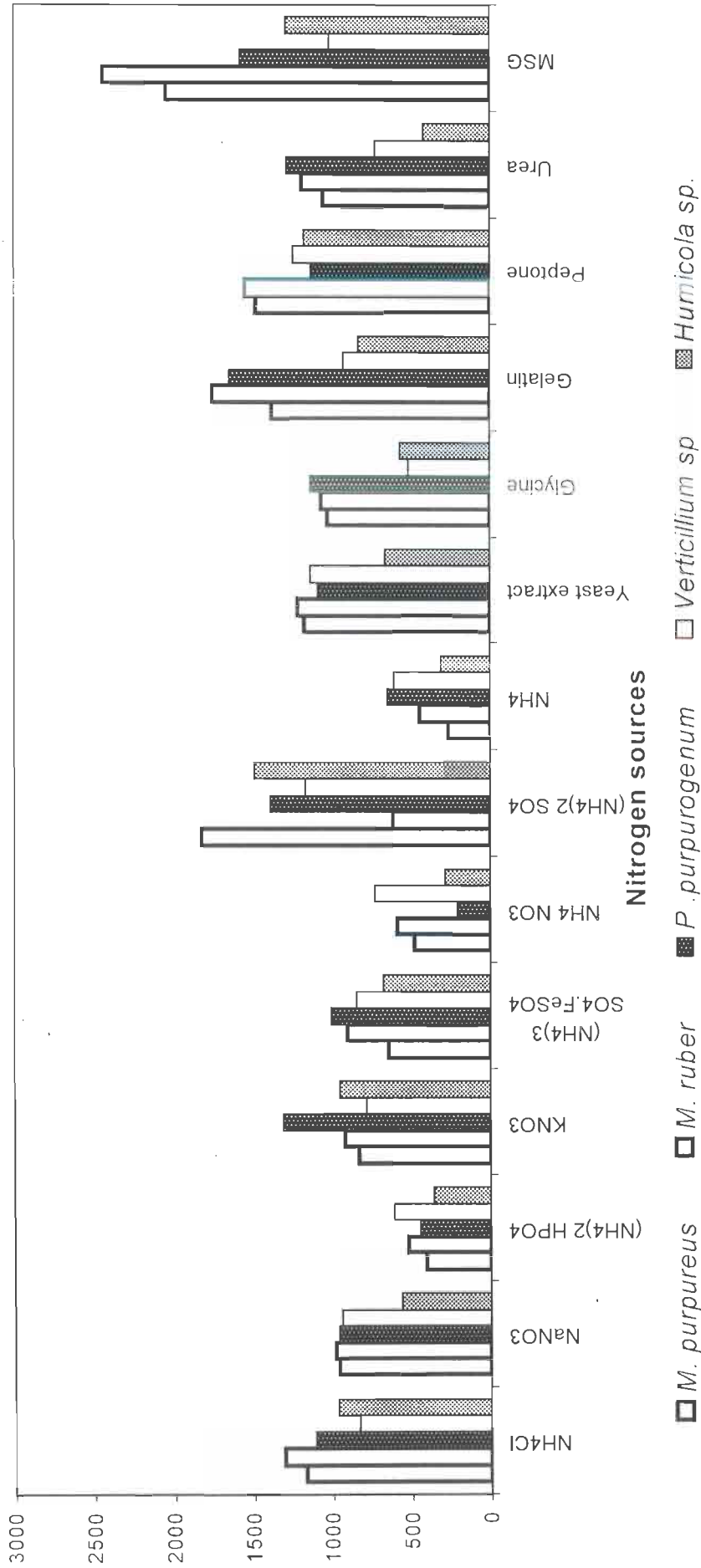
pH of the growth medium has a direct impact on biomass (growth) of fungi (Table 13).

Table 12. Effect of different sources of nitrogen on the extracellular pigment production by fungi

No	Sources of nitrogen	Extracellular pigments in units*									
		<i>M. purpureus</i>		<i>M. ruber</i>		<i>P. purpurogenum</i>		<i>Verticillium</i> sp.		<i>Humicola</i> sp.	
		300 nm	500 nm	300 nm	500 nm	300 nm	500 nm	300 nm	500 nm	300 nm	500 nm
1	Ammonium chloride	236	933	319	983	246	861	513	319	236	731
2	Sodium nitrate	414	547	467	514	147	814	674	264	148	416
3	Di ammonium phosphate	138	267	336	185	134	312	375	231	216	143
4	Potassium nitrate	367	466	384	537	396	913	517	267	317	634
5	Ammonium ferrous sulphate	161	481	345	563	263	742	682	165	264	412
6	Ammonium sulphate	167	315	376	213	67	141	611	118	58	225
7	Ammonium nitrate	415	1406	468	150	418	974	755	413	417	1074
8	Ammonia solution	265	—	232	216	186	463	476	132	163	147
9	Yeast extract	412	764	544	673	370	715	767	364	246	416
10	Glycine	331	695	555	514	367	769	368	145	254	317
11	Gelatin	436	946	612	1143	413	1236	646	276	217	613
12	Peptone	513	967	814	732	220	906	767	475	233	941
13	Urea	364	687	473	715	365	920	511	211	274	144
14	Monosodium glutamate	533	1512	757	1687	263	1316	768	244	319	967
SE		37.28	6.783	14.522	6.864	6.749	7.394	36.697	28.596	10.253	6.164
CD($p = 0.05$)		75.95	13.81	29.582	13.98	13.74	15.064	74.752	58.250	20.886	12.55

* One unit of pigment is defined as equivalent to 0.1 OD at 300 nm or 500 nm light absorption

Fig.6. Effect of different nitrogen sources on the extracellular pigment production in fungi.



In general, pH of the medium set at 5.0 and at 6.0 supported higher biomass production in the fungi. A slight acidic pH of the medium decreased biomass production in the same way as alkaline pH of the medium reduced biomass yield.

Critical analysis of data has shown that the increase in pH was negatively correlated with biomass production (Appendix II c)

4.4.3.2. Effect of pH of growth medium on intracellular pigment production

The data on the influence of pH of growth medium on pigment production (intracellular) are presented in Table 14 and Figure 7.

Yellow pigment production significantly encouraged at pH of 5.0, where as red pigments were high at pH 6.0 in *M. purpureus* and *M. ruber*. But in the case of *Penicillium purpurogenum*, *Verticillium* sp. and *Humicola* sp. pH 6.0 appeared conducive for yellow and red pigment accumulation.

Critical scrutiny of the data further indicated that the accumulation of internal pigments in the fungal mycelium is negatively correlated with increase in the pH of the growth medium (Appendix II c).

4.4.3.3. Effect of pH of growth medium on the accumulation of extracellular pigments

The data on the influence of pH of the growth medium on the accumulation of extracellular pigments are set out in the Table 15 and Figure 8.

Table 13. Effect of pH on biomass production by the fungi

pH of the medium	Biomass production (g l ⁻¹ on dry weight basis)				
	<i>M. purpureus</i>	<i>M. ruber</i>	<i>P. purpurogenum</i>	<i>Verticillium</i> sp.	<i>Humicola</i> sp.
4.0	4.16	5.13	3.14	4.13	2.17
5.0	6.78	7.81	4.16	5.16	3.68
6.0	7.69	6.63	3.14	4.78	4.79
7.0	5.14	5.47	2.67	3.74	3.68
8.0	4.03	5.14	2.46	3.41	2.18
9.0	3.14	4.36	2.03	2.96	1.36
SE	1.312	1.123	1.245	1.472	1.213
CD(<i>P</i> ₀ =0.05)	2.859	2.447	2.714	3.208	2.644

Table 14. Effect of different medium pH on the intracellular pigments production in the pigment producing fungi.

pH	Intracellular pigments in units*									
	<i>M. purpureus</i>		<i>M. ruber</i>		<i>P. purpureogenum</i>		<i>Verticillium sp</i>		<i>Humicola sp.</i>	
	300 nm	500 nm	300 nm	500 nm	300 nm	500 nm	300 nm	500 nm	300 nm	500 nm
4.0	41.6	68.4	42.6	73.4	17.4	74.5	46.7	24.6	13.7	76.3
5.0	36.7	71.9	56.7	98.2	18.6	73.6	48.3	27.3	14.8	79.4
6.0	31.4	78.3	30.6	113.4	21.8	86.7	81.7	36.1	18.2	86.7
7.0	26.3	64.5	21.6	71.9	16.7	66.4	56.8	25.4	12.6	61.3
8.0	21.4	59.6	18.3	68.8	11.4	58.7	36.1	21.5	10.3	62.4
9.0	16.8	41.1	17.8	51.7	8.7	55.3	27.4	18.6	8.5	58.3
SE	5.386	2.614	2.742	2.662	2.725	2.848	2.733	2.853	2.733	2.607
CD($P=0.05$)	11.736	5.695	5.975	5.800	5.937	6.206	5.956	6.216	5.956	5.680

* One unit of pigment is defined as equivalent to 0.1 OD at 300 nm or 500 nm light absorption

Table 15. Effect of pH on the extracellular pigments production by fungi.

pH	Extracellular pigments in units *									
	<i>M. purpureus</i>		<i>M. ruber</i>		<i>P. purpureogenum</i>		<i>Verticillium sp</i>		<i>Humicola sp.</i>	
	300 nm	500 nm	300 nm	500 nm	300 nm	500 nm	300 nm	500 nm	300 nm	500 nm
4.0	234	461	443	649	176	796	617	316	157	814
5.0	436	536	516	786	216	861	746	417	263	816
6.0	361	749	417	983	181	871	816	517	276	831
7.0	214	689	347	786	156	643	704	403	214	746
8.0	189	436	276	659	101	561	547	336	186	836
9.0	67	291	196	346	71	291	370	259	112	514
SE	7.273	7.570	7.299	58.114	8.028	7.931	7.992	12.700	13.312	7.903
CD($P=0.05$)	15.847	16.495	15.904	126.621	17.492	17.280	17.415	27.672	29.005	17.220

* One unit of pigment is defined as equivalent to 0.1 OD at 300 nm or 500 nm light absorption

Fig.7. Effect of pH on the production of intracellular pigments in fungi

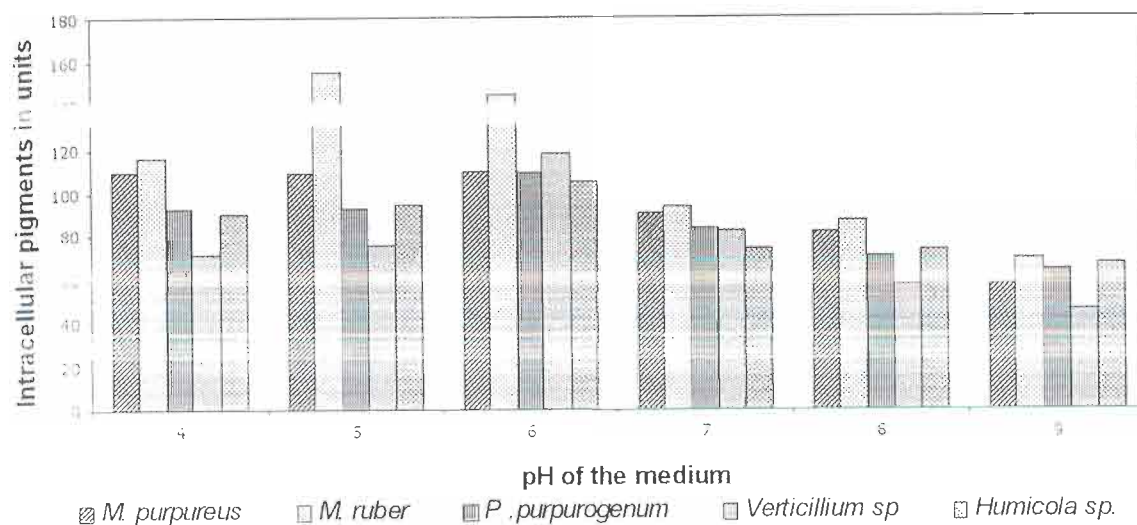
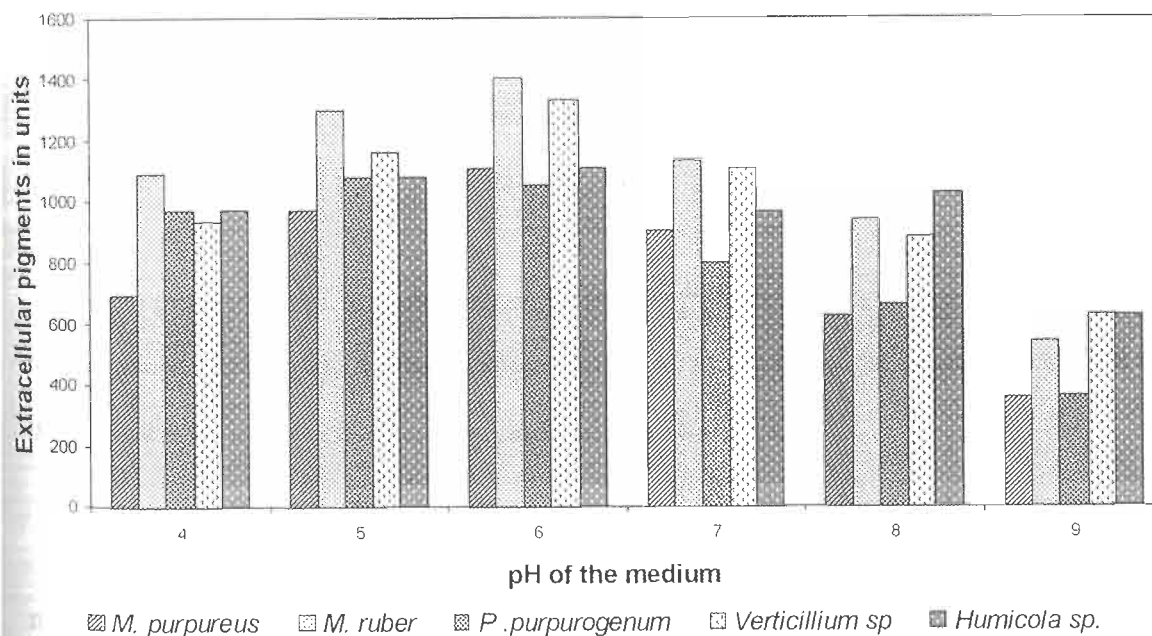


Fig. 8. Effect of pH on the extracellular pigments production in fungi.



pH 6.0 appears significantly better for the production of pigments in the medium of the fungi. The yield of yellow pigment was however high at pH 5.0 in *M. purpureus*, *M. ruber* and *P. purpurogenum*. Other pH levels of the medium reduced pigment yield.

Correlation analysis of the data revealed that the extracellular pigments accumulation is highly negatively correlated with pH levels (Appendix II c).

It may be inferred from this experiment that at pH 5.0 and 6.0, the biomass yield of fungi was high. In addition, intracellular pigments and extracellular pigments production were high.

4.4.4. Effect of temperature on pigment production

The data on the effect of incubation temperature on pigment production and growth of fungi are presented in Tables 16 to 18 and Figures 9 and 10.

4.4.4.1. Effect of temperature on biomass production

Table 16 reveals the influence of growth temperature on biomass production of the selected fungi.

In general, 35°C appears optimum for the growth of all the selected fungi. Low temperature (15°C) and higher temperature (45°C) reduced biomass yield. It is of interest to note that *Verticillium* sp. does not grow at 15°C.

Critical scrutiny of the data shows that growth temperature of fungi bore a negative correlation with biomass yield of fungi (Appendix II d).

Table 16. Effect of temperature on biomass production by fungi.

Temperature (°C)	Biomass production (g l ⁻¹ dry weight)				
	<i>M. purpureus</i>	<i>M. ruber</i>	<i>P. purpurogenum</i>	<i>Verticillium</i> sp	<i>Humicola</i> sp.
15	3.59	2.63	1.96	-	1.41
35	7.29	6.87	3.31	3.78	3.85
45	1.36	1.71	1.61	2.16	1.03
SE	0.156	0.128	0.158	0.040	0.158
CD($P_0 = 0.05$)	0.382	0.315	0.387	0.099	0.387

4.4.4.2. Effect of temperature on intracellular pigment production

The data on the effect of growth temperature on the accumulation of cellular pigments in the mycelium of fungi are set out in the Table 17 and Figure 9.

All the fungi, in general produced both yellow and red pigments in high quantity at a growth temperature of 35°C. At 15°C and 45°C, accumulation of pigments was markedly affected.

Correlation analysis of the data revealed that temperature increase and pigment accumulation within the mycelium of fungi were negatively correlated (Appendix II d).

4.4.4.3. Effect of temperature on extracellular pigments

The data on the effect of growth temperature on extracellular pigments production are presented in Table 18 and Figure 10.

For all the fungi studied, growth temperature of 35°C appears optimum for the secretion of pigments in maximum quantity. *M. ruber* interestingly at 45°C, produced higher quantity of pigments than the others.

Statistical scrutiny of the data has indicated that the extracellular pigments production is negatively correlated with the increase in temperature (Appendix II d).

Summing up the results it may be said that, the optimum temperature for the highest biomass production and pigment production is 35°C. Other temperature levels (15°C and 45°C) significantly reduced the growth and pigment yield of the selected fungi.

Table 17. Effect of temperature on intracellular pigments production in fungi

Temperature (°C)	Intracellular pigments in units *									
	<i>M. purpureus</i>		<i>M. ruber</i>		<i>P. purpureogenum</i>		<i>Verticillium</i> sp		<i>Humicola</i> sp.	
	300 nm	500 nm	300 nm	500 nm	300 nm	500 nm	300 nm	500 nm	300 nm	500 nm
15	23.6	41.6	28.7	64.9	11.6	54.6	-	-	11.6	14.6
35	48.3	71.6	56.3	86.3	14.1	73.4	31.3	23.6	21.6	73.6
45	13.7	21.8	12.6	28.4	8.11	26.7	16.4	14.3	17.4	24.8
SE	6.004	0.765	1.114	2.103	2.733	0.940	0.876	0.816	0.884	1.066
CD($P_o = 0.05$)	14.692	1.871	2.727	5.147	6.689	2.301	2.145	1.998	2.164	2.608

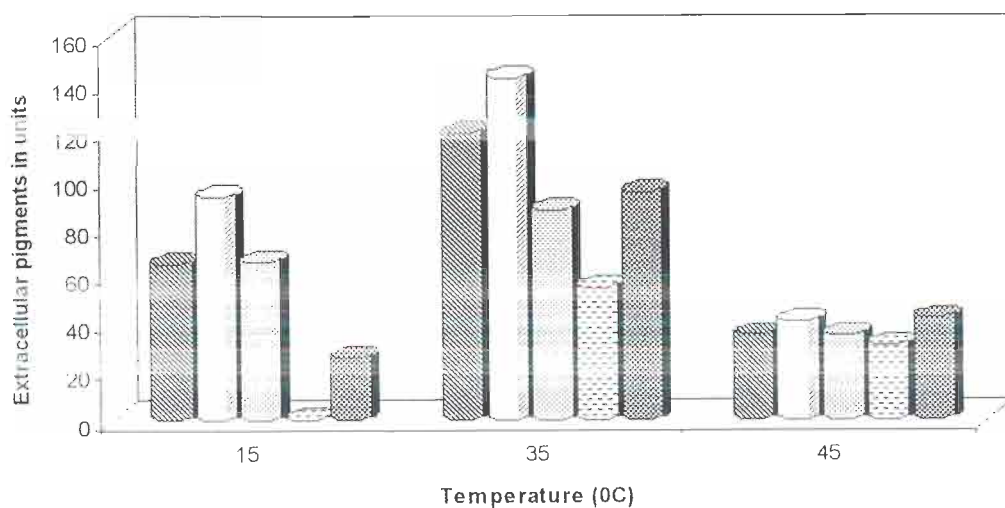
* One unit of pigment is defined as equivalent to 0.1 OD at 300 nm or 500 nm light absorption

Table 18. Effect of temperature on the extracellular pigments production in fungi.

Temperature (°C)	Extracellular pigments in units *									
	<i>M. purpureus</i>		<i>M. ruber</i>		<i>P. purpureogenum</i>		<i>Verticillium</i> sp		<i>Humicola</i> sp.	
	300 nm	500 nm	300 nm	500 nm	300 nm	500 nm	300 nm	500 nm	300 nm	500 nm
15	167	446	272	778	169	668	-	-	187	367
35	468	873	678	1318	236	1216	889	436	232	1147
45	238	113	434	763	178	478	387	316	158	432
SE	7.542	7.333	8.249	7.333	7.800	6.825	6.908	5.784	6.825	19.200
CD($P_o = 0.05$)	18.455	17.945	20.186	17.945	19.088	16.701	16.904	14.153	16.701	46.984

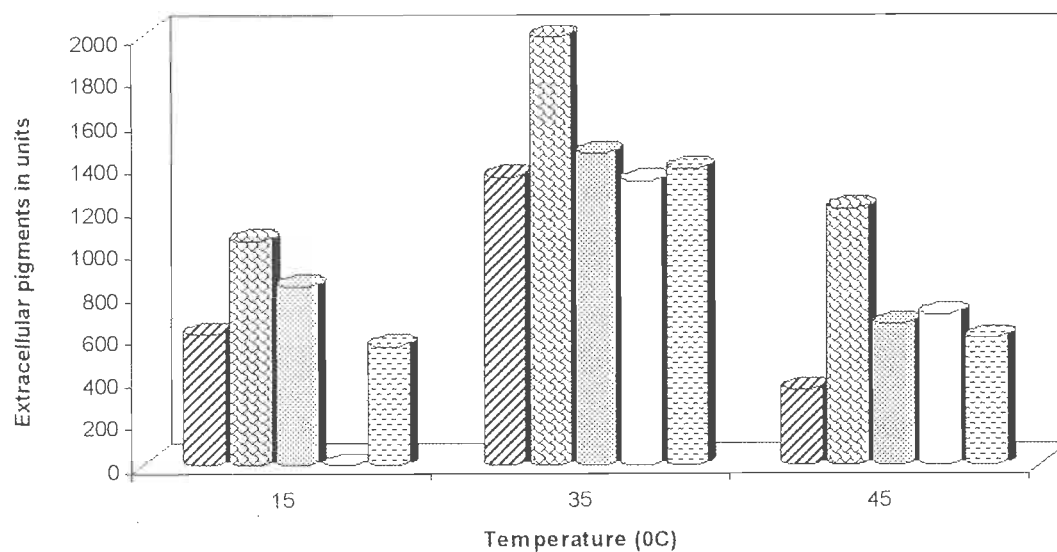
* One unit of pigment is defined as equivalent to 0.1 OD at 300 nm or 500 nm light absorption

Fig. 9 . Effect of temperature on the production of intracellular pigments in fungi



▨ *M. purpureus* □ *M. ruber* ▩ *P. purpureogenum* ▤ *Verticillium sp* ▦ *Humicola sp.*

Fig.10 . Effect of different temperature on the production of extracellular pigments production in fungi.



▨ *M. purpureus* □ *M. ruber* ▩ *P. purpureogenum* ▤ *Verticillium sp* ▦ *Humicola sp.*

4.4.5. Effect of agitation/aeration on pigment production in fungi

The data on agitation or aeration of growth medium on the growth and pigment production of fungi are presented in Tables 19 to 22 and Figures 11 and 12.

4.4.5.1. Effect of agitation on the biomass production

The data on the agitation of growth medium on the biomass production in the selected fungi was presented in the Table 19.

In general, aeration helped fungi to produce more biomass as the rate of agitation increased from 100 to 150 strokes per min. Further increase in agitation tended to decrease fungal growth. The results have further indicated that 150 revolutions per min was good for biomass yield.

Statistical analysis of data revealed that the agitation speed is negatively correlated with the biomass yield (Appendix II e).

4.4. 5. 2. Effect of agitation on the morphology of fungi

The observations on the morphological changes on the mycelium of fungi at different agitation rates are presented in Table 20.

In general, as agitation speed increased the fungal mycelium undergoes pelletization. At lower speed, however the biomass assumed a pulp consistency. Further, the size of the mycelial pellet tended to decrease with speed of agitation. Compactness of the pellets also increased with speed of agitation.

4.4.5.3. Effect of agitation on intracellular pigment production

The data on the effect of agitation speed on the production cellular pigments are presented in Table 21 and Figure 11.

Table 19. Effect of agitation on the biomass production by fungi.

Agitation (strokes per minute)	Biomass production (g l ⁻¹ dry weight)				
	<i>M. purpurineus</i>	<i>M. ruber</i>	<i>P. purpurogenum</i>	<i>Verticillium</i> sp	<i>Humicola</i> sp.
100	5.78	5.68	4.57	2.64	5.14
150	7.27	8.61	7.57	4.16	3.61
200	3.85	6.80	5.73	2.16	2.61
250	3.34	3.47	3.16	2.14	1.43
SE	0.639	0.349	0.354	0.272	0.273
CD(<i>Pc</i> = 0.05)	1.474	0.805	0.816	0.628	0.630

Table 20 . Effect of agitation on the morphology of selected fungi.

Strokes per min	<i>M.purpureus</i>	<i>M. ruber</i>	<i>P. purpurogenum</i>	<i>Verticillium</i> sp.	<i>Humicola</i> sp.
100	Pulp	Pulp	Pulp	Pellet	Pulp
150	Big and loose pellet	Big and loose pellet	Small and loose pellet	Small and compact pellet	Small pellet
200	Small and compact pellet	Small and compact pellet	Small and loose pellet	Small and compact pellet	Small and compact pellet
250	Small and compact pellet	Small and compact pellet	Small and compact pellet	Small and compact pellet	Small and compact pellet

Table 21. Effect of agitation on intracellular pigments production by fungi.

Agitation (strokes per minute)	Intracellular pigments in units *									
	<i>M. purpureus</i>		<i>M. ruber</i>		<i>P. purpureogenum</i>		<i>Verticillium</i> sp		<i>Humicola</i> sp.	
	300 nm	500 nm	300 nm	500 nm	300 nm	500 nm	300 nm	500 nm	300 nm	500 nm
100	18.6	31.6	26.9	43.6	16.4	48.7	24.6	17.6	27.3	58.7
150	7.8	27.6	23.8	40.7	10.5	43.6	28.7	17.7	13.6	46.4
200	6.3	23.1	18.5	26.4	5.7	23.7	26.4	10.6	10.1	14.7
250	6.1	18.4	15.4	27.8	4.4	16.3	17.7	8.3	8.3	10.8
SE	1.421	1.342	2.059	1.396	1.190	1.472	1.373	1.373	1.312	1.194
CD($P_o = 0.05$)	3.277	3.095	4.479	3.220	2.744	3.392	3.166	3.166	3.025	2.754

* One unit of pigment is defined as equivalent to 0.1 OD at 300 nm or 500 nm light absorption.

Table 22. Effect of agitation on extracellular pigments production by fungi.

Agitation (strokes per minute)	Extracellular pigments in units *									
	<i>M. purpureus</i>		<i>M. ruber</i>		<i>P. purpureogenum</i>		<i>Verticillium</i> sp		<i>Humicola</i> sp.	
	300 nm	500 nm	300 nm	500 nm	300 nm	500 nm	300 nm	500 nm	300 nm	500 nm
100	249	367	246	766	176	684	478	416	434	618
150	414	873	347	936	467	765	614	613	168	837
200	567	923	668	1106	368	647	987	498	176	964
250	314	714	233	869	213	613	943	414	184	234
SE	1.446	1.571	1.220	1.232	1.342	1.232	1.232	1.264	2.406	1.401
CD($P_o = 0.05$)	3.334	3.624	2.815	2.843	3.095	2.843	2.843	2.916	5.549	2.232

* One unit of pigment is defined as equivalent to 0.1 OD at 300 nm or 500 nm light absorption.

Fig.11. Effect of agitation on intracellular pigment production in fungi

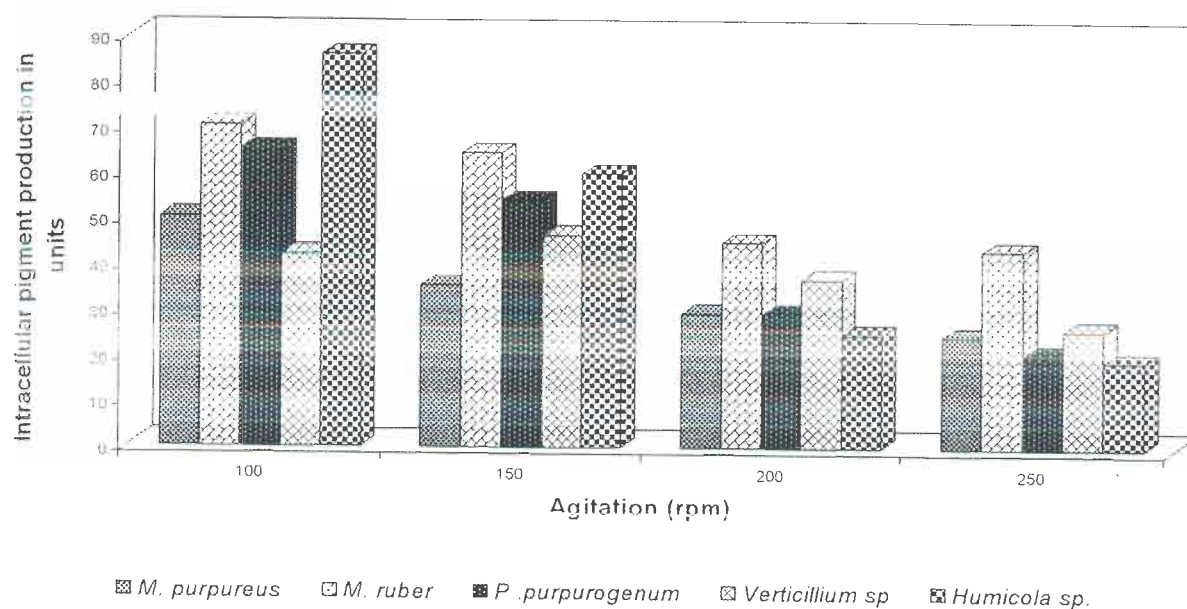
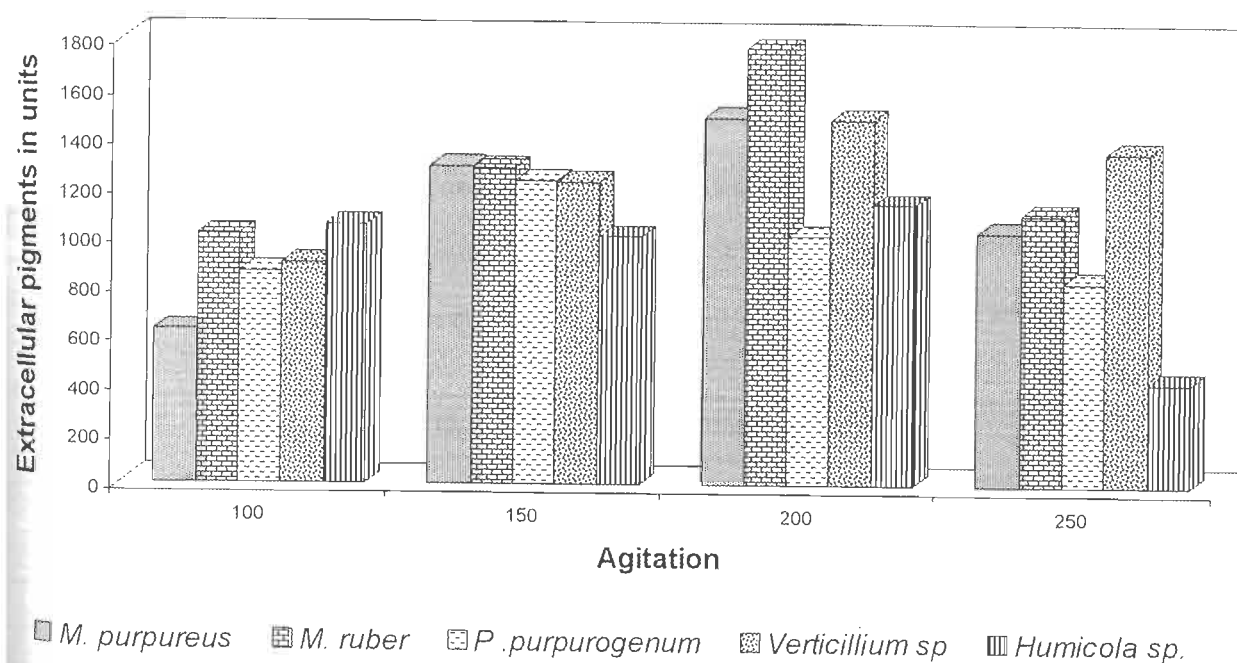


Fig.12. Effect of agitation on extracellular pigments production by fungi



The data revealed as the speed of agitation increased the yield of internal pigments of the fungi got reduced.

Statistical scrutiny of data revealed that the agitation speed and the yield of internal pigments of the mycelium were negatively correlated (Appendix II e).

4.4.5.4. Effect of agitation on extracellular pigment production

The results on the influence of agitation speed on the production of extracellular pigments are presented in the Table 22 and Figure 12.

As the rate of agitation increased, the extracellular pigments yield of the selected fungi significantly increased.

Further, analysis of data also indicated that the speed of agitation is positively correlated with the accumulation of pigments (Appendix II e)

Summing up, it may be concluded that agitation of the growth medium at 200 strokes per min. significantly increased biomass production and intra and extra cellular pigments. The biomass production was high at 150 strokes per min.

4.4.6. Effect of light on growth pigment production in fungi

The data on the effect of different wavelength of light on growth and pigment production in fungi are presented in Tables 23 to 25, Figures 13, 14 and Plate 32.

4.4.6.1. Effect of light on the biomass production in fungi

Table 23 illustrates the effect of different lights on the production of fungal biomass. Under unscreened white light and darkness all the four fungi produced the highest biomass. However under red, yellow, green and blue light the biomass yield was comparatively low.

Table 23 . Effect of light on biomass production by fungi.

Light	Biomass production (g l ⁻¹ dry weight)				
	<i>M. purpureus</i>	<i>M. ruber</i>	<i>P. purpurogenum</i>	<i>Verticillium</i> sp	<i>Humicola</i> sp.
Darkness (No light)	7.57	8.26	5.26	8.17	5.71
White (mixed unscreened)	7.14	7.56	4.14	6.79	3.14
Blue	7.36	6.14	3.54	5.14	3.78
Green	6.74	7.35	4.63	6.17	4.56
Yellow	5.16	6.71	3.74	7.64	3.70
Red	4.36	5.49	3.78	7.56	3.74
SE	0.096	0.129	0.113	0.991	0.707
CD(<i>P</i> _o = 0.05)	0.209	0.281	0.247	2.159	1.540



Plate 32. Experimental set up to study the impact of different light on pigment production.

The statistical scrutiny of the data revealed that biomass yield is negatively correlated with the increase in wavelength of the light (Appendix II f).

4.4.6.2. Effect of light on the intracellular pigment production

The data on the influence of intracellular pigment production are presented in Table 24 and Figure 13.

It is interesting from the data that intracellular pigments accumulated in all the fungi significantly higher in darkness and unscreened white light. Red light and yellow light caused a reduction in pigment production.

4.4.6.3. Effect of light on the extracellular pigment production

The data on the influence of extracellular pigment production are presented in Table 25 and Figure 14.

It is evident from the data that extracellular pigments production in the selected fungi has been significantly increased when incubated under darkness followed by unscreened white light. Red light caused a reduction in pigment production.

The correlation coefficients of the parameters were presented in the table (Appendix II f).

It may be concluded briefly that incubation of fungal cultures under darkness favoured biomass production and pigment production. Blue and green light also supported pigmentation and biomass production in the four fungi.

Table 24. Effect of light on intracellular pigments production by fungi.

Light	Intracellular pigments in units*									
	<i>M. purpureus</i>		<i>M. ruber</i>		<i>P. purpureogenum</i>		<i>Verticillium</i> sp		<i>Humicola</i> sp.	
	300 nm	500 nm	300 nm	500 nm	300 nm	500 nm	300 nm	500 nm	300 nm	500 nm
Darkness(No light)	61.5	68.5	76.5	69.7	21.1	68.3	41.3	22.3	14.8	63.5
White(Mixed unscreened)	39.4	73.4	41.5	86.3	27.4	78.0	57.6	38.7	26.0	78.4
Blue	28.6	63.4	47.8	77.5	25.3	56.2	37.1	31.8	15.2	76.2
Green	36.8	61.1	47.2	74.4	29.7	53.0	36.9	28.2	10.2	77.4
Yellow	26.7	54.6	27.8	51.5	16.1	43.4	26.4	23.4	10.4	56.1
Red	16.3	41.7	21.2	46.2	13.6	38.4	21.5	16.7	13.1	65.9
SE	3.365	2.278	8.889	12.207	2.337	2.347	2.498	2.498	2.465	2.215
CD($P_0 = 0.05$)	7.331	4.965	19.368	26.598	5.092	5.115	5.443	5.443	5.372	4.826

* One unit of pigment is defined as equivalent to 0.1 OD at 300 nm or 500 nm light absorption.

Table 25. Effect of light on the extracellular pigment production by fungi.

Light	Extracellular pigments in units *									
	<i>M. purpureus</i>		<i>M. ruber</i>		<i>P. purpureogenum</i>		<i>Verticillium</i> sp		<i>Humicola</i> sp.	
	300 nm	500 nm	300 nm	500 nm	300 nm	500 nm	300 nm	500 nm	300 nm	500 nm
Darkness (No light)	414	831	447	914	344	567	657	261	206	614
White (Mixed unscreened)	361	736	386	816	214	747	561	314	216	746
Blue	163	673	176	845	189	547	574	297	176	519
Green	131	683	186	747	143	569	471	306	187	514
Yellow	146	661	171	616	113	418	343	224	131	461
Red	141	514	136	716	110	314	213	213	146	417
SE	41.14	7.329	8.028	7.144	30.362	37.2	7.700	7.700	7.329	7.700
CD ($P_0 = 0.05$)	89.638	15.969	17.492	15.565	66.153	81.223	16.778	16.778	15.969	16.778

* One unit of pigment is defined as equivalent to 0.1 OD at 300 nm or 500 nm light absorption

Fig.13. Effect of light on intracellular pigments production by fungi

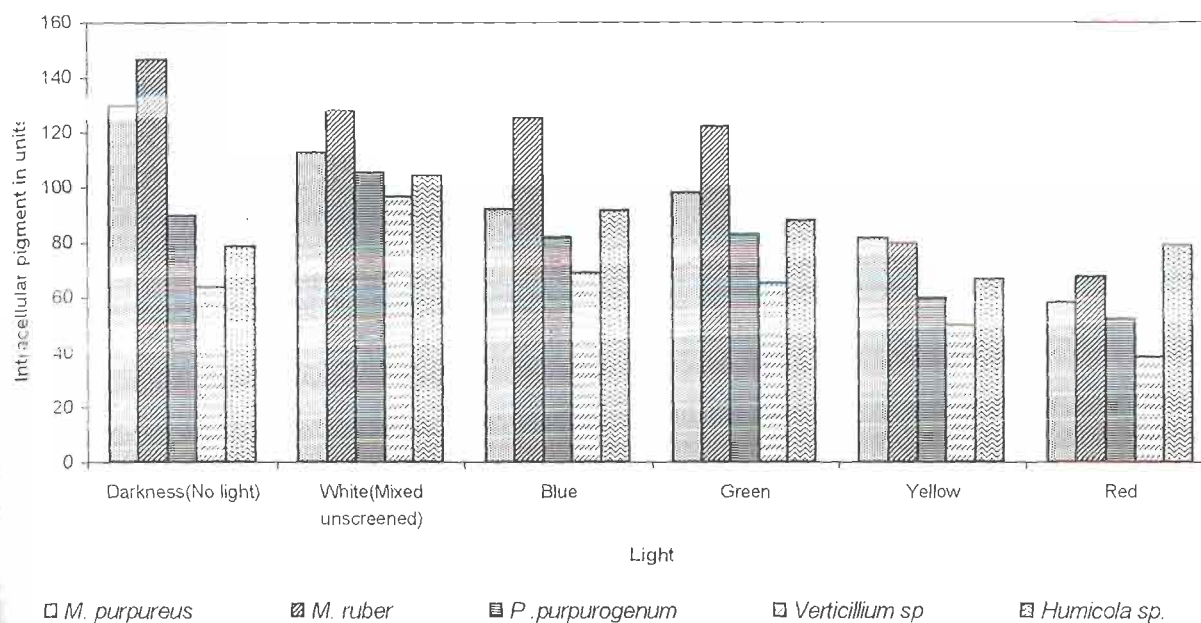
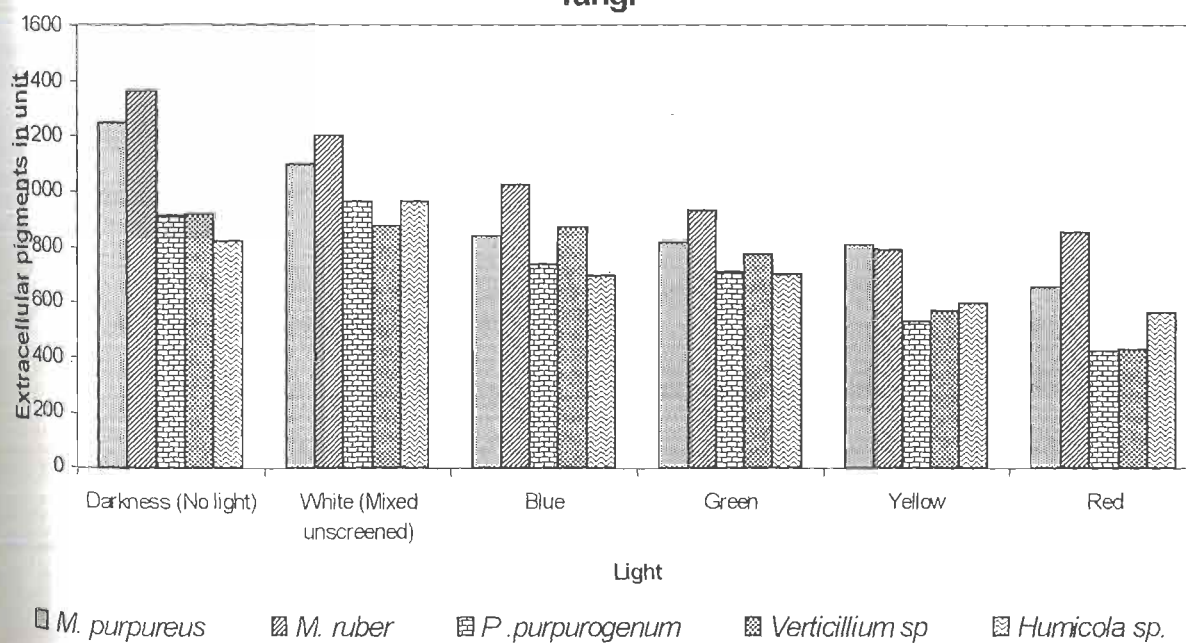


Fig.14. Effect of light on the extracellular pigment production by fungi



4.5. Pigment production Technology

The selected fungi were subjected to both liquid state fermentation and solid-state fermentation for pigment production as detailed earlier. Different natural substrates were utilized for pigment yield. Tables 26 and 27 and Figure 15 illustrate data on pigment production through solid and liquid fermentation techniques.

4.5.1. Solid state fermentation

The data on the influence of different agricultural products and by products on the production of fungal pigment are presented in Table 26 and Plates 36 to 49.

In general, all substrates used enhanced pigments yield except pulse waste. The fungi *Penicillium purpurogenum* produced enormous amount of spores upon solid-state fermentation. As this is an undesirable trait, it was deleted. The fungus *Humicola* sp preferred rice grain and sorghum grain for maximum growth but with reduced pigment production. The quantity of red pigments increased due to solid-state fermentation in *Monascus purpureus* and *M. ruber*.

Among the agricultural products used as substrates, rice grain registered higher pigment yield in all the three fungi tested. In the list of agricultural by products/washes, rice bran and sugarcane bagasse supported heavy yield of pigments.

4.5.2. Liquid fermentation technology

The five selected fungi were cultivated in Czapek Dox broth under static conditions as detailed under materials and methods and the pigment yield was observed. The data are set out in Table 27 and Plates 33 and 34.

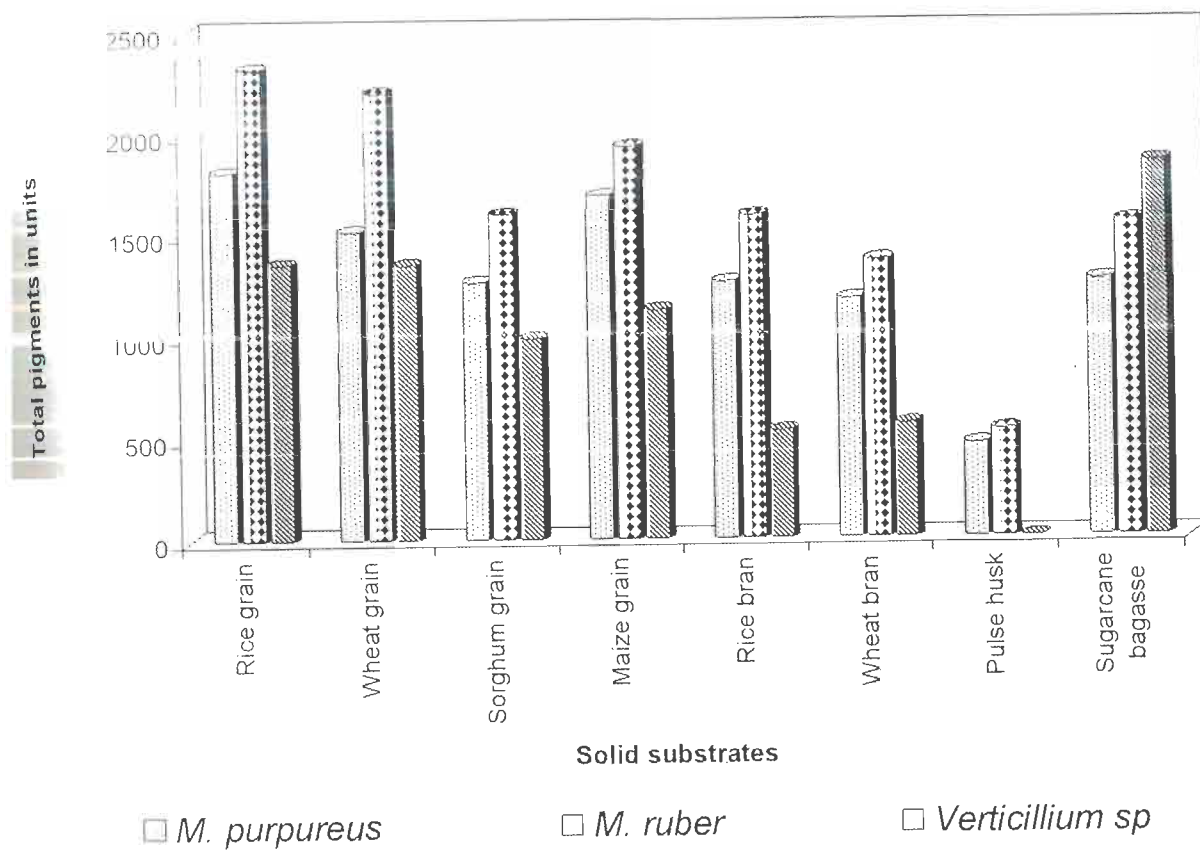
Table 26. Effect of different solid substrates on pigment production.

No	Solid Substrates	Pigments in units*									
		<i>M. purpureus</i>		<i>M. ruber</i>		<i>P. purpurogenum</i>		<i>Verticillium</i> sp.		<i>Humicola</i> sp.	
		300 nm	500 nm	300 nm	500 nm	300 nm	500 nm	300 nm	500 nm	300 nm	500 nm
1	Rice grain	468	1345	678	1642	-	-	917	438	114	678
2	Wheat grain	346	1167	547	1652	-	-	816	536	-	-
3	Sorghum grain	317	948	461	1138	-	-	613	376	123	776
4	Maize grain	446	1245	551	1381	-	-	714	414	-	-
5	Rice bran	326	938	571	1018	-	-	314	216	-	-
6	Wheat bran	313	863	381	986	-	-	216	345	-	-
7	Pulse husk	113	347	114	416	-	-	-	-	-	-
8	Sugarcane bagasse	378	887	571	982	-	-	1107	741	-	-
SE		7.70	7.70	7.78	7.90			7.07	49.91	3.54	3.54
CD($p_{0=0.05}$)		16.338	16.33	16.49	16.75			15.00	105.81	7.50	7.50

* One unit of pigment is defined as equivalent to 0.1 OD at 300 nm or 500 nm light absorption.

- No pigment production

Fig. 15. Effect of different solid substrates on pigment production by fungi



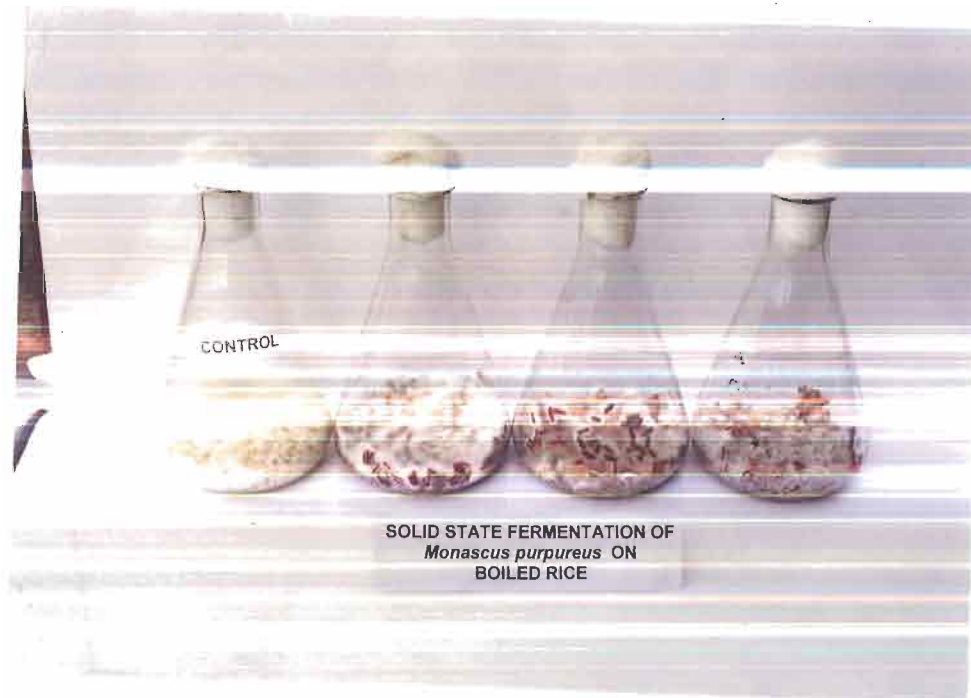


Plate 36. Solid state fermentation of *Monascus purpureus* on boiled rice.

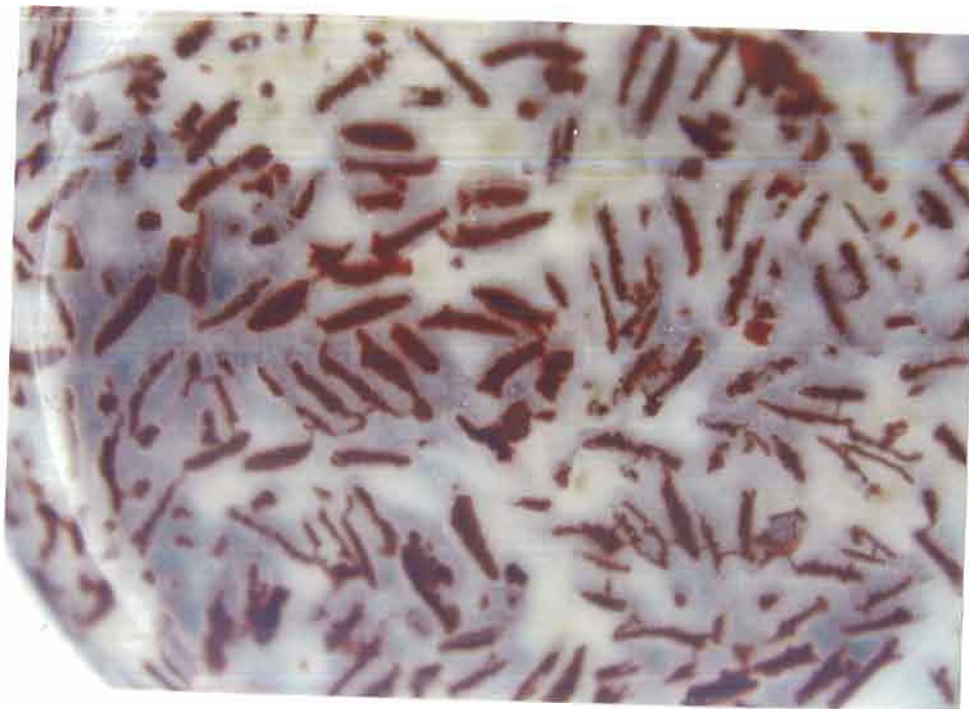


Plate 37. A close up view of cooked rice grains turned brick-red by the growth of *Monascus purpureus*.

Monascus purpureus GROWN
IN RICE



Plate 38. Rice grains turned red by the growth of *Monascus purpureus* (Red rice).



Plate 39. Growth and pigment production by *Monascus ruber* and *Monascus purpureus* on cooked rice grains (SSF).



Plate 40. Close up view of raw rice grains turned red due to pigments secretion by *Monascus purpureus*.



Plate 41. Close up view of Bamathi rice grains turned red due to pigments secretion by *Monascus purpureus*

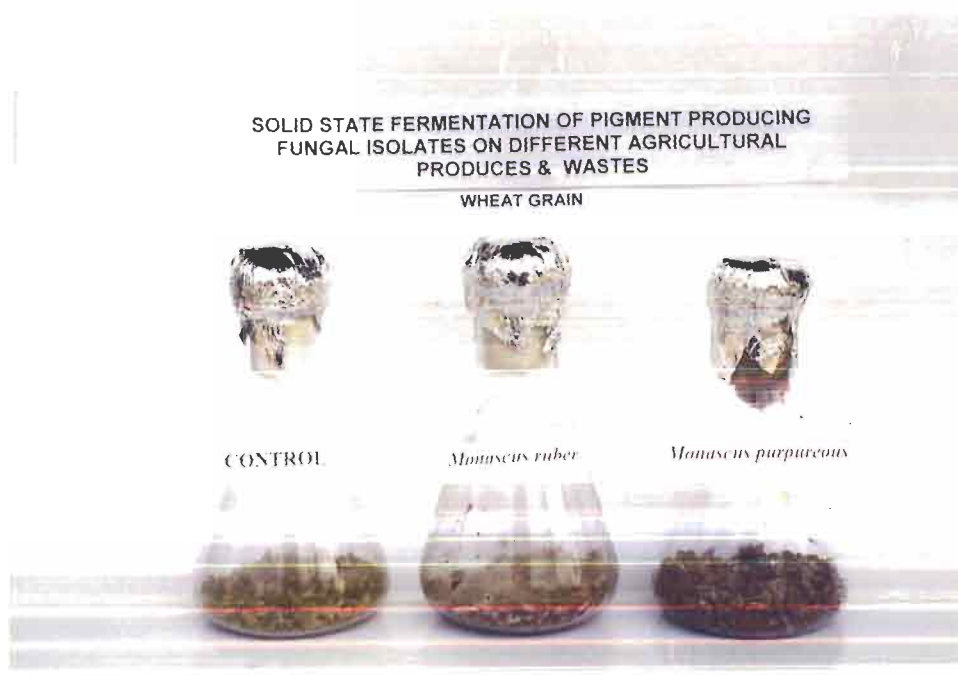


Plate 42. Pigment production by *Monascus* sp on wheat grains.

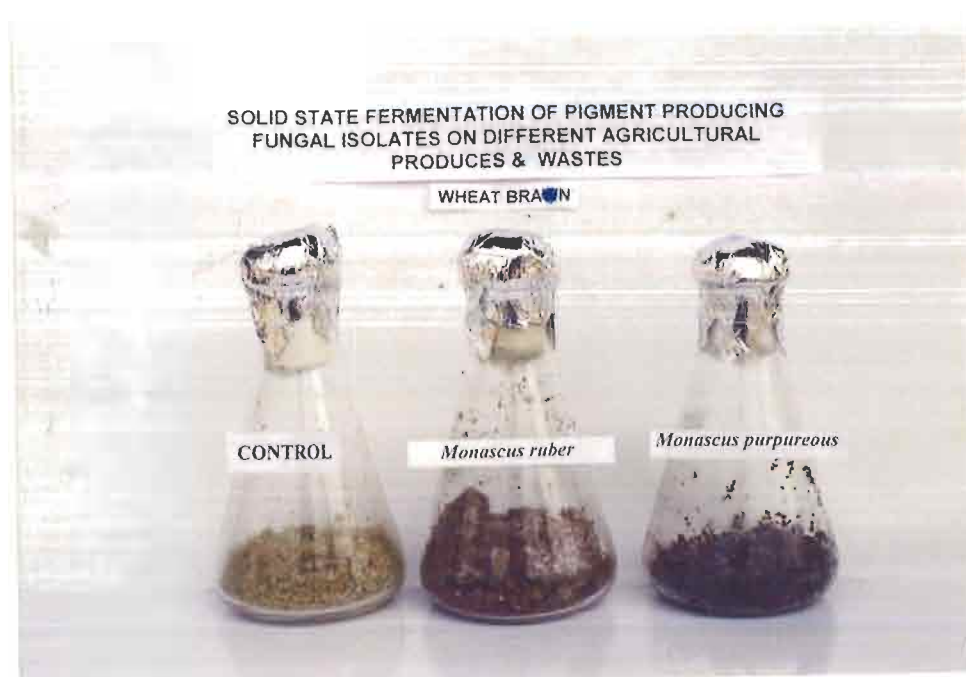


Plate 43. Pigment production by *Monascus* sp on wheat bran substrate.

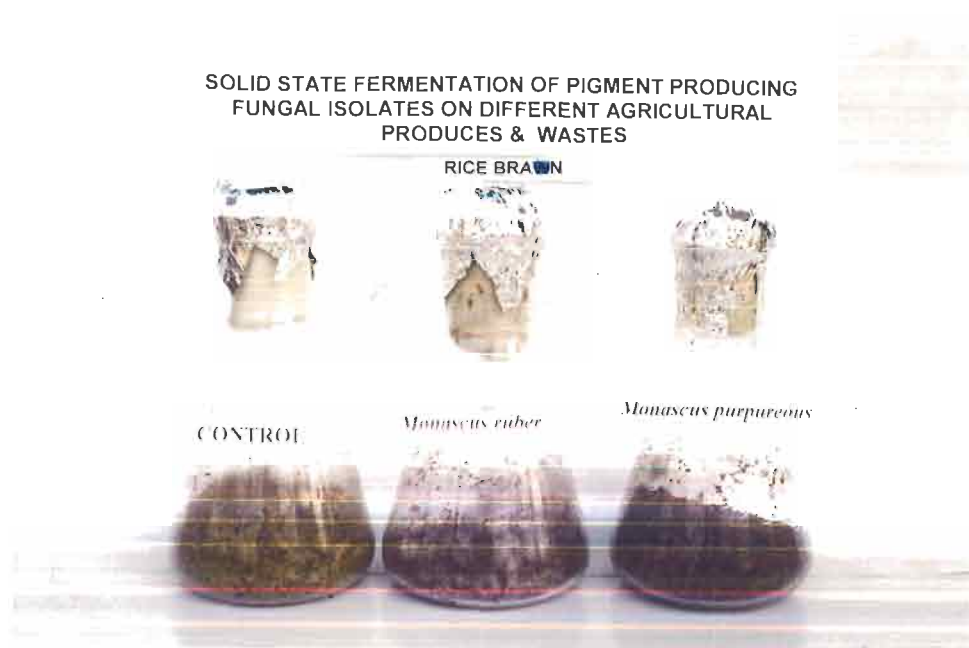


Plate 44. Pigment production by *Monascus* sp on rice bran.



Plate 45. Pigment production by *Monascus* spp. and *Verticillium* sp. (PF-13) on sugarcane bagasse.

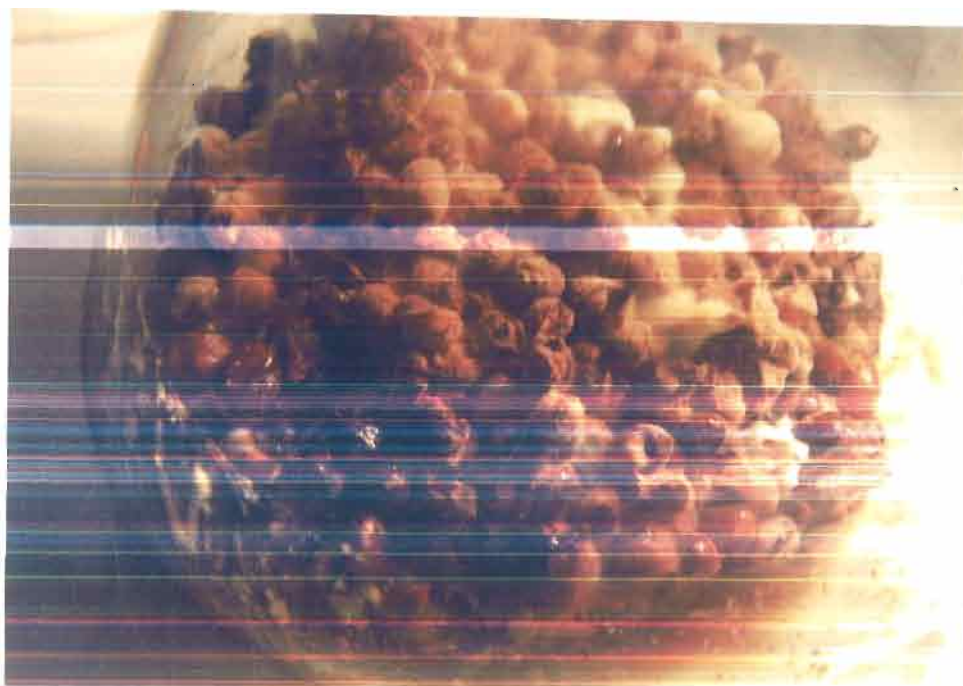


Plate 46. A close up view of pigment production by the fungus *M. ruber* on sorghum grains.

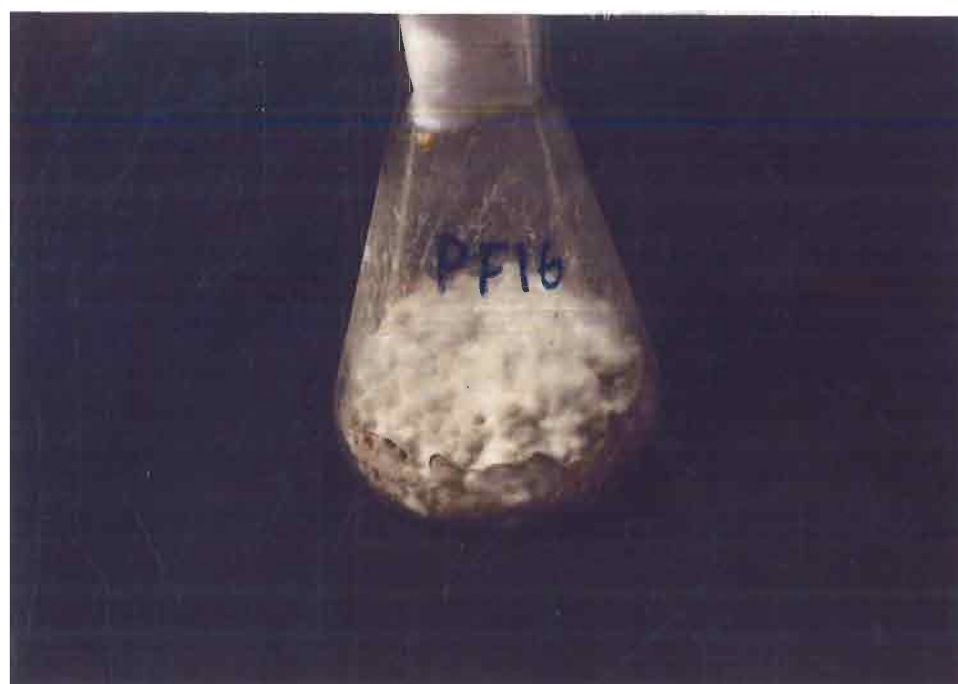


Plate 47. Growth of the fungus *Humicola* sp (PF-16) on sorghum grains.



Plate 48. Pigment production on solid substrates by *Monascus ruber* and *Monascus purpureus*.



Plate 49. Pigment production by *Verticillium* sp on cooked rice grains.

Table 27. Pigment production by fungi in liquid fermentation using Czapek-Dox broth.

Fungi used	Biomass production*	PH shifts @	Pigments in units [#]				Day of pigment excretion
			Intracellular		Extracellular		
			300 nm	500 nm	300 nm	500 nm	
<i>M. purpureus</i>	9.34	7.37	187.3	416.5	446	1083	4
<i>M. ruber</i>	10.61	7.58	213.9	489.5	483	1415	5
<i>P. purpurogenum</i>	6.35	6.81	131.3	354.4	249	867	3
<i>Verticillium</i> sp.	8.73	6.92	331.6	114.3	738	251	5
<i>Humicola</i> sp.	5.08	6.83	231.2	383.7	167	847	6

* g l⁻¹ dry weight

@ Initial pH : 5.5

One unit of pigment is defined as equivalent to 0.1 OD at 300 nm or 500 nm light absorption

All values represent mean of three observations.



Plate 33. Bio colour production by different fungi in liquid medium.

In general, all the fungi produced higher biomass yield than the earlier conical flask experiments. *M. ruber* produced maximum quantity of pigments and also biomass.

4.6. Large scale production of fungal pigments

4.6.1. Developing a low cost growth medium

The pigments from the selected fungi produced on a large scale by following solid-state fermentation and liquid fermentation techniques. For higher pigment yield and reducing cost of production some modifications were introduced. The results are presented in Tables 28 to 31 and Figures 16 to 18.

Low cost polypropylene bags were used as mini fermentor. To compose a cost effective mechanism for higher pigment production rice kanji was included along with complex nutrient broth (Potato dextrose broth) and a mineral inorganic medium (Czapek - Dox broth). Data on pigment yield are presented in the Tables 28 to 30 and Figures 16 and 17.

Pigment production and biomass yield were maximum in potato dextrose medium. However, rice kanji as a cheap alternative can be used as a low cost medium. It produced considerable amount of pigments and biomass production.

The data indicated that rice kanji even after two folds of dilution encouraged pigment production as much as other conventional growth media. Hence rice kanji could serve as a fermentation medium for pigment production.

The fungus, *M. ruber* produced significantly higher quantities of pigment than others in rice kanji medium.

Table 28 . Effect of different low cost liquid media on the biomass production by fungi.

Liquid media	Biomass production (g l ⁻¹ dry weight)				
	<i>M. purpureus</i>	<i>M. ruber</i>	<i>P. purpurogenum</i>	<i>Verticillium</i> sp	<i>Humicola</i> sp.
Potato Dextrose broth	13.67 ^a	18.13 ^a	11.14 ^a	15.71 ^a	10.59 ^a
Czapek-Dox broth	11.36 ^c	15.68 ^b	10.13 ^b	11.36 ^b	6.21 ^c
Rice kangi	12.16 ^b	12.46 ^c	8.76 ^c	10.78 ^c	7.36 ^b

Data followed by the same alphabet are statistically on par

Table 29. Effect of different low cost liquid media on the intracellular pigment production by fungi.

Liquid media	Intracellular pigments in units*									
	<i>M. purpureus</i>		<i>M. ruber</i>		<i>P. purpurogenum</i>		<i>Verticillium</i> sp		<i>Humicola</i> sp.	
	300 nm	500 nm	300 nm	500 nm	300 nm	500 nm	300 nm	500 nm	300 nm	500 nm
Potato Dextrose broth	146.2 ^a	314.7 ^a	156.4 ^a	416.7 ^a	111.2 ^a	181.8 ^a	210.6 ^a	146.3 ^a	81.3 ^a	113.6 ^a
Czapek-Dox broth	136.4 ^b	216.2 ^b	114.6 ^c	345.5 ^b	86.8 ^c	161.4 ^b	116.7 ^b	128.1 ^b	74.6 ^b	104.7 ^b
Rice kangi	101.6 ^c	176.4 ^c	131.6 ^b	246.2 ^c	91.3 ^b	81.5 ^c	80.7 ^c	117.8 ^c	34.3 ^c	71.3 ^c

* One unit of pigment is defined as equivalent to 0.1 OD at 300 nm or 500 nm light absorption.

Table 30. Effect of different low cost liquid media on the extracellular pigment production by fungi.

Liquid media	Extracellular pigments in units*									
	<i>M. purpureus</i>		<i>M. ruber</i>		<i>P. purpurogenum</i>		<i>Verticillium</i> sp		<i>Humicola</i> sp	
	300 nm	500 nm	300 nm	500 nm	300 nm	500 nm	300 nm	500 nm	300 nm	500 nm
Potato Dextrose broth	476 ^a	874 ^a	674 ^a	1317 ^a	214 ^a	861 ^a	946 ^a	231 ^a	176 ^a	871 ^a
Czapek-Dox broth	371 ^b	816 ^b	561 ^b	941 ^b	203 ^b	814 ^b	861 ^b	214 ^b	136 ^b	763 ^b
Rice kangi	343 ^c	764 ^c	413 ^c	817 ^c	186 ^c	767 ^c	781 ^c	171 ^c	112 ^c	413 ^c

* One unit of pigment is defined as equivalent to 0.1 OD at 300 nm or 500 nm light absorption

Fig. 16. Effect of different low cost liquid media on the intracellular pigment production by fungi

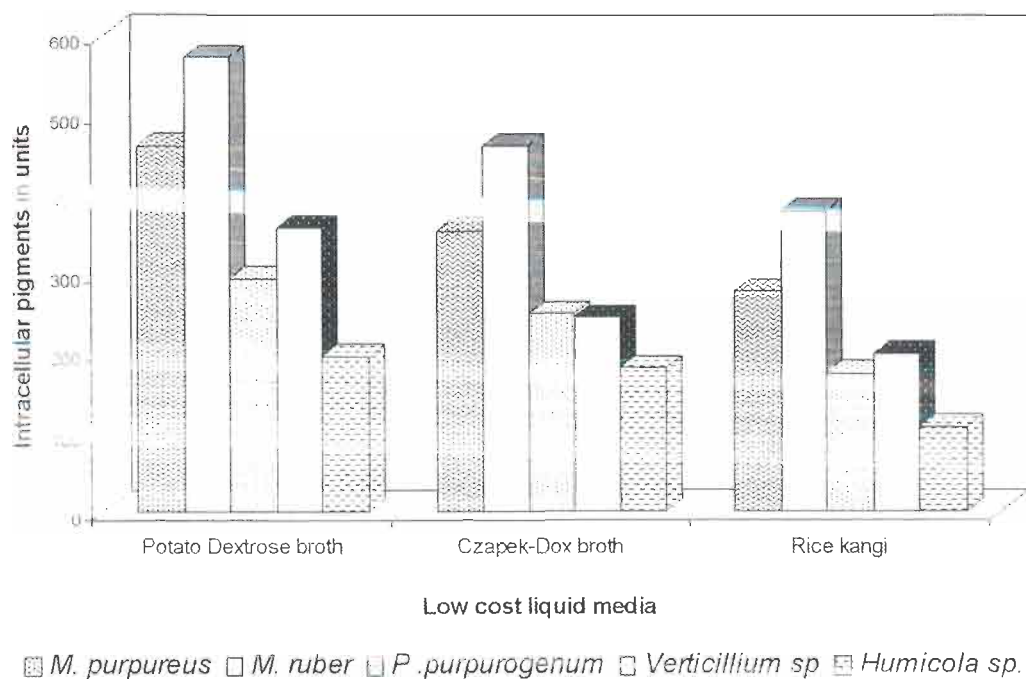
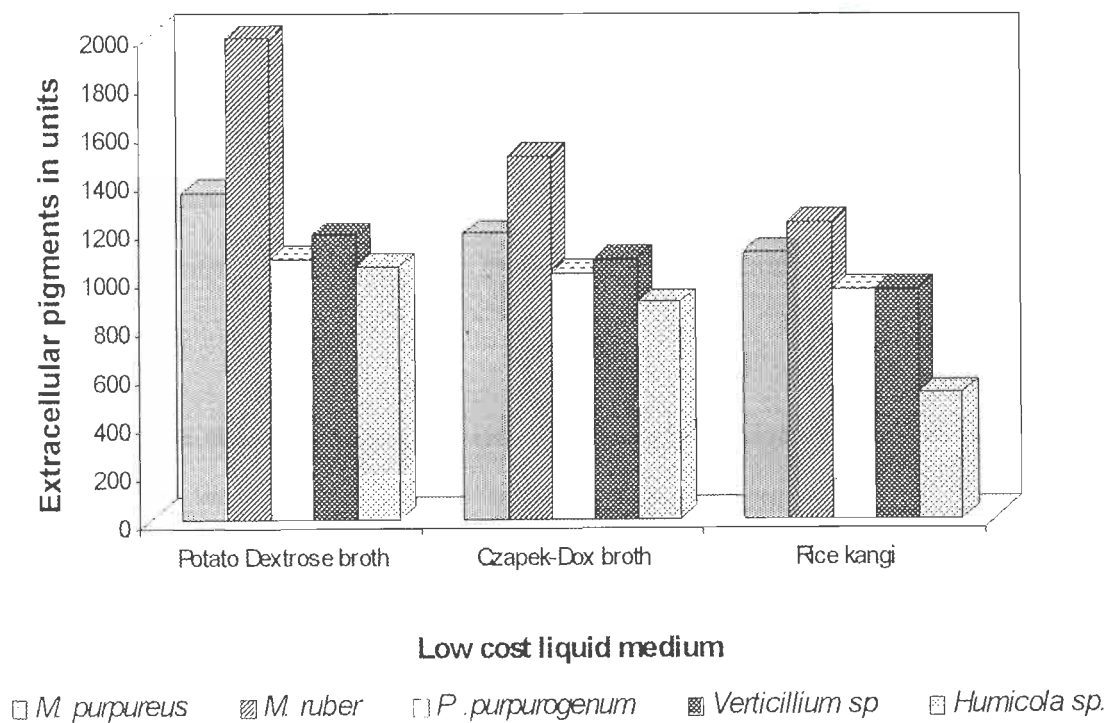


Fig.17. Effect of different low cost liquid medium on the extracellular pigment production by fungi



To compose a low cost solid-state fermentation medium, different kinds of rice were utilized. The results were presented in the Table 31 and Figure 18.

Among the different substrates, raw rice was quite suitable for more pigments yield. Basmati rice yielded higher yields of yellow and red pigments. For economising production cost, raw rice was selected as substrate for solid-state fermentation.

4.6.2. Pigment production in polybags

Poly bag fermentors were successfully used to produce fungal pigments. The data are presented in the Tables 32 and 33 and plate 35.

It is inferred from the data that rice kanji produced more pigment yield and biomass in *M. ruber*. As a whole, compared to glass vessel fermentation, polybags helped for producing higher yields of pigments.

The data have further indicated that raw rice substrate in polybags also yielded higher quantities of pigments.

Summing up, it may be said that polybags can serve as convenient, low cost, and handy fermentors for pigment production either as liquid fermentation or solid-state fermentation. Raw rice and rice kanji offer good scope for fermentation substrates.

4.7. Studies on the genetic improvement in fungi for higher pigment production

Studies were undertaken to improve the pigment yields in fungi through genetic manipulation. As detailed earlier, they were exposed to the chemical mutagen and several mutant clones were obtained (Plates 50 to 68).

Table 31. Effect of different low cost solid substrates for the pigment production by *Monascus* spp.

Low cost substrate	Pigments in units g of substrate*			
	<i>M. purpureus</i>		<i>M. ruber</i>	
	300 nm	500 nm	300 nm	500 nm
Par boiled rice	671.3 ^b	983.9 ^c	763.8 ^c	1130.5 ^c
Raw rice	738.4 ^a	1111.4 ^b	814.6 ^a	1481.6 ^b
Broken rice	631.4 ^c	1101.6 ^b	803.9 ^b	1461.5 ^b
Cheeraga Samba rice	743.1 ^a	1146.3 ^a	784.3 ^c	1631.4 ^a
Basmathi rice	746.5 ^a	1136.6 ^a	844.9 ^a	1736.7 ^a

* One unit of pigment is defined as equivalent to 0.1 OD at 300 nm or 500 nm light absorption

Fig. 18. Different low cost solid media for pigment production in *Monascus* spp.

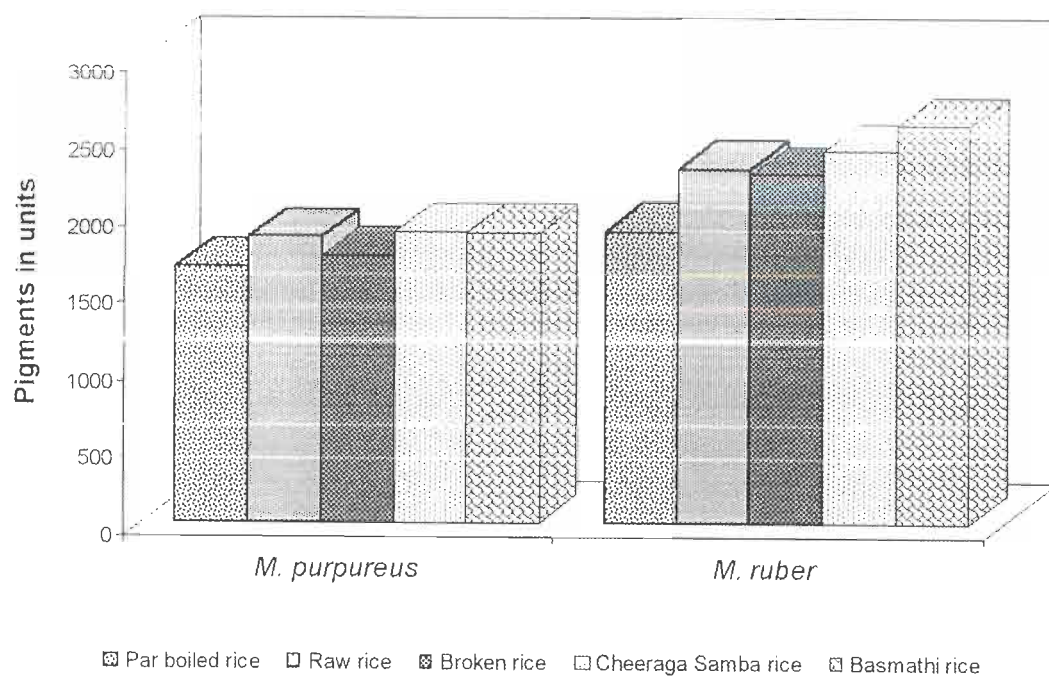


Table 32 . Pigment production by fungi in poly bag fermentation using raw rice.

Fungi used	Pigments in units l ⁻¹ *	
	300 nm	500 nm
<i>Monascus purpureus</i>	530	1155
<i>M. ruber</i>	807	1305
<i>Verticillium</i> sp	863	415

- One unit of pigment is defined as equivalent to 0.1 OD at 300 nm or 500 nm light absorption.

Table 33 . Pigment production by fungi in poly bag fermentors using rice kangi.

Fungi for mass multiplication	Biomass production	Pigments in units l ⁻¹ *				Days of pigment excretion
		Intracellular		Extracellular		
		300	500	300	500	
		nm	nm	nm	nm	
<i>M. purpureus</i>	13.93	77.2	115.6	458	895	4
<i>M. ruber</i>	15.83	99.8	189.2	521	1054	4
<i>P. purpurogenum</i>	10.20	46.9	118.8	141	745	3
<i>Verticillium sp.</i>	14.84	514.6	117.3	684	514	4
<i>Humicola sp.</i>	7.56	71.4	102.5	65	756	5

* One unit of pigment is defined as equivalent to 0.1 OD at 300 nm or 500 nm light absorption.



Plate 35. Bio colour production in poly bags.

Mutagenesis in *M. purpureus*, *M. ruber* and *Penicillium purpurogenum* yielded several interesting mutants. Among the three fungi used, *Penicillium purpurogenum* yielded more number of mutants than in other fungi. Several growth and albino mutants were obtained (Plates 51 to 64). Survival concentration (LD 50) of NTG to this fungi is $150 \mu\text{g ml}^{-1}$ (Plate 50). The mutation frequency observed is 0.017 per cent.

Mutagenesis of the fungus, *Monascus purpureus* resulted in the isolation of several mutants. (Plates 65 and 66). The effective concentration of $100 - 100 \mu\text{g ml}^{-1}$ g NTG was chosen for inducing mutation. The mutation frequency observed for the fungi is 0.016 per cent.

In the case of *M. ruber*, the optimum concentration for the mutagenesis is $50 - 100 \mu\text{g ml}^{-1}$ and the mutation frequency observed is 0.004 per cent. NTG mutagenesis in pigment producing fungi yield a large number of mutant clones that exhibited distinct differences in growth and pigment production. Incidentally bleached mutants (albino) and pleomorphic mutants were also encountered; these mutants were discarded as they were devoid of economic characters (Plates 67 and 68).

4.7.1. Evaluation of mutants of the selected fungi for higher pigment production

The data on the pigment yielding efficiency of the mutants of *Penicillium purpurogenum*, *Monascus purpureus* and *Monascus ruber* are set out in Tables 34 to 36 and Figure 19.

4.7.1.1. Mutants of *Penicillium purpurogenum*

The improved characters of the mutants of *Penicillium purpurogenum* compared with the parent strain are furnished in the Table 34.

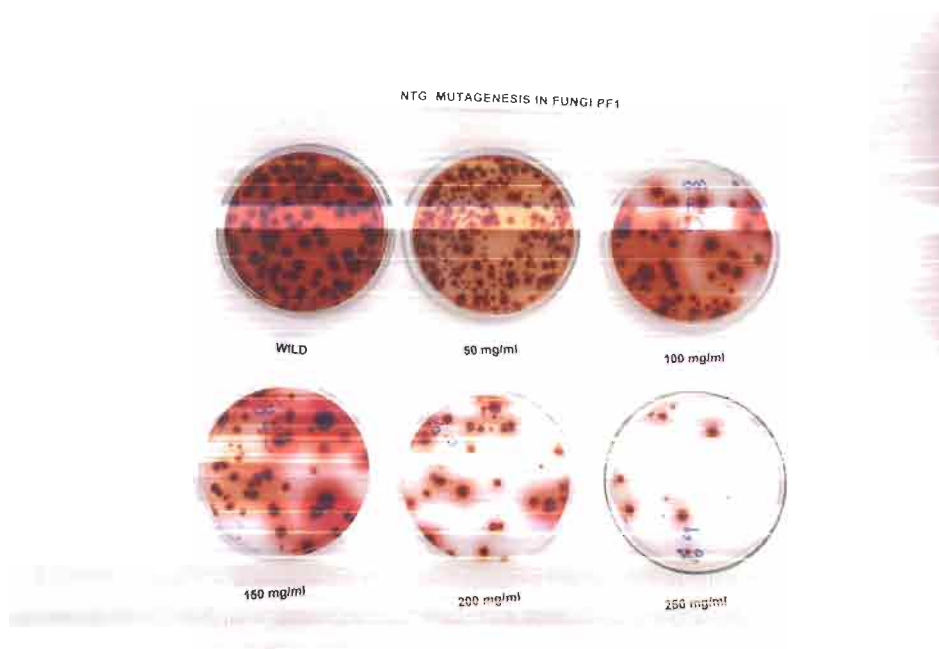


Plate 50. Genetic improvement in *Penicillium purpurogenum* (NTG Mutagenesis).



Plate 51. Parent strain of *Penicillium purpurogenum*.

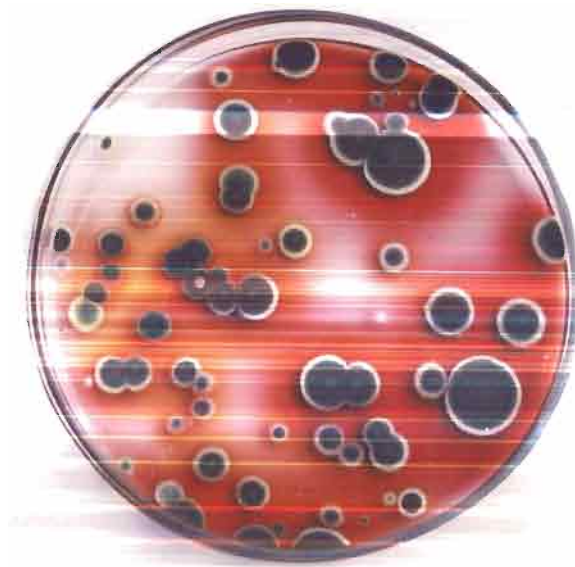


Plate 52. Mutant clones of *Penicillium purpurogenum* exhibiting variations in pigment synthesis.

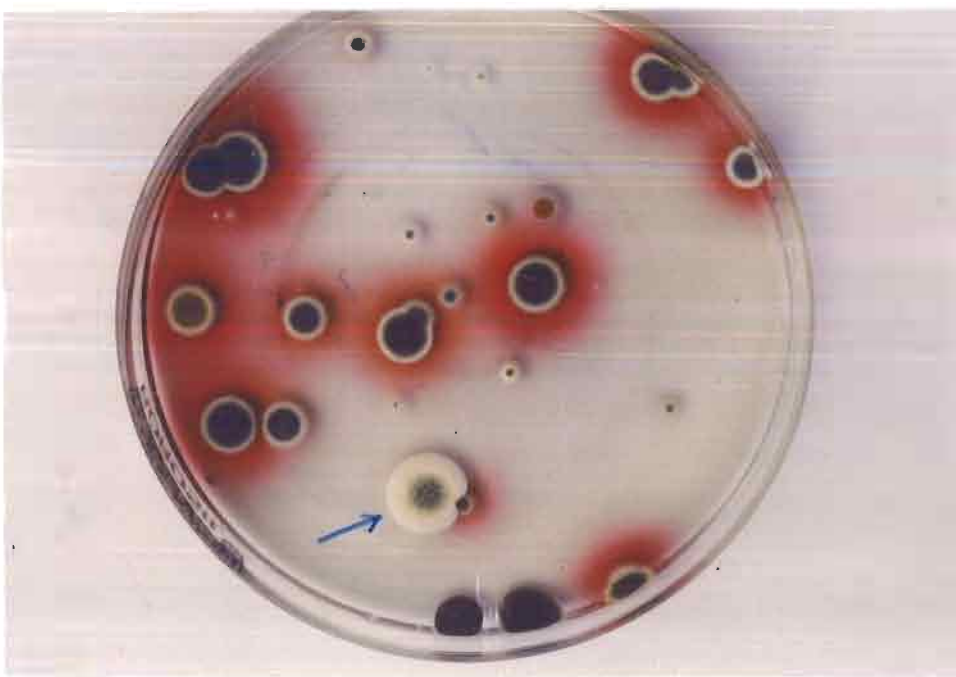


Plate 53. Mutant clones of *Penicillium purpurogenum* (Albino mutants).

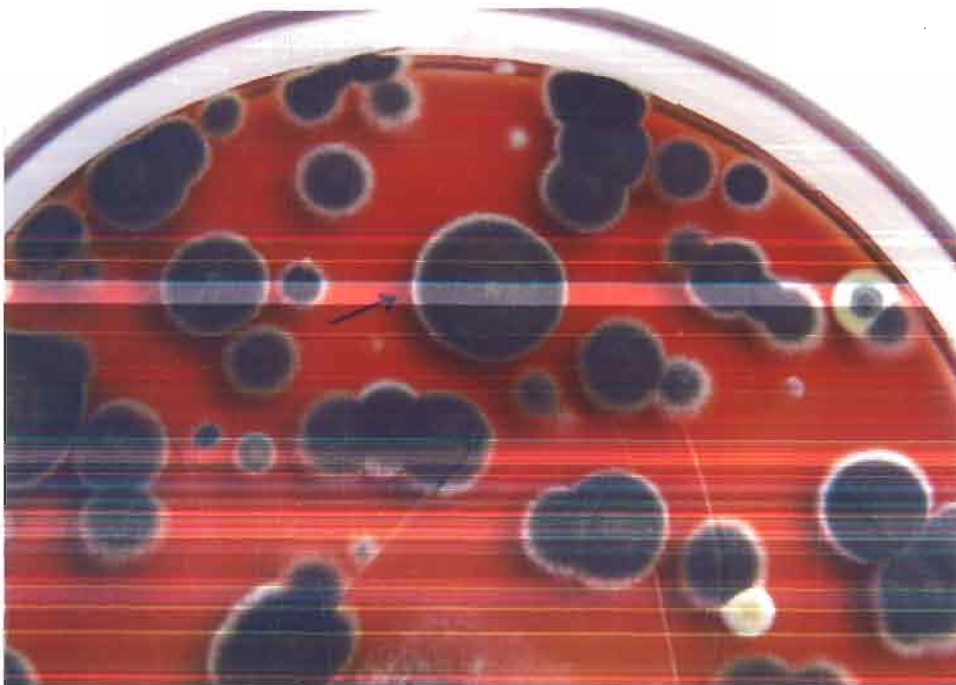


Plate 54. A view of the mutant clones of *Penicillium purpurogenum* (Growth mutants).

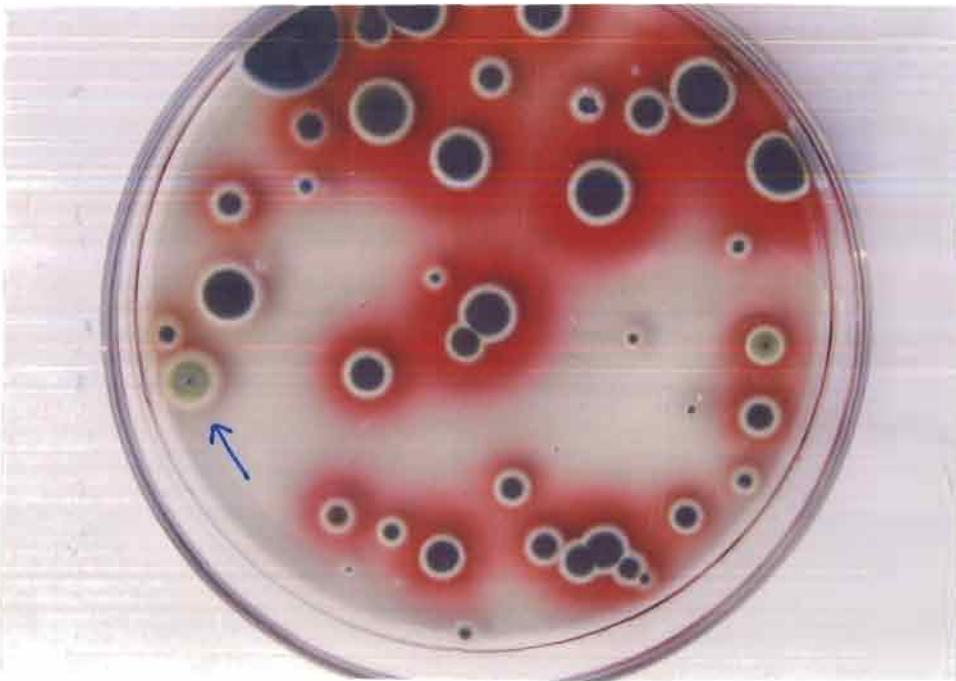


Plate 55. Mutant clones of *Penicillium purpurogenum* (Albino mutants)

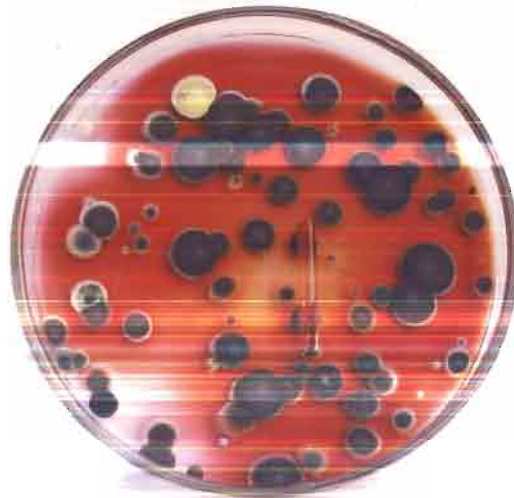


Plate 56. Albino mutants in *Penicillium purpurogenum* among pigment producing clones.

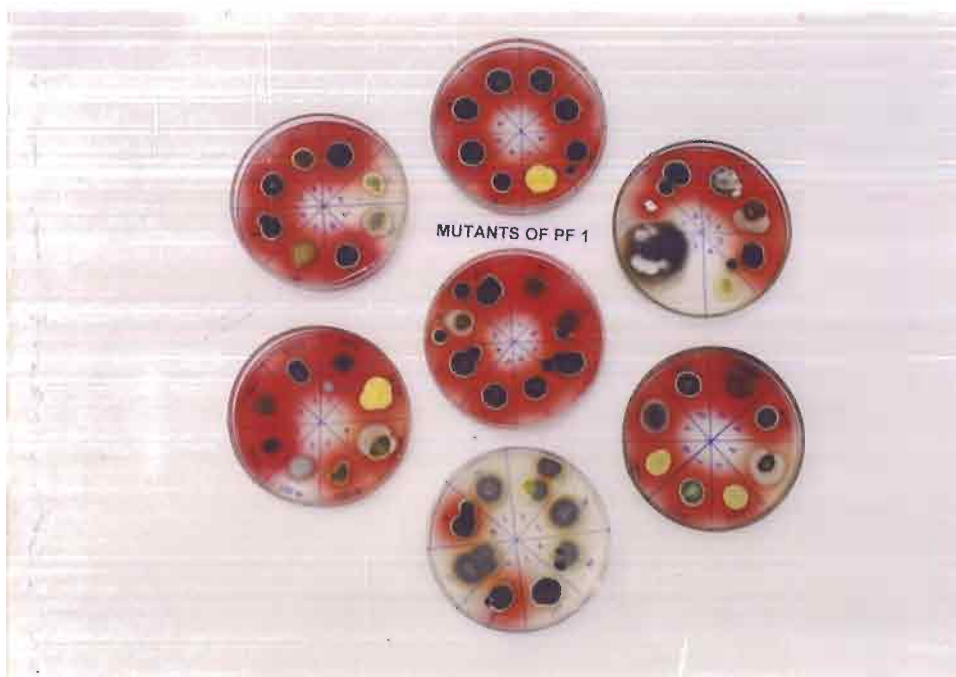


Plate 57. Growth and pigment secretion in the mutant clones of *Penicillium purpurogenum*.

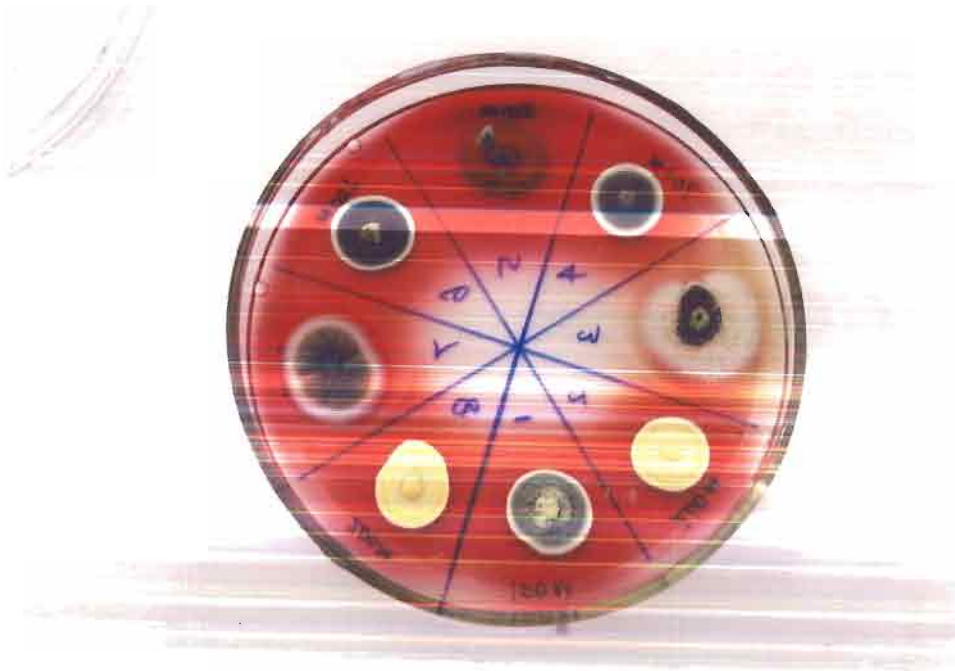


Plate 58. A few promising mutant clones of *Penicillium purpurogenum*.

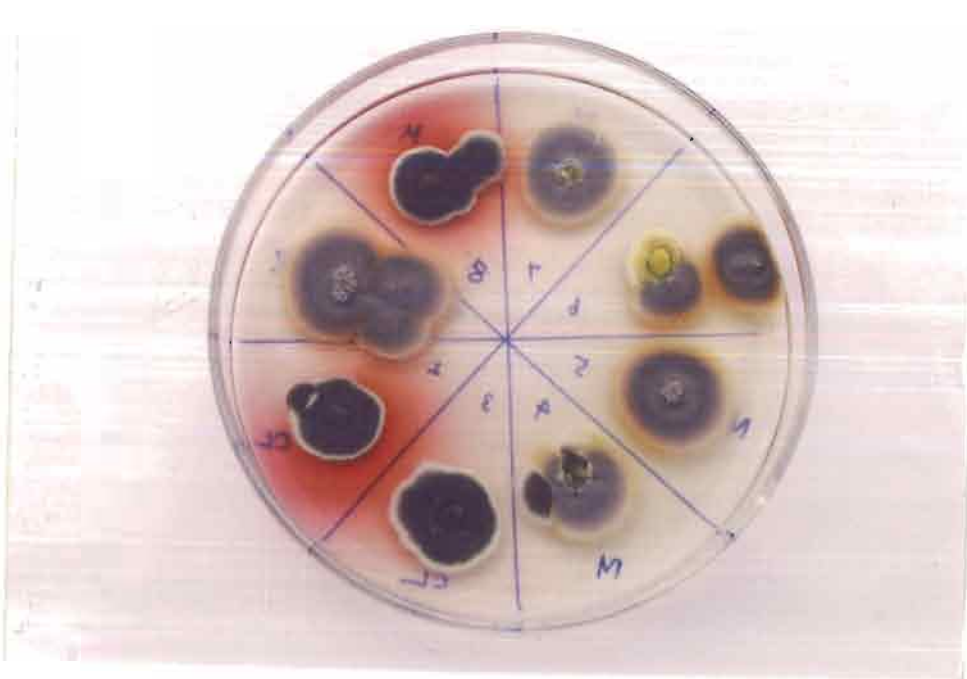


Plate 59. Non pigment producing mutants in *Penicillium purpurogenum*.

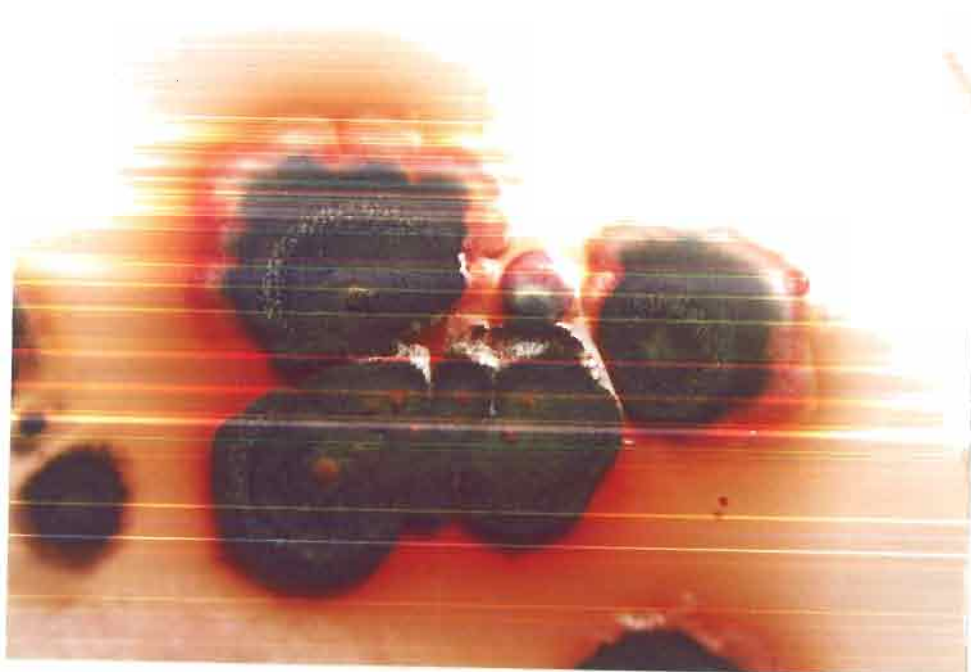


Plate 60. Pigment over producing mutants of *Penicillium purpurogenum*.

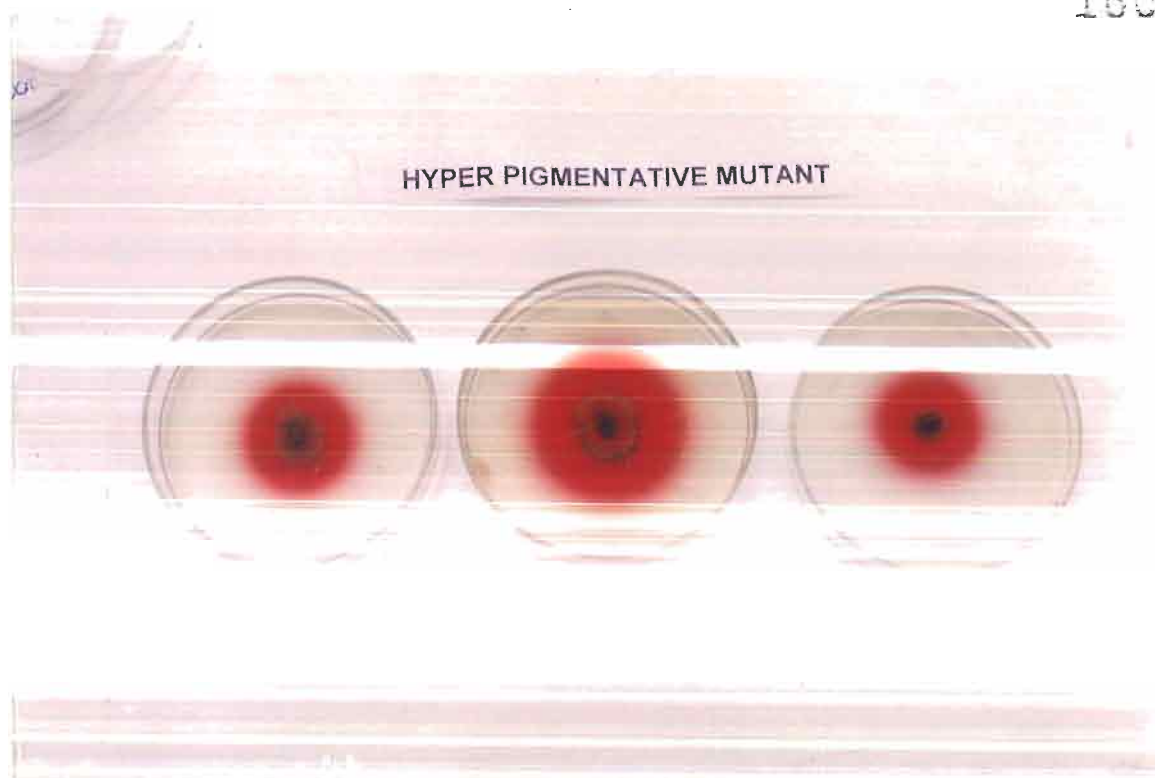


Plate 61. Hyper pigmentative Mutants of *Penicillium purpurogenum* expressing excess pigment secretion.

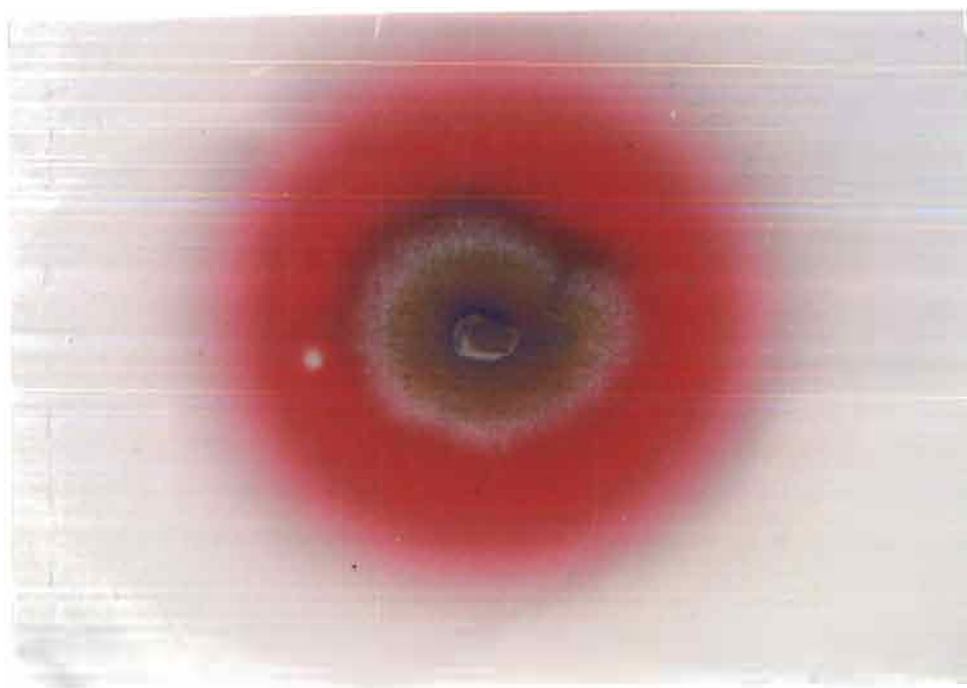


Plate 62. A close up view of pigment over producing mutants of *P. purpurogenum*.

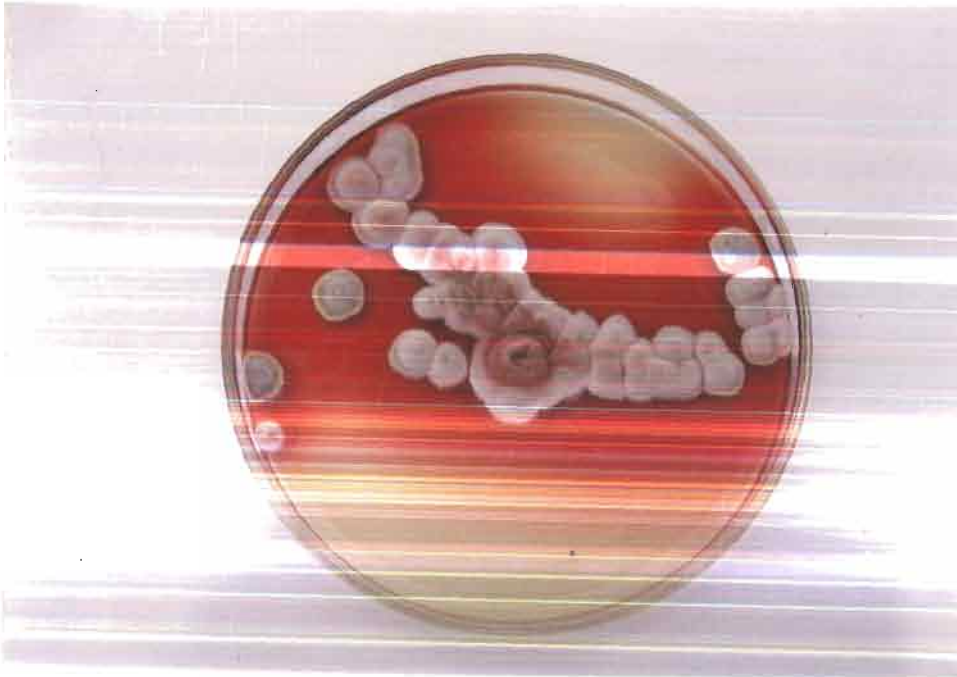


Plate 63. Growth and pigment production in morphological mutant of *P. purpurogenum*



Plate 64. Pigment bearing mycelium of *P. purpurogenum* (300 x)

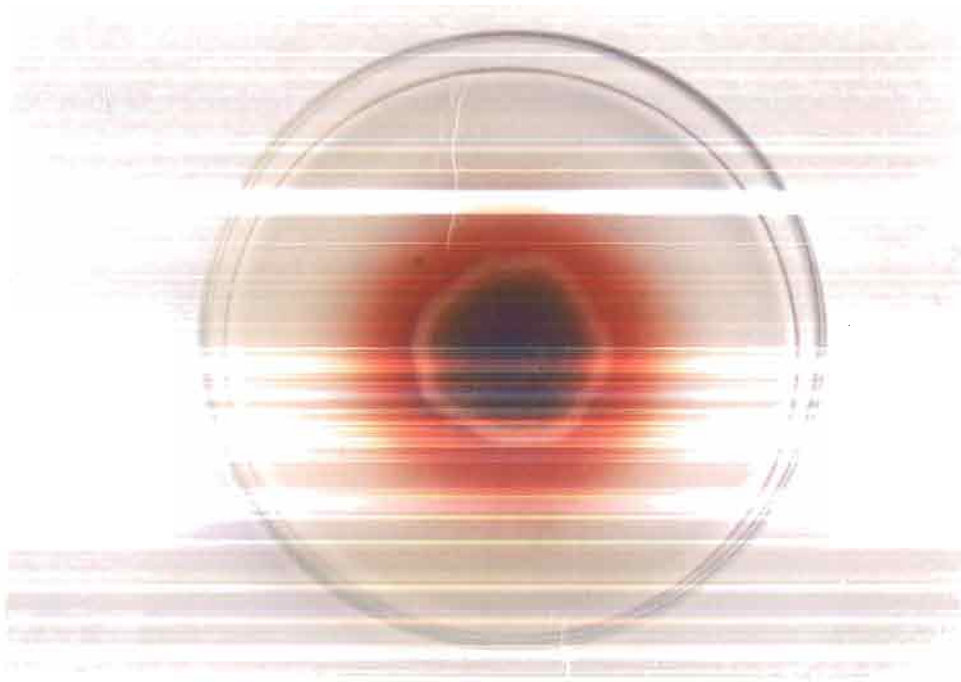


Plate 65. Mutants of *Monascus purpureus*

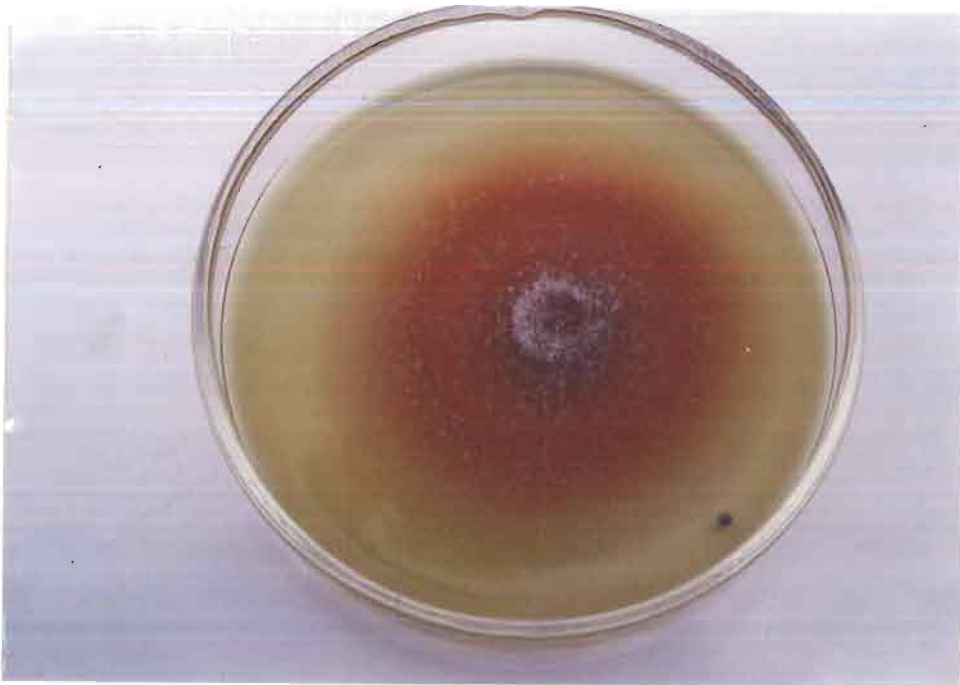


Plate 66. Mutants of *M. purpureus* expressing profuse pigmentation.

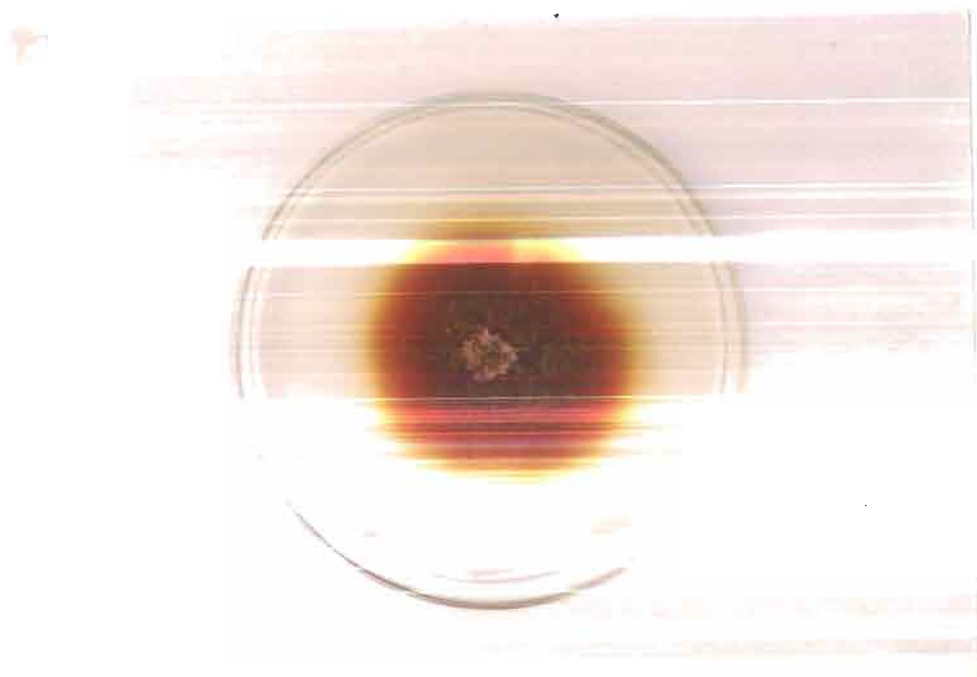


Plate 67. Mutants of *Monascus ruber*

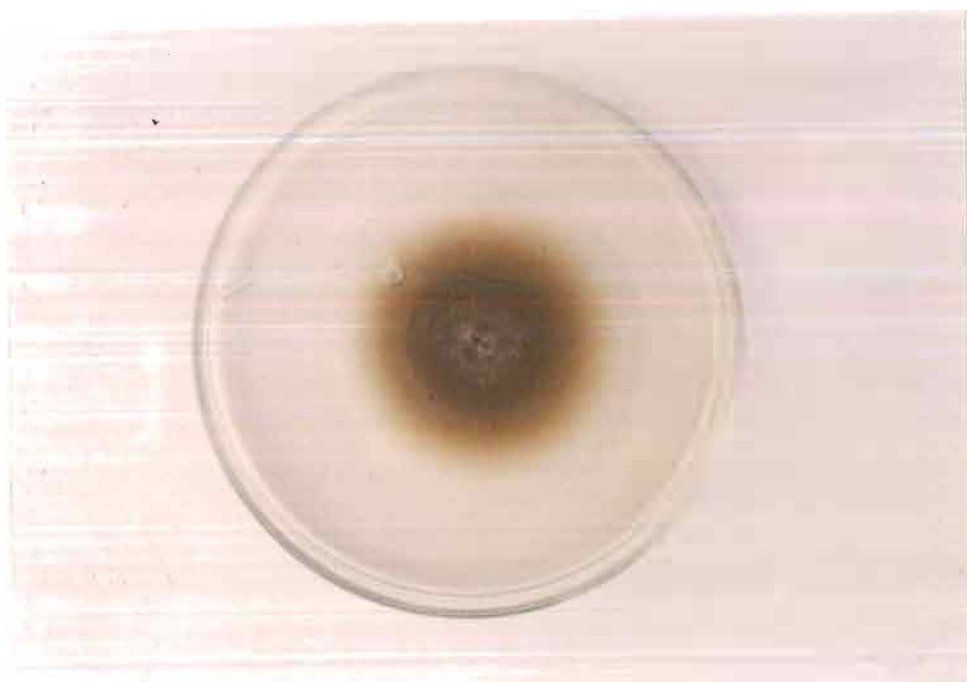


Plate 68. Morphological mutant of *M. ruber*.

When compared with the wild strain, the mutants of *P. purpureogenum* recorded a low biomass yield (-63.4%). However, mutants PFI - 200M-7, 150 M-4 and 200 M-6 produced higher (+56.6%) pigment yield. In general, all the mutants produced more pigments than the wild strain.

Interestingly, the mutant PFI-150M-4 recorded the highest yield of pigments. The study further revealed that the yield of pigments improved by 14.8 to 28.8 per cent under identical growth conditions.

4.7.1.2. Mutants of *Monascus purpureus*

Mutants of *M. purpureus* and the wild strain in respect of pigment yield is set out in Table 35 and Figure 19.

Out of five mutants selected for detailed study three mutants produced lower biomass than the parent strain. Interestingly, these mutants produced 60 per cent increased yield of pigment over wild strain. The mutant MP-M-2 even though biomass production was low exhibited a considerable enhanced yield of pigment. In general, all the three mutants secreted registered a striking improvement in the yield of pigment with less of biomass. Further, in mutant MP-M-7, the excretion time of pigment was short.

4.7.1.3. Mutants of *Monascus ruber*

The data on the characterization of mutants of *M. ruber* are presented in Table 36 and Figure 19.

It is of interest to observe that the mutants in general characteristics produced lower biomass yield than was the wild strain of *M. ruber*. Further the mutants without

Table 34. Pigment production by the mutants of the fungus *Penicillium purpurogenum*.

No	Mutants of <i>P. purpurogenum</i>	Characters of mutants				
		<i>Biomass</i> *	Pigments in units * *		% increase over the parent	Day of pigment excretion
			Intra cellular	Extra cellular		
1	PF1 - wild	3.64	67.3	714	-	3
2	PF1- 200 M-7	1.33	81.3	916	27.6	4
3	PF1- 150 M-4	1.47	88.6	926	29.8	4
4	PF1- 150 M-1	2.51	83.9	838	17.9	4
5	PF1- 200 M-4	5.61	81.5	816	14.8	3
6	PF1 - 200 M-6	4.74	83.8	914	27.7	3
7	PF1 200 M-8	3.71	73.2	843	17.2	3
8	PF1 200 M-5	4.68	86.5	879	23.5	4
9	PF1 150 M-2	5.71	82.4	849	19.2	3
10	PF1 200 M-3	3.81	42.2	914	22.3	3
	SE	0.324	2.417	7.633		
	CD($P_o = 0.05$)	0.677	5.041	15.923	-	-

* gram per litre on dry weight basis.

** One unit of pigment is defined as equivalent to 0.1 OD at 300 nm or 500 nm light absorption

Table 35. Pigment production by the mutants of *Monascus purpureus*.

Mutants of <i>M.purpureus</i>	Biomass production (g l ⁻¹ dry wt.)	Pigments in units *		% increase over the parent	Day of pigment excretion
		Intracell ular	Extracell ular		
MP-Wild	6.81	71.6	714	-	4
MP-M-1	6.16	68.3	986	34.2	4
MP-M-2	6.31	81.5	1176	60.0	4
MP-M-4	4.37	88.7	813	14.7	5
MP-M-7	7.68	69.6	914	25.2	3
MP-M-11	7.46	88.3	1116	53.2	4
SE	1.031	8.008	6.985		
CD(<i>Po</i> = 0.05)	2.247	17.448	15.220		

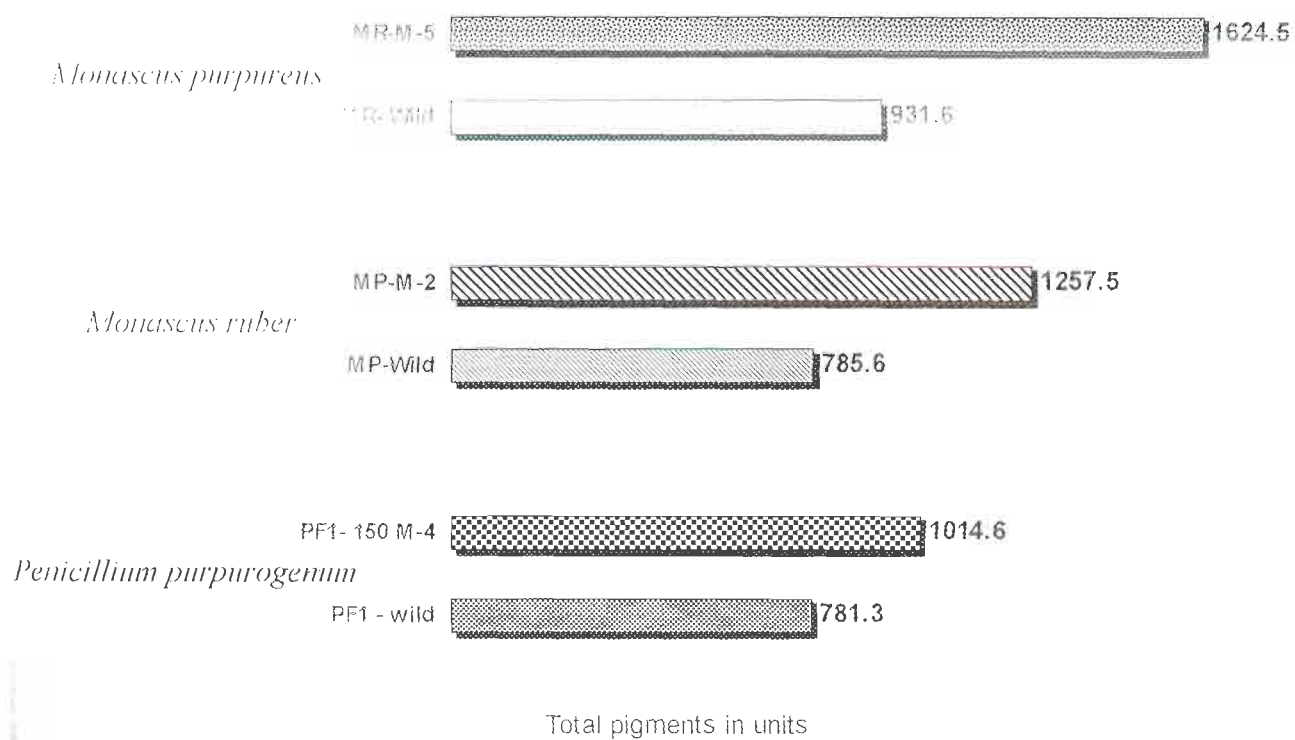
* One unit of pigment is defined as equivalent to 0.1 OD at 300 nm or 500 nm light absorption.

Table 36. Pigment production by the mutants of *Monascus ruber*.

Mutants of <i>M.ruber</i>	Biomass production (g l ⁻¹ dry wt.)	Pigments in units*		% production over wild parent	Day after pigment excretion
		Intra cellular	Extra cellular		
MR- Wild	8.14	114.6	817	-	4
MR-M-1	7.16	101.3	983	16.3	4
MR-M-3	8.15	121.4	1106	31.7	3
MR-M-5	8.16	146.5	1478	74.3	4
MR-M-6	6.14	103.3	986	16.9	4
MR-M-9	7.16	113.6	948	13.9	3
SE	1.411	2.643	7.084		
CD(<i>Po</i> =0.05)	3.075	5.760	15.434		

* One unit of pigment is defined as equivalent to 0.1 OD at 300 nm or 500 nm light absorption

Fig.19. Response of mutants over parents in total pigments production



exception produced higher yields pigments than ^{the} parent strain. The yield of red pigments was more than yellow pigments. Among the mutants studied, MR-M-5 registered much higher production of pigments than the parent strain.

4.8. Purification and characterization of pigments

Pigments excreted out in the liquid fermentors were collected and purified. Pigment extraction was carried out both by airdrying the culture filtrate and by solvent extraction - producers as described earlier.

4.8.1. Extraction of fungal Pigments

In a clean enamel tray, the culture filtrate containing the pigment was taken and dried under sun. Dried pigments were collected and freeze dried in the lyophilizer (Plate 59).

The suitability of solvents for pigment extraction was ^Sdetermined. The results are furnished in Table 37.

Ethyl alcohol and methyl alcohol were suitable for very good pigment extraction from the solid state fermented material. It is inferred from the table that ethyl alcohol and methyl alcohol were suitable for all the ^{SP}five fungi. Preferably, chloroform was used for the extraction from culture filtrate of all the five fungi. Acetonitrile propanol, acetic acid were good in extraction of all fungi.

4.8.2. Physio-chemical characterization of pigments

Data on the pigments and their physical characteristics are presented in Tables 38 , 39 and Figures 20 to 22.



Plate 69. Biocolourant powder obtained from *Monascus ruber*.

Table 37. Extractability of fungal pigments in different solvents.

No	Solvents	<i>Monascus purpureus</i>	<i>Monascus ruber</i>	<i>P. purpureogenum</i>	<i>Verticillium sp.</i>	<i>Humicola sp.</i>
1.	Water	+	+	+++	+++	+++
2.	Methyl alcohol	+++	+++	+++	+++	+++
3.	Ethyl alcohol	+++	+++	++	+++	+++
4.	Chloroform	+++	+++	-	+	-
5.	Carbon tetra chloride	-	-	-	+	-
6.	Hexane	-	-	-	-	-
7.	Benzene	-	-	-	+	-
8.	n-heptane	-	-	-	+	-
9.	Cyclohexane	-	-	-	+	-
10.	Acetonitrile	++	++	+	++	++
11.	iso-propanol	++	++	+	+	-
12.	Propan-2-ol	++	+	+	++	-
13.	Petroleum ether	++	++	-	-	-
14.	Ethyl acetate	-	-	+	+++	-
15.	Ethyl aceto acetate	-	-	+	+++	-
16.	iso-butyl alcohol	-	-	+	+	-
17.	Lactic acid	+	+	-	+++	+
18.	Acetic acid	+++	+++	+	+++	+
19.	Acetone	+++	+++	++	+++	-
20.	Nitrobenzene	-	-	-	-	-
-	Un extractable		+	poor extraction		
++	Good extraction		+++	Very good extraction		

All the pigments from selected fungi were stable at temperature of 50°C and 70°C. However, at 120°C there was a slight decomposition of pigments. Pigments from *M. ruber* were highly stable in all the temperatures tested.

The stability of the pigment at different pH levels was examined. In general, the fungal pigments were quite stable at alkaline and acid pH. It may be inferred that changes in pH of the pigments caused little damage to the pigments.

Different sources of light like sunlight, UV light and fluorescent light were used for the stability analysis. Exposure to fluorescent light did not affect pigment stability, however under UV light and under maximum sunlight there was deterioration of pigments.

The study on the addition of food preservatives to pigments indicated that sodium benzoate and potassium meta bisulphite had no effect on the stability of pigments. Citric acid does not cause any effect on the pigments.

These studies have indicated that the fungal pigments can be safely used under different sources of light and food preservatives.

4.8.2.1. Spectrophotometric scanning of pigments

The scanning studies on the pigments of the selected fungi are presented in the figures 21a to 21e.

In general, all the pigments studied had their maximum absorbance both in the UV range and also in the visible range. Between 200 nm to 300 nm (UV

Table 38. Stability of the fungal pigments under stress conditions.

No	Treatments	Per cent stability after treatment*				
		<i>M. purpureus</i>	<i>M. ruber</i>	<i>P. purpurogenum</i>	<i>Verticillium</i> sp	<i>Humicola</i> sp
1	Pigments in units	930	1360	860	730	1260
2	Temperature treatment					
	Moist heat					
	50°C	96.7	98.5	96.5	97.2	93.6
	70°C	92.4	95.5	87.2	86.3	84.1
	120°C	89.2	88.2	81.8	73.9	74.6
	Dry heat	71.5	76.4	71.6	65.7	75.2
3	pH treatment					
	pH 2.0					
	15 min	93.5	98.5	93.0	95.8	97.6
	30 min	92.4	94.1	93.0	93.1	95.2
	pH 10.0					
	15 min	100.0	99.2	96.6	95.8	98.4
	30 min	98.8	97.0	95.3	89.0	96.8
4	Light treatment					
	Sun light	90.3	91.1	83.7	95.8	80.1
	Fluorescent light	98.9	99.2	97.6	95.8	99.2
	UV light	92.4	88.2	82.5	87.6	89.6
5	Food preservatives					
	Citric acid	100.0	100.0	98.8	97.2	100.0
	Sodium benzoate	100.0	100.0	100.0	100.0	100.0
	Potassium meta bisulphate	92.5	96.3	81.3	95.8	96.0

Fig. 20 a. Effect of temperature on the stability of fungal pigments

Fig. 20a. Effect of heat on the stability of fungal pigments

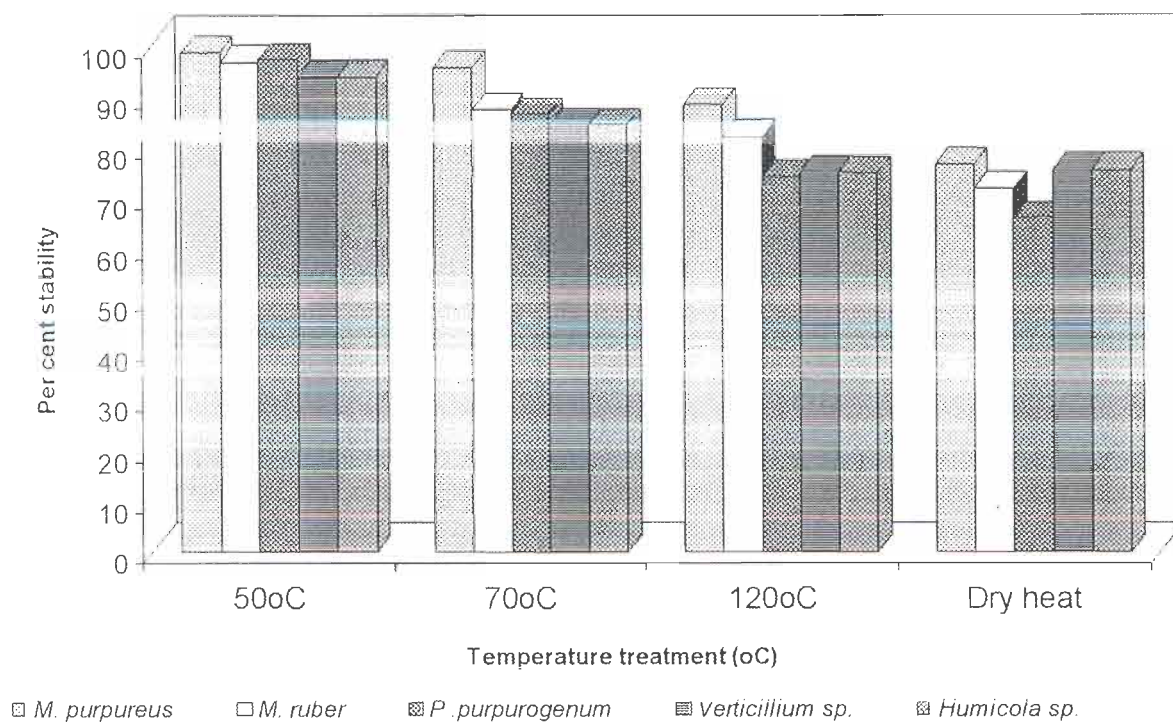


Fig.20b. Effect of pH on the stability of fungal pigments

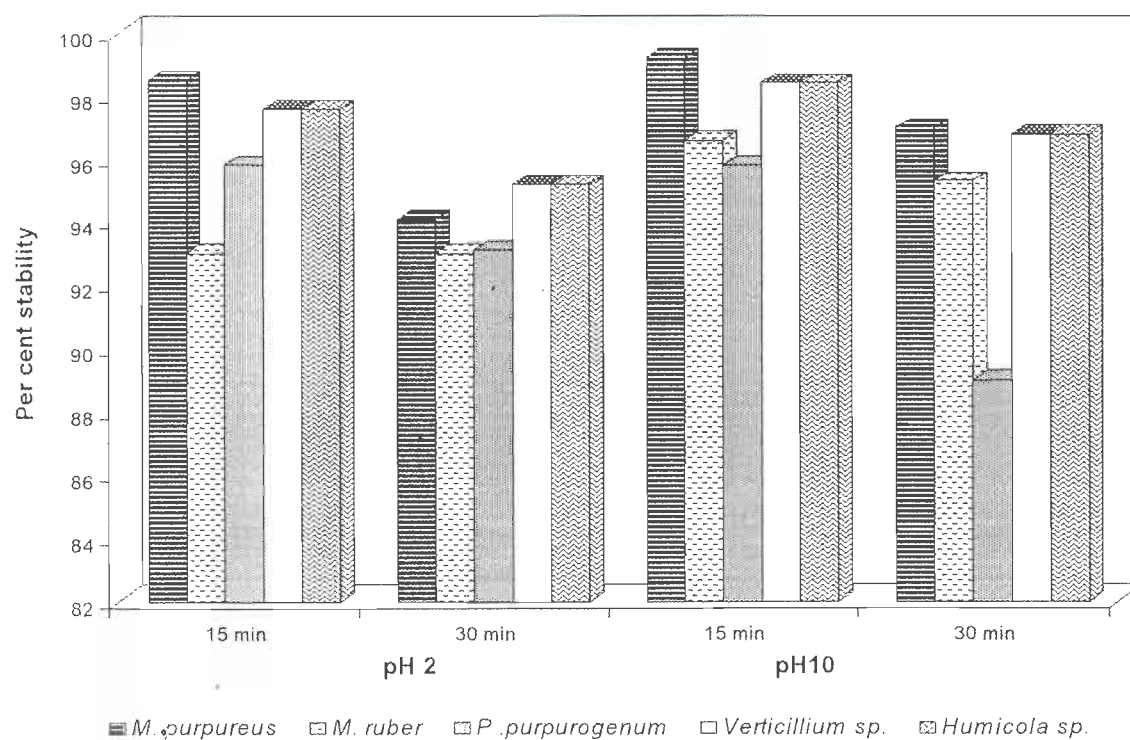


Fig. 20c. Effect of light on the stability of fungal pigments

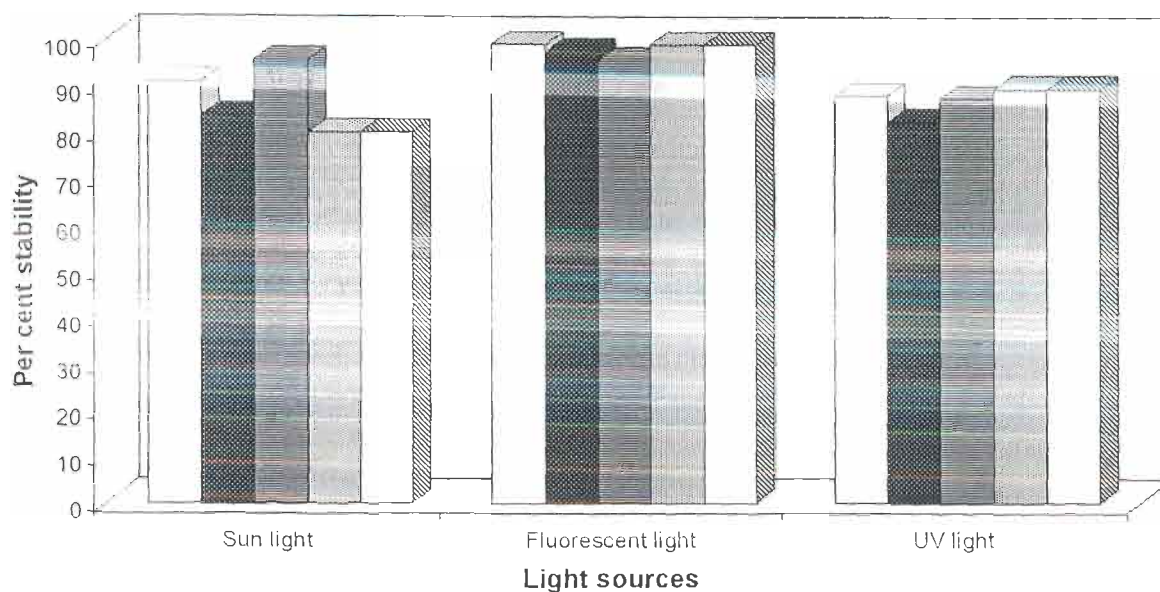
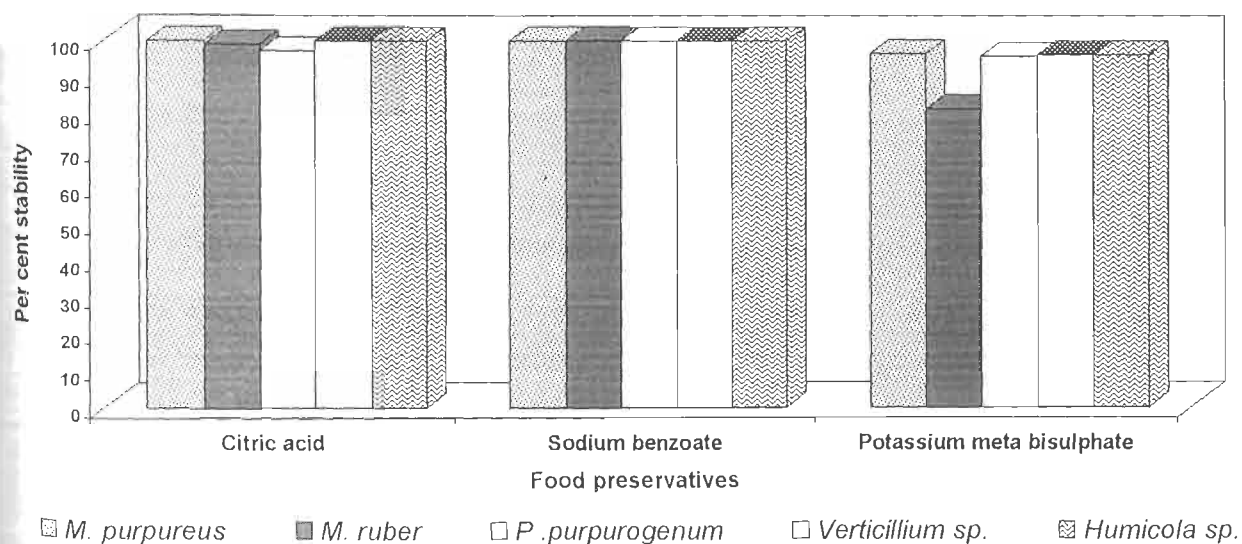


Fig. 20d. Effect of food preservatives on the stability of fungal pigments



range) there was maximum absorption invariably in all the samples of pigments. Pigments of *Monascus* spp. exhibited absorption peak at visible range at 500 nm and 350 nm. Pigments of *Penicillium purpurogenum* absorbed maximally at 490 nm pigments for *Verticillium* sp. and *Humicola* sp. exhibited absorption peaks at 350 nm and at 500 nm.

Absorption maximum at UV range is of little significance for colours. More of the bright colours crimson red, brick red, orange, yellow, etc. are visible colours usable in food industry. UV absorption spectrum indicates that apart from the visible colours, the pigment preparation contains other interesting compounds. perceivably secondary metabolites of pharmaceutical importance as Monacolin - K.

4.8.2.2. Thin layer chromatography of fungal pigments

The fungal pigments were partly purified using Thin layer chromatography. The crude pigments of fungi were resolved into discrete bands of different colours. The pigments from *Monascus ruber* recorded 13 bands depicting different colours of yellow, orange and red (Plate 70). Their R_f values are presented in the Table 39.

Pigments from *Monascus purpureus* exhibited 3 separate bands on TLC plates (Table 39). They were yellow, pink and orange pigments.

Pigments from *Penicillium purpurogenum* displayed two distinct bands of yellow and deep red.

Interestingly, pigments of *Verticillium* sp. were separable into five individual bands with yellow, orange and pink colours.

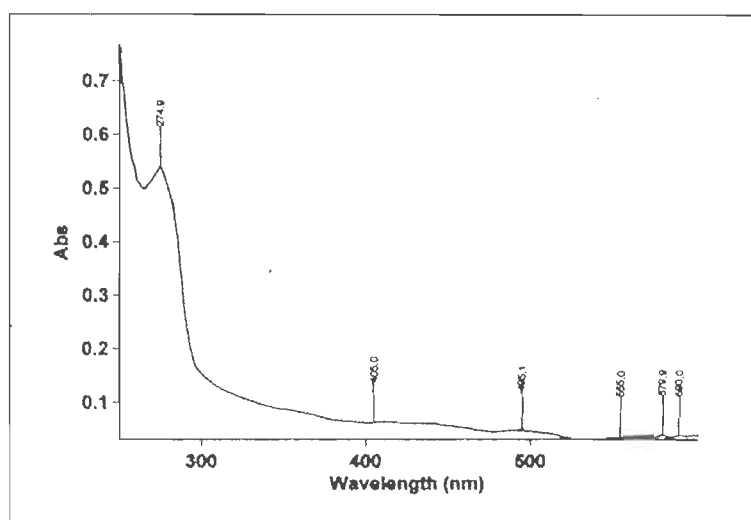
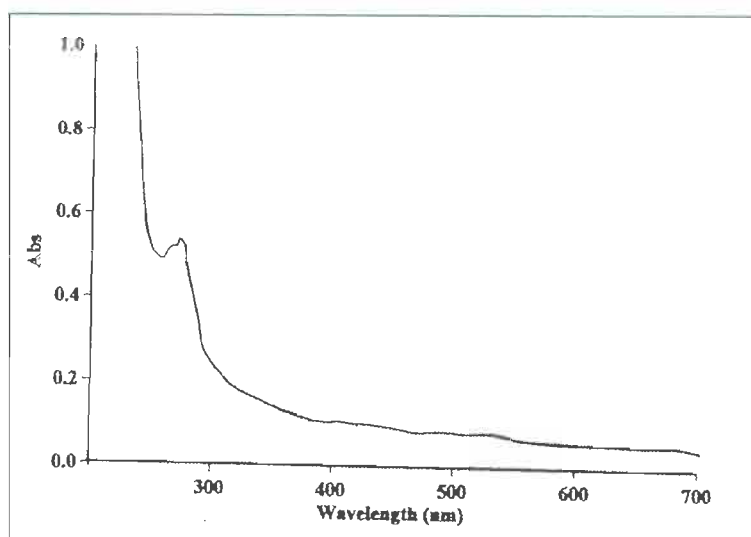
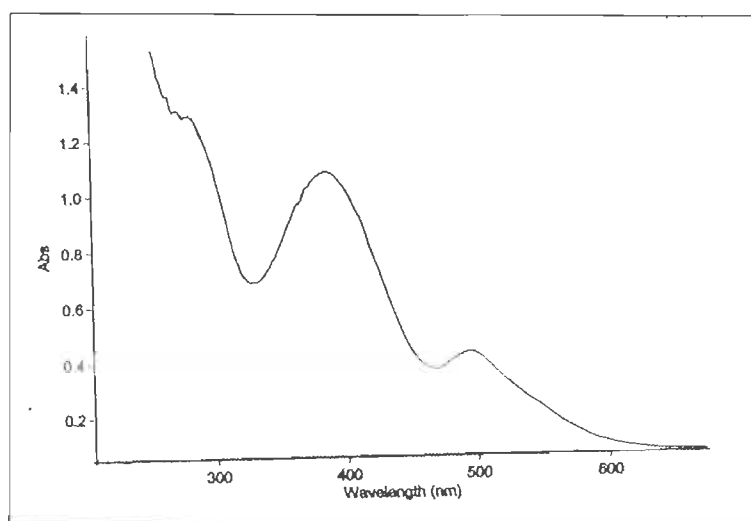


Fig. 21a. Spectrophotometric scanning of pigments of *Monascus purpureus*.

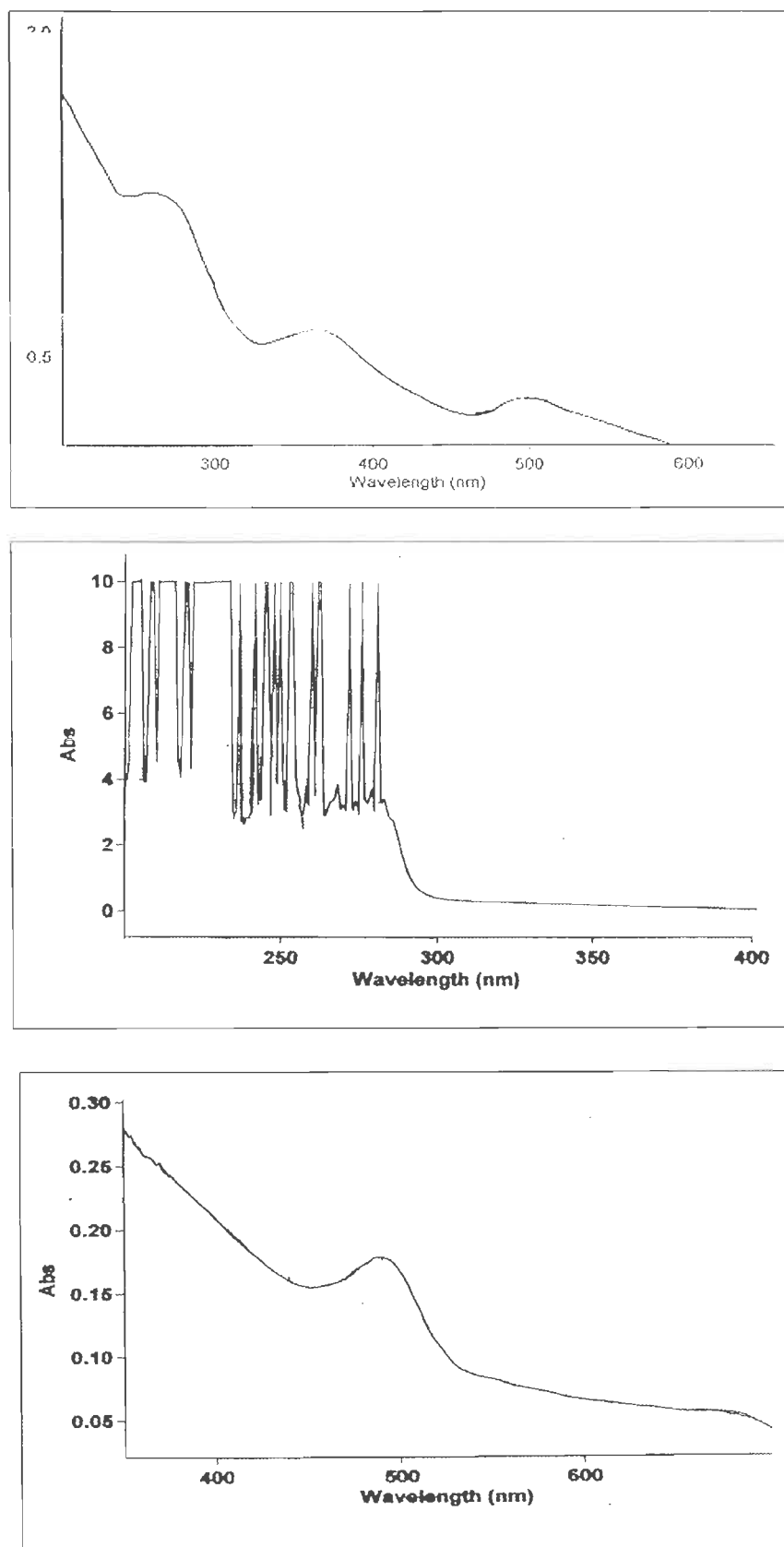


Fig. 21b . Spectrophotometric scanning of pigments of *Monascus ruber*.

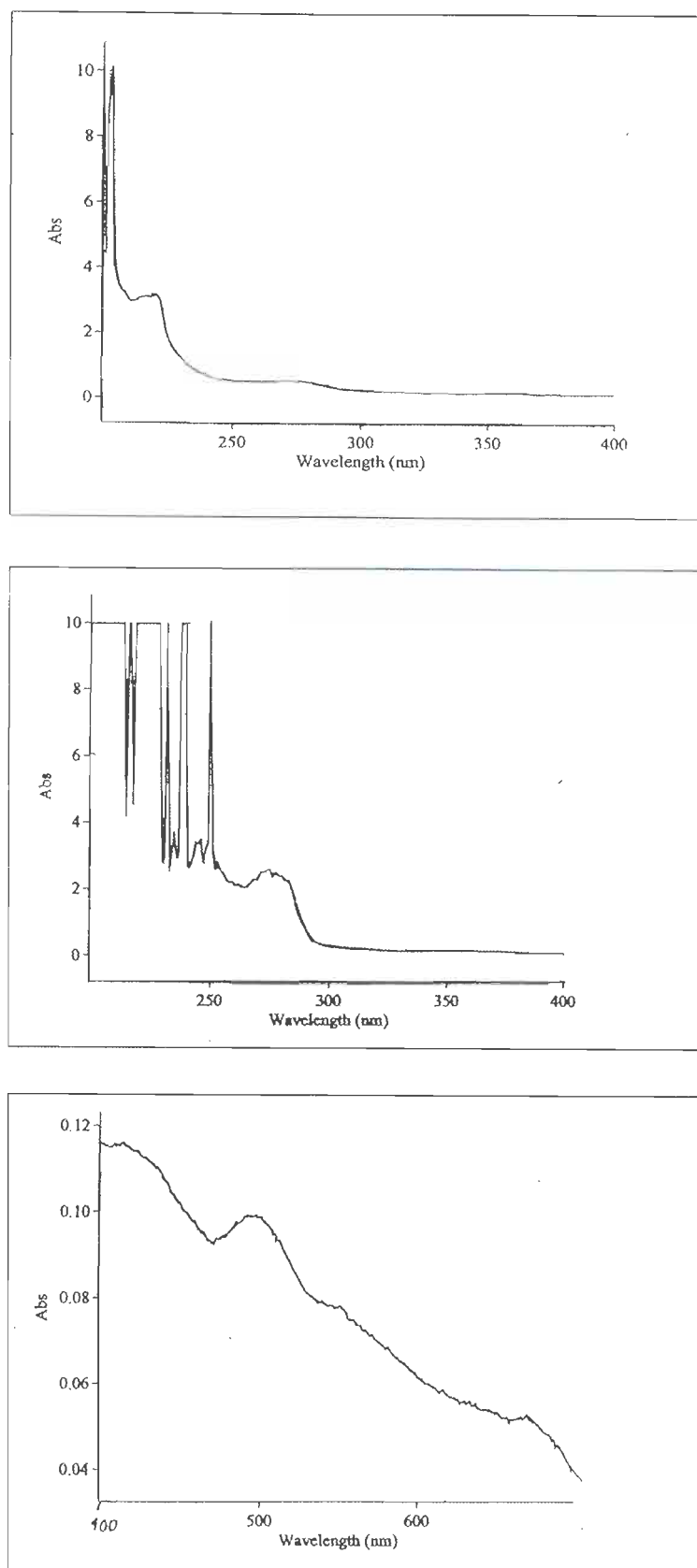


Fig. 21c. Spectrophotometric scanning of pigments of *P. purpurogenum*.

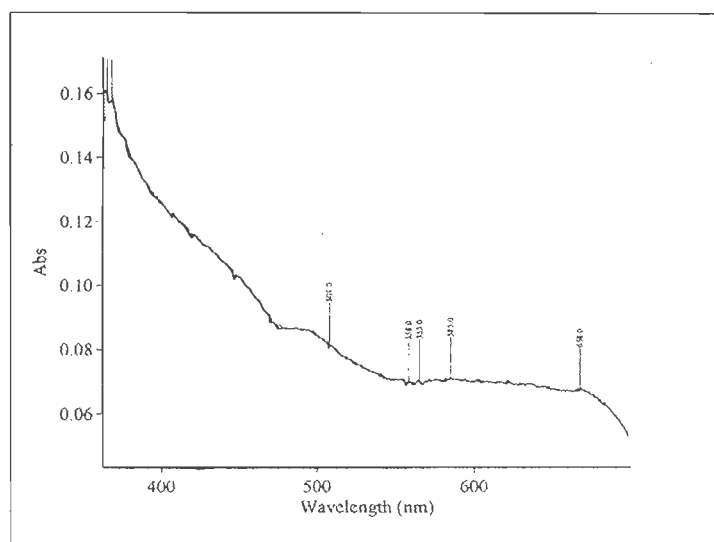
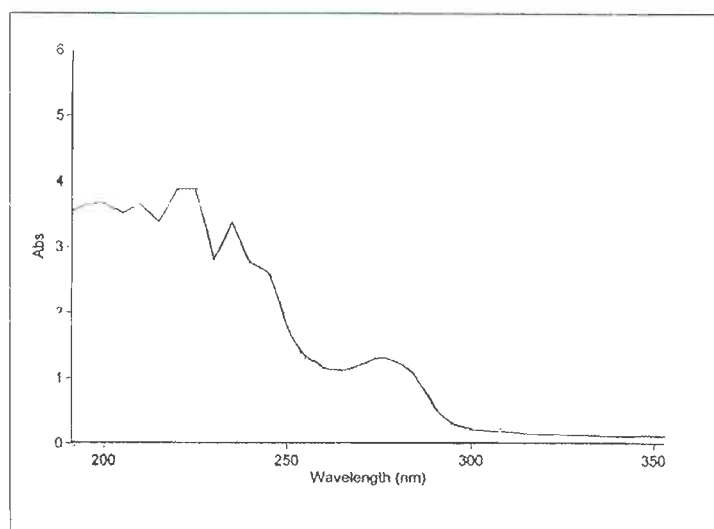
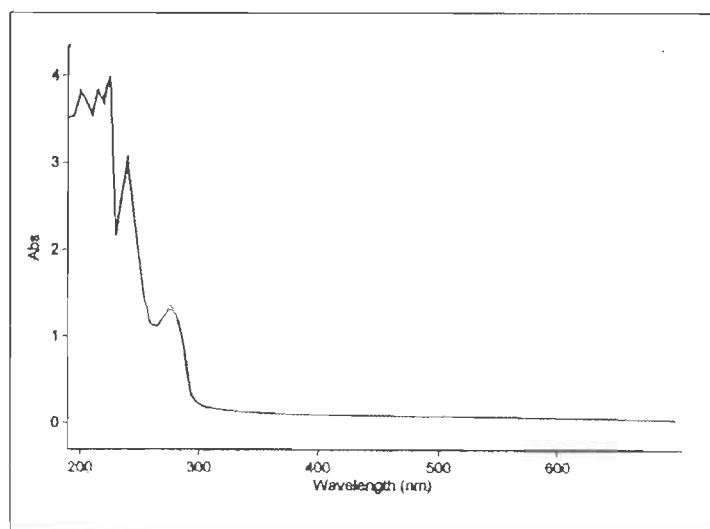


Fig. 21d. Spectrophotometric scanning of pigments of *Verticillium* sp.

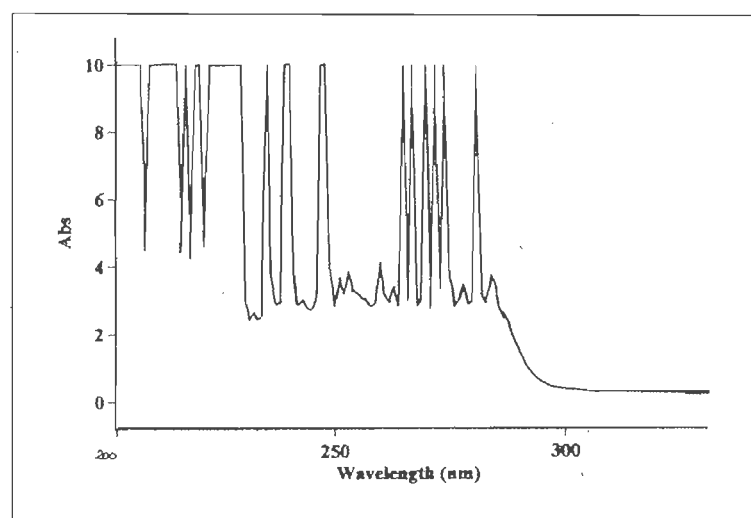
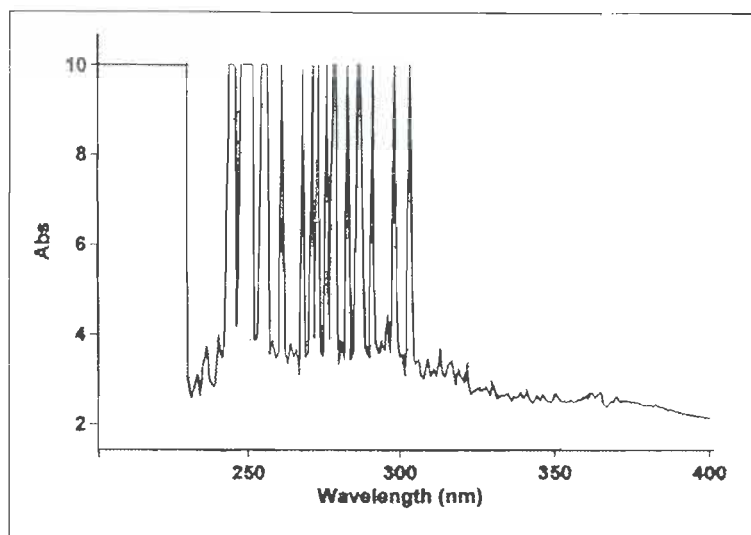
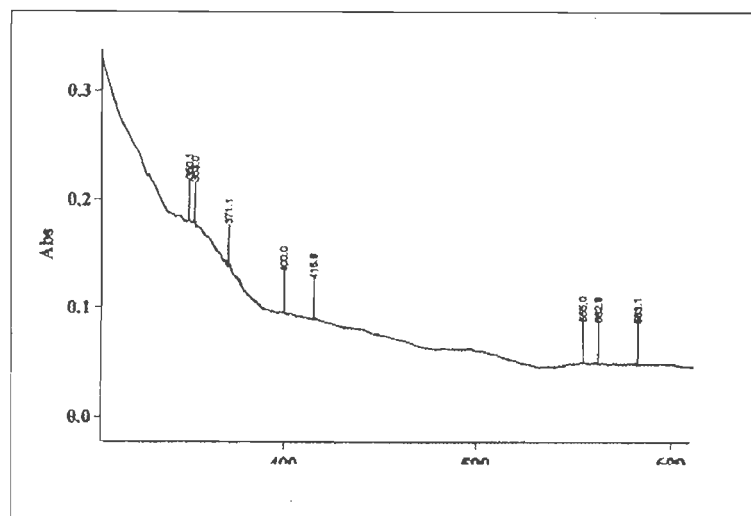


Fig. 21e. Spectrophotometric scanning of pigments of *Humicola* sp.

Table 39 . Thin layer chpomatographic separation of pigments.

No	Source of fungal pigments	Band No.	R _f value	Colour of the bands
1	<i>M. purpureus</i>	1	0.97	Yellow
		2	0.68	Pink
		3	0.17	Orange
2	<i>M. ruber</i>	1	0.97	Red
		2	0.86	Red
		3	0.75	Orange
		4	0.71	Pink
		5	0.66	Pink
		6	0.58	Pink
		7	0.50	Pink
		8	0.45	Pink
		9	0.41	Red
		10	0.33	Red
		11	0.15	Pink
		12	0.11	Pink
		13	0.75	Pink
3	<i>P. purpurogenum</i>	1	0.97	Yellow
		2	0.85	Red
4	<i>Verticillium</i> sp.	1	0.72	Yellow
		2	0.65	Orange
		3	0.21	Orange
		4	0.11	Pink
		5	0.05	Pink
5	<i>Humicola</i> sp.	1	0.94	Brown
		2	0.59	Red

* Solvent system used is Chloroform : Methyl alcohol : Water :: 65: 20: 4



Plate 70. TLC separation of pigment from *Monascus ruber*.

The crude pigments from the fungus *Humicola* sp. upon TLC showed two discrete bands of dark brown and red colours.

From these analysis, it may be inferred that predominating pigments in the four fungi were yellow and red pigments. Red pigments predominated in all the fungi.

4.8.2.3. HPLC analysis of pigments

HPLC analysis were carried out to know the retention time and similarities between the pigments. The results of the analysis are set out in Figure 22.

From the studies conducted with HPLC, the fungi *Monascus purpureus* and *M. ruber* exhibited peaks at 14 and 15 minutes and at 2 min. One peak was registered in both fungi. *Penicillium purpurogenum* recorded peaks at 0.9 min and 1.33 min. *Verticillium* recorded peak at 0.8 min and 1.3 min. This shows that these pigments are quite similar in chemical structure. *Humicola* sp. produced 3 distinct peaks at 1 min, 1.5 min and 2.5 min. which is characteristically different from the other fungi.

It may be summed up that all the five species of fungi produced distinct pigments separable into several fractions. Apart from the colouring matter, there might be compounds of pharmaceutical importance which require further study.

4.9. Toxicological studies on fungal pigments

Toxicity of fungal pigments if any was tested in a sensitive insect silkworm as detailed earlier. The data are presented in Table 40.

Pigments from the five fungi where administered with the normal diet, mulberry leaves caused no toxicity. The movement of the worms was normal. There was no symptom of ill health and abnormal secretion. Cent per cent survival was recorded in the worms in all treatments. The worms underwent normal moulting.

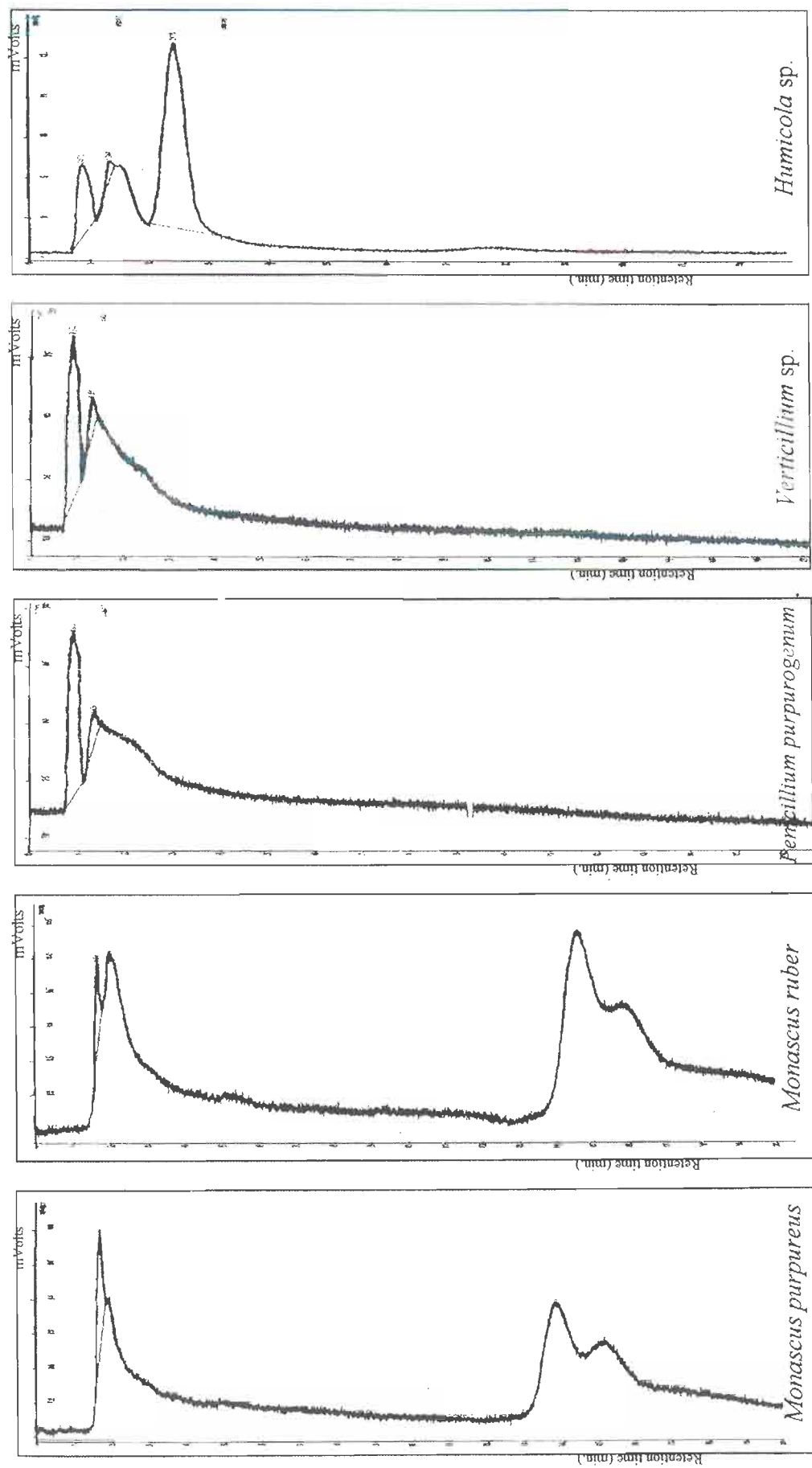


Fig. 22. HPLC analysis of pigments from the selected fungi.

Table 40. Toxicological studies of fungal pigments on the silkworm.

Source of fungal pigments	Silk worm activity				
	Movement	Feeding rate	Symptoms of ill health	Abnormal excretion	Death
Untreated	Normal	Voracious	Nil	Nil	Nil
<i>Monascus purpureus</i>	Normal	Voracious	Nil	Nil	Nil
<i>Monascus ruber</i>	Normal	Voracious	Nil	Nil	Nil
<i>Penicillium purpurogenum</i>	Normal	Good	Nil	Nil	Nil
<i>Verticillium</i> sp.	Normal	Good	Nil	Nil	Nil
<i>Humicola</i> sp.	Normal	Low	Nil	Nil	Nil

Each treatment consisted of one hundred larvae.

These observations suggest that the fungal pigments, at the concentration used were not toxic to silkworm larvae.

4.10. Product Development using *Monascus* pigments

Finely powdered *Monascus* pigments were utilized as colour additive for the development of food products. To enhance the appearance of foodstuff and acceptability, *Monascus* pigments were added.

Three products namely halwa, cake and squash were developed with *Monascus* pigments (Plate 71 to 73). A panel of judges critically assessed the quality, acceptability of the food preparations. The scores are presented in Table 41.

The results indicated that addition of pigments of *Monascus* enhanced the acceptability and also their appearance. By the use of pigments, the squash colour improved very much.

4.11. Analysis for *Monacolin K* and product development

Spectrophotometric and HPLC analysis of fungal broth and pigment extracts were done to detect the presence of the therapeutic compound Monacolin K. This compound absorbs maximally, at 237 nm.

The results have indicated that Monacolin - K was very much present in the preparations. Further HPLC analysis has confirmed the presence.

4.11.1. Spectrophotometric analysis of monocolin-K

The results of the spectrophotometric scanning of the pigments obtained from the figure 23.

Table 41. Organoleptic test for the *Monascus* pigments added food products.

No	Characters	Products scoring*					
		Wheat Halwa		Plum Squash		Soft Cake	
		A	B	C	D	E	F
1	Colour and Appearance						
	<u>9 8 7 6 5 4 3 2 1</u> Good Poor	7.1	3.9	8.1	4.6	8.5	6.5
2	Flavour						
	<u>9 8 7 6 5 4 3 2 1</u> Good Poor	7.4	3.8	7.9	5.1	7.8	7.6
3	Texture						
	<u>9 8 7 6 5 4 3 2 1</u> Good Poor	6.2	4.2	8.2	7.5	8.5	7.5
4	Taste						
	<u>9 8 7 6 5 4 3 2 1</u> Good Poor	8.1	3.8	8.0	6.8	8.6	8.0
5	Overall acceptability						
	<u>9 8 7 6 5 4 3 2 1</u> Good Poor	7.8	4.0	8.6	6.5	9.0	7.5

* Scores are the means of ten-member judge panel

<i>Monascus</i> Pigment added		Un pigmented control	
A	Wheat Halwa	B	Wheat Halwa
C	Plum Squash	D	Plum Squash
E	Soft Cake	F	Soft Cake

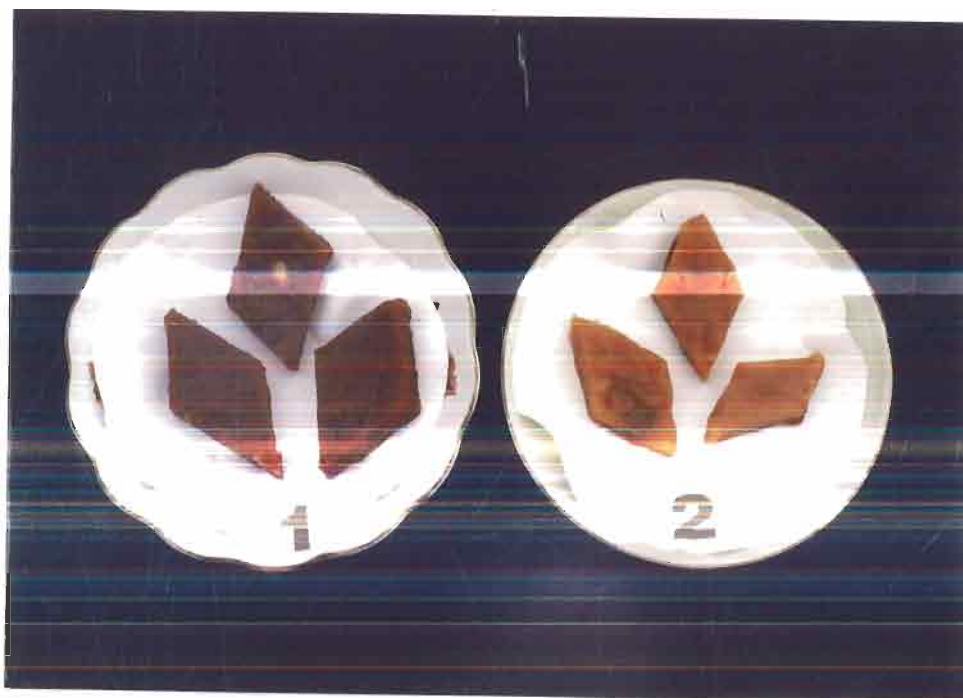


Plate 71 . Product development with *Monascus* pigment.
1. Biocolour fortified Halwa 2. Halwa without biocolour



Plate 72. Product development with *Monascus* pigment
1. Fortified with biocolourant plum squash 2. Plum squash (Control)

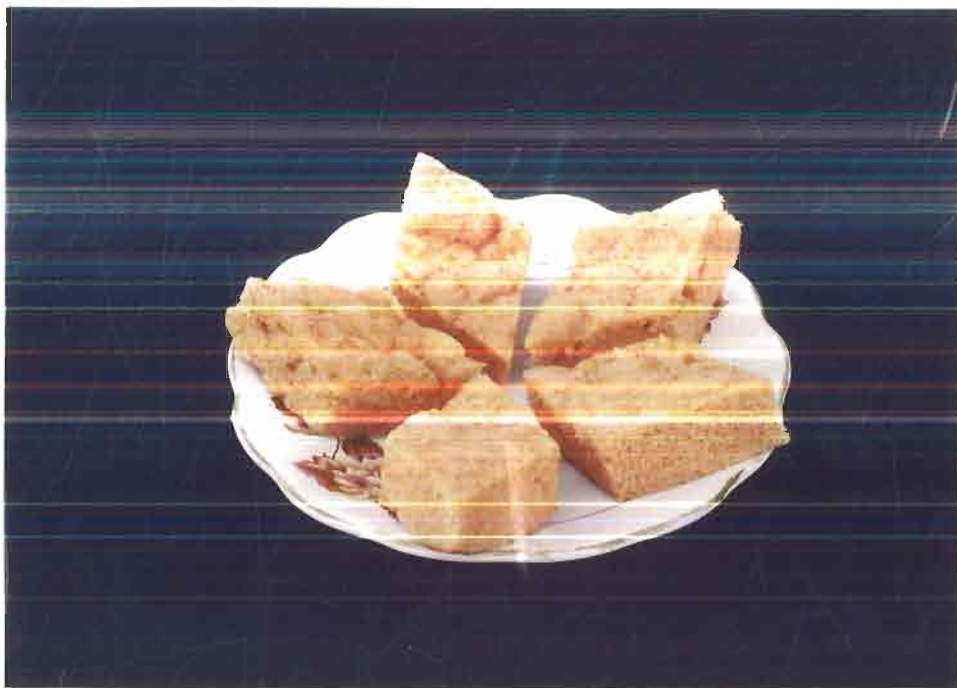


Plate 73. Product development with *Monascus* pigments
Biocolourant fortified soft-cake.

The results clearly indicated that the 'Lovastatin', commercial tablet of Monacolin-K was detected at 237 nm and 254 nm. The extracts of *Monascus purpureus* and *Monascus ruber* also registered corresponding peaks at this wavelength. This clearly indicates the presence of the wonder drug Monacolin-K in the *Monascus* spp. pigment preparation.

4.11.2. HPLC analysis of Monacolin-K

HPLC analysis of Monacolin-K is presented in the figure 24.

The results clearly suggest that the extracts of *Monascus* spp. recorded peaks at the retention time of 3.0 min, which corresponded to the commercial Monacolin-K suggesting that conditions favoured pigment production also have favoured the production of Monacolin-K and both the economic products could be isolated from the fungi.

The study concludes that the presence of Monacolin-K in the extracts of *Monascus* spp. offers wide scope in both food and pharmaceutical industries.

4.11.3. Product Development

The rice based powdered *Monascus* pigments were stuffed into gelatin capsules for the purpose of medication as a source of 'Lovastatin' (Plates 74 and 75). The capsules are stored for future use.

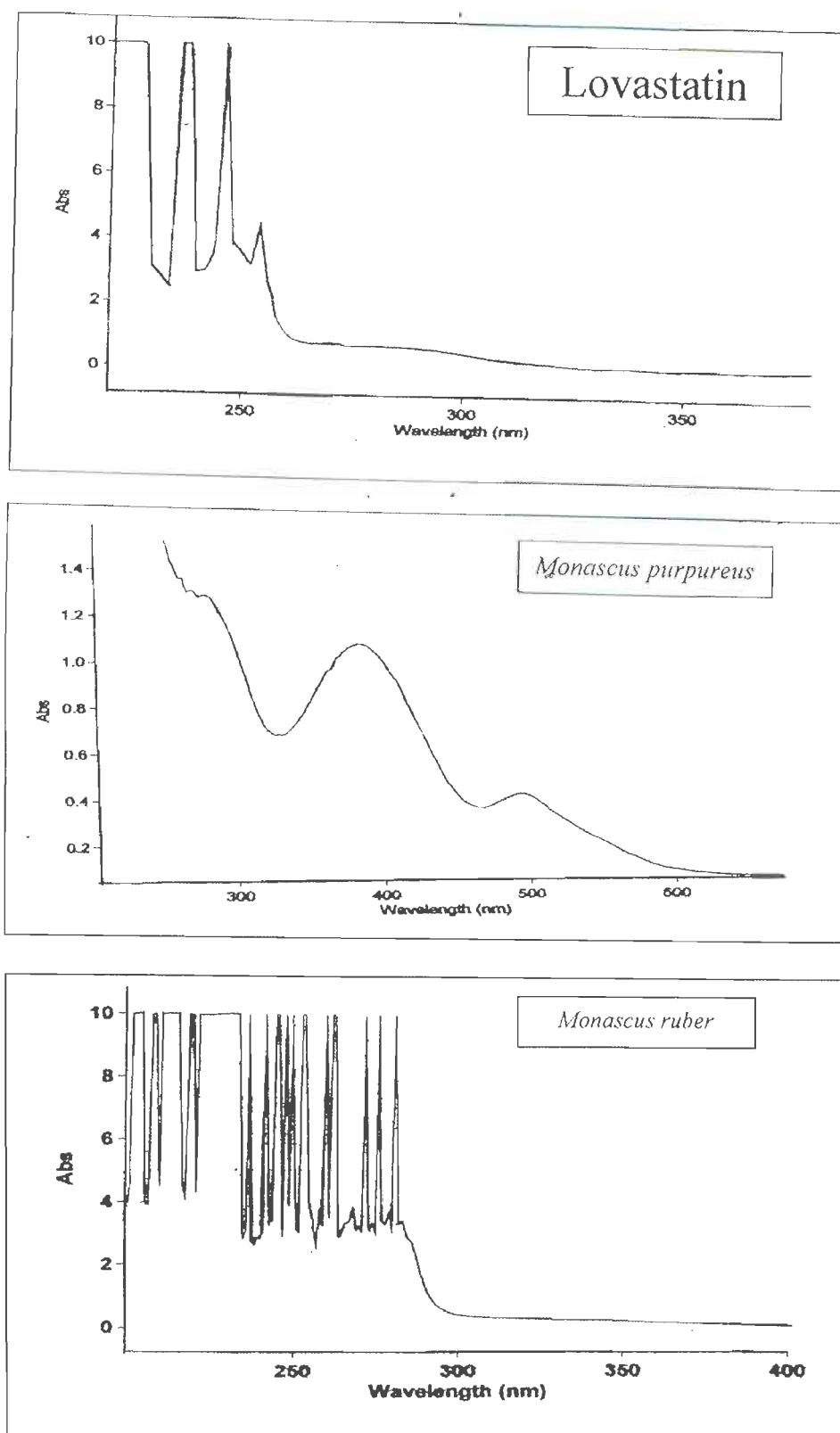


Fig. 23. Spectrophotometric analysis for the presence of Monacolin-K drug in the *Monascus* spp.



Fig. 24. HPLC analysis of *Monascus* spp. for the presence of Monacolin-K



Plate 74. Red rice powder used for MONACOLIN K capsule production.



Plate 75. Monacolin K in capsule form.

Discussion

Chapter V

DISCUSSION

Ever since the microorganisms were discovered, their usefulness to humanity has been slowly unfolded. With the dawn of Microbial Biotechnology greater impetus has been given to microorganisms. It is amazing to see scores upon scores of newer species of microorganisms are recognized and being utilized in several industrial processes.

Since long time, a variety of colours, have been used in food preparation and in making of soft drinks. An appealing and pleasing colour of food and beverages is an important characteristic that goes a long way with the aesthetic value and ready acceptance by people. Many a synthetic dye have crept into food industry in the middle of last century and assumed monopoly.

Though they are cheap, fast acting, stable, attractive with innumerable shades, people have realized their ill effects. Many cause cancer and attendant hazards to human health (Singh, 1997). Therefore, the trend of the day is to abandon synthetic colours and use colours of natural origin. Various plant parts, animal products, insects and naturally occurring minerals were used over hundreds of years as natural colours. Microorganisms occupy a distinct place as source of natural colours. More than bacteria fungi display a wide range of fascinating colours. However, till recently fungi have remained unexplored for colour production. This is perhaps due to their association with mycotoxins and aflatoxins. In the present study, an attempt has been made to identify species of pigment producing fungi and to make use of the biocolourants in food preparations. The well known pigments producing fungi like, *Monascus purpureus* and *M. ruber* have also been included.)

5.1. Source and isolation of pigment producing fungi

As soil is regarded as the "treasure house" of microorganisms, soil samples were collected from different parts of India and from the rhizosphere of crops. The purpose of selecting different soil samples was to explore the biodiversity as much as possible. Western Ghats of south India is considered to be rich in biodiversity of plants, animals and microorganisms. Rhizosphere soils samples, by virtue of their richness in nutrients would provide a variety of fungi.

The study revealed that the population of fungi was much higher in the forest soil samples than in cultivated soils presumably due to rich organic matter content of the former. Though the number of pigment producing fungi in general was low, there existed considerable biodiversity in pigment producing fungi in forest soils.

5.2. Screening of fungi for pigment production

Though all the isolates of fungi were pigment producing in nature a screening schedule was necessary to recognize the potential pigment producer. Under a given set of growth conditions the quantity of extracellular pigments produced was examined for newer isolates. This vigorous screening yielded three isolates, PF-1, PF-12 and PF-16. These isolates excreted very intense colours of red, orange and reddish brown pigments respectively. As these three isolates consistently registered very high quantity of pigment excretion, they were included for detailed studies further.

5.3. Taxonomic identification and characterization of fungal isolates

The three newer isolates of fungi obtained in the present study were characterized. Identity of the standard cultures was confirmed by their reproductive structures with the help of key provided by Hawksworth and Pitt (1983).

The three newer isolates were studied using moisture chamber technique. The morphological features of the isolates were critically examined and their taxonomic positions were determined as *Penicillium purpurogenum* (PF1) *Verticillium* sp. (PF12) and *Humicola* sp. (PF16) as per the detailed description provided by Gilman (1957), Domsch and Gams (1972), Ainsworth *et al.* (1973), Cannon *et al.* (1985) and Dennis (1968).

5.4. Certain cultural and physiological characters of the selected fungi

In order to recognize the optimum growth condition for maximum pigment production, the selected fungi were grown with various sources of carbon and nitrogen and at different temperature and pH levels. The isolates were grown under different light and speed of agitation.

5.4.1. Carbon sources

The study has revealed that *Monascus* spp. grew well and produced higher mycelial biomass with carbon sources like glucose, starch and mannitol. Pigment production was recorded high with the carbon sources as glucose, maltose and mannitol. Broder and Koehler (1980) observed that maltose yielded higher red pigments than the yellow pigments in *Monascus*, glucose and fructose recorded low red pigments. Further they stated that more red pigments were recorded when the growth media contained high levels of ammonium nitrate with low glucose. *Monascus* spp. have been cultivated in media with mannitol (Yamanaka *et al.*, 1975), ethyl alcohol (Yoshimura *et al.*, 1975 and Yamanaka *et al.*, 1975), rice powder (Lin and Suen, 1973, Su and Huang, 1980 and Miyake *et al.* 1982) as carbon sources. Chen and Johns (1994) confirmed that glucose and maltose yielded high pigment production in *M. purpureus*. Carbon sources of the medium greatly influence growth and pigment production. Glucose and starch were preferred for growth while maltose yielded higher quantity of pigments in fungi.

5.4.2. Nitrogen sources

In the present study, out of 14 nitrogen sources tested for growth and pigment production, yeast extract and ammonium nitrate were preferred by fungi. Peptone, monosodium glutamate and sodium nitrate yielded good biomass accumulation and pigment yield. Yoshimura *et al.* (1975) found that yeast extract amended medium registered good growth of *Monascus* spp. The present results are in agreement with those of Yoshimura *et al.* (1975). In that yeast extract, nitrates, monosodium glutamate favoured high pigment production. Carels and Shepherd (1977) discussed that yeast extract increased red pigments and ammonium nitrate produced yellow pigments.

Chen and Johns (1993) reported in contrast to the earlier observation that ammonium and peptone served as good nitrogen sources that yielded better growth and pigmentation. They obtained the highest concentration of pigments in glucose-peptone medium at pH 6.5. Organic nitrogen sources favoured red pigment production in *Monascus* spp. because it serves also as a carbon source and promoted the protein bound dissolution of red pigment into the culture broth (Broder and Koehler, 1980). These studies clearly point out that by providing in the growth medium appropriate sources of carbon and nitrogen one can harvest heavy yields of biocolourants. Organic sources of nitrogen are rapidly absorbed without much biochemical transformation.

The pH of the fermenting broth could influence the pigment production and also the quality of the pigment (Morton and Macmillan, 1954). When fungi metabolize carbon and nitrogenous compounds in the medium, the pH of the medium is bound to change. This cannot be avoided until one uses a highly buffered growth medium.

5.4.3. Temperature

Temperature of incubation has profound influence on pigment production and fungal biomass yield. Generally, at 35°C there was higher biomass and pigment production. Many scientists working with *Monascus* spp. observed that the fungus preferred 35°C as its optimum temperature (Wong and Bau, 1977; Bau and Wong 1979, Broder and Koehler, 1980; Wong and Koehler, 1981a, b and Wang *et al.*, 1981). Maintenance of 35°C in the fermentors is a cost intensive process. During the hot period of the year, the ambient temperature goes upto 40-45°C and during winter temperature falls to < 18°C. It becomes necessary to keep the temperature at 35°C for harvesting pigments from fungi.

5.4.4. pH of the growth medium

Fungi generally prefer an acidic pH for good growth and activity. The selected fungi were grown at a pH of 5.5 for all experiments. In the present study different pH levels from pH 4.0 to pH 9.0 were set for culturing fungi. The results have shown that an acid pH range of 5.0- 6.0 enhanced higher biomass and pigment yields. pH of the growth medium has got a far reaching consequences on the physiology of fungi. Conidial development and pigment synthesis are influenced by different pH levels; at acidic range reduced the conidiation and increased pigmentation. At alkaline ranges, growth and pigmentation got reduced. Conway and Downey (1950) have shown that the intracellular pH of the microorganism did not vary much in response to changes of the extracellular pH. However, the external pH affected the transport of materials from extracellular to intracellular and *vice versa*.

Chen and Johns (1993) explored that fungal growth and ankaflavin synthesis were favoured at low pH where as the production of other pigments was relatively independent of pH. They have concluded that the nature of nitrogen source decided

the growth and pigmentation. They also observed a high ratio of extracellular to intracellular pigment absorbance at 500 nm at the pH 6.5. Evans and Wang (1984) were of the opinion that pH of the growth medium pH 5 - 6.0 favoured maximum pigment production. The present results are in accordance with their views.

5.4.5. Agitation/aeration of growth medium

The result of the present study have revealed that agitation of the medium has been negatively correlated with the biomass production while it was positively correlated with extracellular pigments production. At low speed of agitation, the fungi produced 'pulp form' of mycelia. When the rate of agitation increased, the mycelia became pellets i.e. spherical.

Agitation is said to improve extracellular pigmentation. This may be due to the centrifugal force acting on the mycelium, which throws away the pigment. Moreover, agitation improves O₂ supply to the mycelium which may induce pigment production. Eventhough the biomass yield was low, pigment production improved. In the present study, due to higher speed of agitation compact mycelial pellets were formed with no conidiation. This has improved biomass and pigment production in the selected fungi.

Takahashi *et al.* (1965) and Yoshimura *et al.* (1975) reported many forms of *Monascus* spp. pellets due to agitation of the broth. The forms included were pulp type, pellet type, globose type and intermediary forms. Yoshimura *et al.*, (1975) reported that morphology and physiology of fungi influence the pelletization of mycelium during agitation. According to them, at 300 rpm pulp type of filaments appeared; at 600-800 rpm mycelial growth decreased and pigment production became high and at above 1200 rpm pellets were formed with diminished pigment production.

Hamdi *et al.* (1996) suggested that aeration due to agitation improved pigmentation in *Monascus purpureus*. They further proposed that due to aeration, the ethanol accumulation in the broth reduced leading to accumulation of pigments.

5.4.6. Light

In the present study, the cultures were grown under different spectrum of visible light and also in darkness. Incubation under darkness favoured highest biomass and pigment production. This may be due to the avoidance of light destruction of pigments. White light also supported good growth, may be due to the stimulatory effect of light spectrum. However, incubation under different screened light like blue, yellow, green, red recorded lower accounts of pigment production. Red and yellow lights destroyed the pigments while blue light supported good pigmentation and growth in all the fungi.

5.5. Pigment production through fermentation

Large scale pigment production from fungi can be achieved in two ways; solid state fermentation and liquid state fermentation.

5.5.1. Solid state fermentation

In the present study, different solid substrates along with rice were used. *Penicillium purpurogenum* produced enormous spores on solid substrates and hence it was withdrawn. *Monascus* spp and other fungi were successfully grown in different substrates. Rice bran and sugarcane bagasse were preferred by the fungi. *Verticillium* sp. utilized bagasse better than others. Rice starch being the preferred carbon source for *Monascus* spp., they grew well on rice bran also. *Humicola* sp, though grew well on these substrates, failed to produce pigments in considerable amounts. This may be due to profuse conidiation.

At present, solid state fermentation (SSF) technique has become very popular throughout the world. *Monascus* spp. utilized all solid substrates viz., rice, maize, corn, wheat, and their by products like bran. Koji fermentation of *Monascus* spp. essentially is solid state fermentation on rice. It is interesting to note that most oriental food fermentations are indeed solid state fermentations. It is advantageous over liquid fermentation in terms of pigment yield but disadvantageous in terms of the downstream process of separation of pigments from substrates.

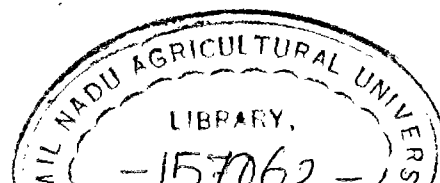
Hesseltine (1977 a and b) utilized different substrates like rice, corn, soybean and millets for the fermentation of *Monascus*. But pharmaceutical fermentations of *Monascus* are not based on SSF. Instead, liquid state fermentation is preferred for the production of pigments and also for other metabolites. Pigment production in *Monascus* fungi, by solid state fermentation always has resulted in 10 fold higher yields (Lin, 1973; Mak *et al.*, 1990).

5.5.2. Liquid state fermentation

Although traditional processes in Asia have predominantly employed solid state fermentation methods, liquid state fermentation or submerged fermentation is the classical method still in use in western countries.

5.5.3. *Monascus* spp. in liquid fermentation

In the present study, pilot scale fermentations were carried out in Haffkin's flasks with large volume of medium. This favoured high pigment yields in short time. Although separation of pigments from the broth is difficult, the amount of pigment secretion is very high. As against solid state fermentation, *Penicillium purpurogenum* produced more pigments in liquid fermentation. In liquid fermentation excess spore production is controlled.



The literature on liquid fermentation is massive. To optimize cultural conditions, various key factors have been modified with an aim of harvesting heavy pigment yields. Lin (1973) demonstrated that *Monascus* spp. could be cultivated as submerged culture and pigment yields could be improved. Yamaguchi *et al.* (1973) developed a method to harvest water soluble pigments in submerged fermentation by adding amino acids with protein as nitrogen source. This would cause rearrangement of bases with the pigment skeleton which produce hygroscopic pigments. The yield of *Monascus* pigments obtained by Lin and Suen (1973) was over 1080 absorbance units per litre broth. However, in the present study the yield of pigment was much higher.

Lin and Iizuka (1982) developed mutants of *Monascus kaoliang* that produced one hundred fold increased pigment yield. Youngsmith *et al.* (1994) determined pure yellow pigments from the culture broth by using a mutant of *Monascus* spp. Lee *et al.* (1998) demonstrated that when incubation time increased pigment production also increased. As an advancement in liquid culture study, coculture of *Saccharomyces cerevisiae* enhanced pigment production 10 folds in the broth (Shin *et al.*, 1998). Lee and Chen (1998) thoroughly studied the effect of aeration in liquid fermentation. Even though many parameters have been optimized for many fold increases in pigment production, extraction of pigments from the broth is still laborious.

5.6. Low cost media for pigment production

In the present study, a few modification were introduced in order to cut down the input cost. In the place of fermentors and large glass vessels, poly propylene bags were experimented upon. Polybags can serve as a mini fermentor for the production of pigments. Polybag fermentors were also helpful for easy handling. This facility can be followed in small scale industries of low capital investment. The yield of pigments from such polybags fermentor, is as good as the yields obtained from conventional fermentation.

A low cost liquid medium for polybag fermentor was also designed. The results have suggested that rice kanji after proper dilution with water can be used for pigment fermentation. Yoshimura *et al.* (1975) also obtained high productivity of *Monascus* pigments with rice medium. As a by-product of rice cooking, rice kanji is a nutritive substrate. With proper dilution rice kanji can serve as a good medium for pigment production. It is interesting to observe that pigment production in rice kanji was higher than that obtained with conventional media.

Many other low cost substrates have been tried earlier. Soy soaking water and waste water of 'temph' were used for *Monascus* pigments production. Hamdi *et al.* (1998) tried prickly pear juice as a low cost input with monosodium glutamate. They harvested significantly higher yields of pigments with prickly pear juice. Yongsmith *et al.* (1994) utilized cassava waste in the liquid medium for the production of *Monascus* pigments especially the yellow pigments.

The study has revealed a low cost solid substrate, different forms of rice in polybags. Palo *et al.* (1960) reviewed the solid substrate fermentation with different rice varieties and they concluded that non glutinous rice varieties gave higher yields of pigments.

5.7. Genetic improvement in pigment producing fungi

Genetic improvement of the selected fungi was carried out by mutagenesis. N methyl N nitro, N-nitrosoguanidine was used as mutagen. The spores of the fungi, *M. ruber*, *M. purpureus* and *Penicillium purpurogenum* were mutated under optimum conditions. A large number of mutants were obtained. Due to the random scissoring action of NTG, more stable mutants with improved pigmentation were obtained.

Many of the mutants exceeded the parent strains in terms of pigment yield. Pigment yield increased by 30 per cent and these mutants were considered as super strains. Besides, many morphological and albino mutants were also obtained. Lin and Suen (1973) treated ascospores of *Monascus* spp. F2 with N methyl - N nitro N-nitrosoguanidine (NTG) and isolated pigments over producing strains. Wong and Bau (1977) irradiated spores of *Monascus purpureus* with X-ray and fast-neutron and obtained six strains with differed morphology, photoresponses and antibacterial activity. Hiroi *et al.* (1978) obtained a hyper pigmentative mutant using NTG. Wong and Koehler (1981) used UV irradiation to get mutants in *Monascus*. Lin and Iizuka (1982) obtained mutants after alternative treatments with NTG and UV irradiation and these mutants produced extracellular pigments on various substrates. They claimed that they obtained mutants with 100 fold yield increase over the parent strain. Recently, Yongsmith *et al.* (1994) obtained mutants of *Monascus* spp. which produced high concentration of yellow pigments with 10 fold increase over the wild parent. In the present study NTG mutagenesis has yielded mutants in the three species of fungi with 30 per cent improved pigment production. These fungi offer great scope for further genetic manipulation to improve upon the pigment yields. Incidentally, all the described mutants are stable without reversion

5.8. Pigments extraction and purification

Separation of pigments from the culture media was attempted by two processes, (i) air drying and (ii) solvent extraction procedures.

In the present study, the cell free culture filtrate obtained through filtration of the broth was air dried under bright sun light and the crude pigment recovered. Pigments were further purified with methyl alcohol or ethyl alcohol. These solvents

were found suitable for all the five fungal pigments. However, extraction of pigments directly from the broth necessitated the use of other non polar solvents like chloroform, acetonitrile and petroleum ether. The crude preparation of pigments were lyophilized and stored. The crude pigments of *Monascus* as it was viscous was made into a fine powder on mixing with rice flour.

Pigments of *Monascus* have been extensively studied by scientists. The initial isolation and characterization of pigments were done by Fielding *et al.* (1960 and 1961), Haws and Holker (1961) and Inouye *et al.* (1962). *Monascus* pigments are readily soluble in ethanol and slightly soluble in water (Su and Huang, 1980).

The red pigment, Monascorubrin from *Monascus* was soluble in ether, methyl alcohol, ethyl alcohol, benzene, chloroform, acetic acid and acetone (Su and Huang, 1980). For the isolation of pigments many methods have been suggested in the literature. The mycelial pigments could be extracted with hot methyl alcohol (Blanc *et al.*, 1994), freeze dried mycelial sonication and liquid nitrogen drying followed by ethyl alcohol extraction (Wong and Koehler (1981) and Martinkova *et al.* (1999). Further purification of pigments was carried out by chromatography and HPLC procedures (Yoshimura *et al.*, 1975; Wong and Koehler, 1983; Blanc *et al.*, 1994 and Martinkova *et al.*, 1999).

In the present study, the extracted crude pigments were separated by thin layer chromatography (TLC) into distinct bands and the bands were eluted and analyzed further. The partially purified pigments were analyzed using HPLC to detect the individual compounds. The methods suggested by Blanc *et al.* (1994) were followed in the HPLC analysis.

5.9. Stability of pigments

Fastness and stability of pigments are major factors in the practical use of biocolours. It is expected that the pigments are highly stable at elevated temperatures and at widely varying pH and also upon exposure to different light rays.

The pigments isolated in the present study were tested for their stability at different temperatures (50, 70 and 120°C), different pH levels (pH 2.0 to 9.0), different light sources (UV, fluorescent and sunlight) and with addition of different chemical preservatives. The results have clearly shown that the pigments are quite stable not only under normal conditions but also at extreme conditions. There was little decomposition or degradation of pigments, while studying the stability of *Monascus* pigments. Broader and Koehler (1980) noticed no colour change after 3 hours of refluxing at 66°C and they noticed cloudy precipitation by autoclaving due to protein-pigment complex formation. Wong and Koehler (1983) added evidence to support that there was little effect on the pigments due to UV and heat treatments. Hajjaj et al. 1997 studied the stability of pigments in sunlight and they confirmed that there was no reduction in pigment intensity. Great damage to pigments occurs after 40 hrs of prolonged exposure to sunlight. The observation of Lee *et al.* (1998) that pigments over 24 hrs of exposure at 80°C were quite stable adds additional support. Lee and Chen (1998) listed all the parameters related to stability of pigments of *Monascus*. They studied light, heat and chemical degradation of *Monascus* pigments extensively and concluded that *Monascus* pigments were stable at all conditions. Lin and Demain (1994) reported that the pigments extracted in 70 per cent methanol was heat stable upto 1000°C, however, n-butanol extracted pigments were stable only upto 60 hrs of exposure to day light (Hajjaj *et al.*, 1997). These studies confirmed that the pigments of *Monascus* spp are very stable.

5.10. Toxicology studies on pigments of fungi

The present study also included observations of the toxicity if any. The pigments were administered along with feed to silkworms to observe the toxicity if any of the crude pigments. The results have clearly shown that the worms were healthy without any symptom of ill health. In fact, worms had a preference of pigment treated leaves over the control. Hence, the pigments are safe to use. It is necessary to extend these tests on rats, rabbits and guinea pigs to ensure the non toxic nature of the pigment. This part of the investigation could not be completed. As far as *Monascus* spp. are concerned, conflicting reports have been published on toxicity. Wang and Hesseltine (1979) straight away declared that there was absence of mycotoxins in the traditional fermented *koji*. Parenthetically, *Monascus* has not been reported to cause any disease in humans (Dubos and Hirsch, 1965). Acute oral and intraperitoneal toxicity on mice was observed at LD 50 values of 33.3 g and 8.7 g / kg body weight respectively (Kaio *et al.*, 1978). Sako *et al.* (1983) reported that toxicity in fetel rat hepatocyte enzymes. John (1991) conducted genotoxicity tests of *Monascus* spp. and showed no toxicity.

Sabater-Vilar *et al.* (1999) after conducting AMES mutagenic tests and teratogenic tests of *Monascus* spp. reported that *Monascus* failed to induce mutagenicity. The antibiotic activity exhibited by *Monascus* poses some problem now a days. In the same way, the fungal metabolite, citrinin has invited debate among Food Technologists. By avoiding citrinin producing *Monascus* strains this problem can be circumvented as suggested by Hajjaj *et al.* (1999 and 2000a). They further concluded that the low levels of citrinin in food applications of *Monascus* would not cause any serious problem. All the five pigments of fungi are confirmed as non toxic and safe for use.

5.11. Product development with *Monascus* pigments

In the East, pigments of *Monascus* are already in active use in several food preparation. Surprisingly it also finds use in human medicine. In the present study, *Monascus* pigment was added to the Indian common sweet, Halwa prepared from wheat milk for improving its colour and aesthetic value. As the pigments were not readily soluble in water, it was first dissolved in ethanol and mixed with the milk. The pigments formed a complex with the foodstuff. A second food product, plum squash was prepared with *Monascus* pigment to impart red colour to the squash. A soft cake was also added with *Monascus* pigment to tone up the colour.

Innumerable food preparations have been developed by using *Monascus* pigments. Traditionally, red rice, red wine, sausages, fish sauces, paste, meat products, soybean curd are prepared in the far East with these pigments. In the Western world these pigments are used in the preparation of ice cream, sauce, bread, curing meat products, candies and confectionaries (Anonymous, 1999a). Palo *et al.* (1960) described the production of red rice and Dizon and Sanchez (1984) prepared *Monascus* pigment fortified banana sausage and 'tocino'. For replacing nitrites in the curing of meat, *Monascus* pigments are utilized (Chen and Ockerman, 1998).

The present study has revealed that *Monascus* pigment fortified food preparations received very high acceptance. It is fervently hoped that in the foreseeable futures these fungal pigments would receive greater attention by Food Scientists in India.

5. 12. Monacolin-K - Analysis and product development

A wonder drug from *Monascus* extracts was recognized as 'Monacolin K' a cardio vascular drug which is suggested for reducing blood cholesterol (HMA-CoA reductase inhibitor) (Anonymous, 1999c).

Extracts of *Monascus* spp. grown red rice has shown authentic peak at 4.00 min (retention time) when analyzed in HPLC which matched well with 'Lovastatin' a commercial grade product of Monacolin K. Absorbance at 237 nm and in spectrophotometric scanning also revealed the presence of lovastatin in ample quantity in red rice. Red rice powder was dispensed as capsules for ready use. Endo (1979) first observed the presence of Monacolin in the extracts of *Monascus* spp. History acknowledges the wisdom of ancient Chinese in Tang Dynasty during 800 A.D. who used red rice to normalize blood circulation. The 'Chinese pharmacopoeia' - '*Ben Cao Gang Mu - Dan Shi Bu Yi*' published during Ming Dynasty (1368-1644) mentioned the medicinal properties of this drug. After Endo's discovery of Monacolin-K, many forms of Monacolin have been developed (Endo, 1979; Endo *et al.*, 1985 and Endo *et al.*, 1996). Recent studies have proved that Lovastatin yields from the extracts of *Monascus* spp. ranged from 0.71 mg to 1.27 mg g⁻¹ of red rice (Garong *et al.*, 1998). In the Western countries, the cost of Red rice capsules is high. As a cheap source of cardio vascular drug, *Monascus* pigment was placed for the scrutiny by the Indian pharmaceuticals. This can also be produced by small scale entrepreneurs. Recently Heber *et al.* (1994) cleared the doubts about Monacolin-K by proving its activity through convincing experimental results.

From the foregoing discussion it becomes clear that fungi like *Monascus* spp. *Penicillium purpurogenum*, *Verticillium* sp. and *Humicola* sp. offer great scope for industrial exploitation not only for biocolourant production but also for synthesizing life saving drugs like Monacolin - K.

Summary

Chapter VI

SUMMARY

The primary objective of this study was to explore fungal pigments as biocolourants for food industry. Owing to the conflicting reports on the safety of permitted food colours and due to the indiscriminate use of non-permitted colours, there is an urgent need to identify natural pigment sources as safe food colourants. This study critically examines the potentials of fungi as sources of biocolourant production. Along with an established pigment producing fungus, *Monascus* newer fungi have been explored and reported. Soil samples from across the country were collected and pigment producing fungi were isolated. In addition to *Monascus* spp., *Verticillium* sp., *Penicillium purpurogenum* and *Humicola* sp. were studied to optimize the production technology for pigments. Genetic improvement of these cultures were also attempted to improve the yield of pigments. Finally, different food products with fungal pigments were prepared and evaluated. The results of the various experiments are summarized below:

1. A total of thirty soil samples were collected from various parts of the country and analyzed microbiologically for their fungal flora. As many as twenty promising isolates of fungi that recorded high pigment producing ability were obtained. A rigorous screening of these isolates helped narrowing down to three promising isolates viz., PF1, PF12 and PF16 that stood out distinctly for pigment production.
2. Taxonomic characterization of the new isolates revealed that they were *Penicillium purpurogenum*, *Verticillium* sp. and *Humicola* sp. *Monascus purpureus* and *Monascus ruber* were also included in the study.

3. For optimizing conditions for maximizing pigment production, the selected five fungi were grown with different carbon and nitrogen sources at different levels of pH, various temperatures, aeration and agitation levels and at different light conditions. Fungal biomass yield, pH shifts in the culture medium, intracellular and extracellular pigment accumulation were studied. Glucose, sucrose, starch and maltose supported good biomass and pigment production in all the fungi. pH 5.0-6.0, temperature 35°C, 100-150 strokes per minute of agitation and incubation in dark were the optimal parameters to harvest higher biomass and pigments from the fungi.
4. Pigment production both by solid state fermentation (SSF) and by liquid fermentation were studied. Various agricultural products and by products were used as substrates for pigment production. In the case of solid substrates, rice was selected due to its capacity to enhance production of pigments. In the case of liquid fermentation, rice kanji was selected.
5. An attempt was made to compose a low cost fermentation medium for pigment production. Rice kanji (ten times diluted with water) and raw rice (cooked) served as better media for biocolour production.
6. Pigments from the fermented broth were extracted and purified based on air-drying process and also by using methyl alcohol solvent extraction. The pigments were lyophilized and stored at 5°C.
7. The partially purified pigments were analyzed for their absorbance at different wavelengths. Pigments were repurified based on their spatial movement on TLC plates. Further, the pigment fractions were analyzed using HPLC technique. The pigments were also studied for their stability in different treatments like pH, temperature, light and chemical preservatives.

8. The parent strain of *Monascus* spp. and *Penicillium purpurogenum* were exposed to N-methyl N-nitro N-nitrosoguanidine (NTG) for developing mutants with increased pigment production. These mutants exhibited genetic stability and recorded significantly higher yield of pigments.
9. Toxicological studies made on the pigment using a sensitive target organism, silkworm, revealed that the pigments were non toxic to silkworms and interestingly the worms preferred the pigments. Mammalian and human toxicity studies on the pigment need to be carried out.
10. Using *Monascus* spp. pigments as a biocolourant, a few food products like halwa, plum squash and soft cakes were prepared. The acceptability of the products was scored with a ten member panel of judges. Pigment fortified halwa, plum squash and soft cakes were adjudged as most preferred products.
11. An important cardio-vascular drug, Monacolin K was analyzed in the extracts of *Monascus* spp. The presence of Monacolin K was detected in the pigments based on the spectrophotometric and HPLC analysis. The “red rice” powder stuffed gelatin capsules were made as a step towards developing a pharmaceutical product of *Monascus* pigment.

From the foregoing summary it becomes amply clear that *Monascus* spp. and other potential fungi like *Verticillium* sp., *Humicola* sp. and *Penicillium purpurogenum* offer good scope for industrial production of biocolourants.

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Appendices

APPENDIX I

COMPOSITION OF GROWTH MEDIA

1. Potato Dextrose agar medium (Riker and Riker, 1936)

Composition

Potato tubers (Peeled)	- 250 g
Dextrose	- 10.0 g
Tap water	- 1000 ml
pH	- 5.5 - 6.5
Agar	- 15 g

Preparation

Well sliced potato tubers were cooked for 15 minutes in 250 ml tap water over a water bath. After cooling, the slices were macerated and filtered through two layers of cheesecloth. The volume of the extract was made upto 1000 ml with water.

2. Martin's Rose Bengal Agar medium (Martin, 1950)

Glucose	- 10.0 g
Peptone	- 5.0 g
Magnesium sulphate	- 0.5 g
Potassium dihydrogen phosphate	- 1.0 g
Rose Bengal	- 33.0 mg
* Streptomycin	- 30.0 mg
Distilled water	- 1000 ml
pH	- 5.5 - 6.5
Agar	- 15 g

* Streptomycin was added just before the plating of the medium.

3. Malt yeast extract medium (Difco manual, 1977)

Malt extract	- 50.0 g
Distilled water	- 1000 ml
pH	- 5.5. - 6.5
Agar	- 15 g

4. Yeast morphology agar medium (Difco manual, 1977)

Yeast extract	- 3.0 g
Malt extract	- 3.0 g
Peptone	- 5.0 g
Glucose	- 1.0 g
Chloramphenicol (Autoclaved)	- 50-500 mg
Distilled water	- 1000 ml
pH	- 5.5 - 6.5
Agar	- 15 g

5. Sabouraud dextrose agar medium - (Difco manual, 1977)

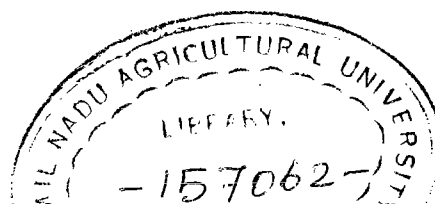
Peptone	- 10.0 g
Dextrose	- 40.0 g
Distilled water	- 1000 ml
pH	- 5.5 - 6.5
Agar	- 15 g

6. Czapek-Dox Agar medium - (Pitt, 1979)

Sucrose	-	30.0 g
Sodium nitrate	-	2.0 g
Dipotassium hydrogen phosphate	-	1.0 g
Magnesium sulphate	-	0.5 g
Potassium chloride	-	0.5 g
Ferrous sulphate	-	0.01 g
Distilled water	-	1000 ml
pH	-	5.5 - 6.5
Agar	-	15 g

7. *Monascus* medium (Lin *et al.*, 1992)

Maltose	-	50.0 g
Monosodium glutamate	-	12.4 g
Dipotassium hydrogen phosphate	-	2.4 g
Potassium dihydrogen phosphate	-	2.4 g
Magnesium sulphate	-	1.0 g
Potassium chloride	-	0.5 g
Zinc sulphate	-	0.011 g
Ferrous sulphate	-	0.01 g
Manganous sulphate	-	0.03 g
Ammonium nitrate	-	0.286 g
Distilled water	-	1000 ml
pH	-	5.5 - 6.5
Agar	-	15 g



8. Mycological Agar Medium (Difco Manual, 1977)

Soytone	-	10 g
Dextrose	-	10 g
Distilled water	-	1000 ml
pH	-	5.5 – 6.5
Agar	-	15 g

APPENDIX II

Appendix II a. Correlation coefficients for the effect of carbon sources on growth and pigment production in fungi.

Character	<i>M. purpureus</i>	<i>M. ruber</i>	<i>P. purpureogenum</i>	<i>Verticillium sp</i>	<i>Humicola sp</i>
Biomass × pH	0.5957	0.8069	0.4480	0.5396	0.3899
Biomass × intracellular pigment	0.7850	0.8812	0.5127	0.6714	0.1409
Biomass × extracellular pigment	0.7709	0.9222	0.3907	0.5284	0.4500

Appendix II b. Correlation coefficients for the effect of nitrogen sources on growth and pigment production in fungi.

Character	<i>M. purpureus</i>	<i>M. ruber</i>	<i>P. purpureogenum</i>	<i>Verticillium sp</i>	<i>Humicola sp</i>
Biomass × pH	0.2687	0.03715	0.3366	0.0859	0.16605
Biomass × intracellular pigment	0.2666	0.4052	0.3811	0.2942	0.3639
Biomass × extracellular pigment	0.2472	0.0963	0.1895	0.3205	-0.0496

Appendix II c. Correlation coefficients for the effect of temperature on growth and pigment production in fungi.

Parameter	<i>M. purpureus</i>	<i>M. ruber</i>	<i>P. purpureogenum</i>	<i>Verticillium sp</i>	<i>Humicola sp</i>
Temp × Biomass	-0.1902	0.0222	-0.0061	-0.7145	0.0656
Temp × pH shifts	0.1990	-0.2068	0.1799	0.8244	0.2755
Temp × intracellular pigment	-0.1634	-0.3466	-0.3466	-0.4018	-0.3148
Temp × extracellular pigment	-0.0681	0.3287	0.3287	-1.000	0.2267

Appendix II d. Correlation coefficients for the effect of pH on growth and pigment production in fungi.

Parameter	<i>M. purpureus</i>	<i>M. ruber</i>	<i>P. purpureogenum</i>	<i>Verticillium sp</i>	<i>Humicola sp</i>
pH × Biomass	-0.4845	-0.5578	-0.8089	-0.7798	-0.4038
pH × pH shifts	-0.9687	-0.9724	-0.9819	-0.9835	-0.9964
pH × intracellular pigment	-0.5762	-0.7225	-0.8840	-0.5603	-0.6278
pH × extracellular pigment	-0.9246	-0.9246	-0.7611	-0.4683	-0.7615

Appendix II e. Correlation coefficients for the effect of agitation on growth and pigment production in fungi.

Parameter	<i>M. purpureus</i>	<i>M. ruber</i>	<i>P. purpureogenum</i>	<i>Verticillium sp</i>	<i>Humicola sp</i>
Agitation × Biomass	-0.7661	-0.5066	-0.4200	-0.4740	-0.9962
Agitation × pH shifts	-0.4085	-0.4959	-0.5006	-0.3822	-0.9719
Agitation × intracellular pigment	-0.9624	-0.9511	-0.8493	-0.9888	-0.9694
Agitation × extracellular pigment	0.4927	0.2889	0.2224	0.8611	0.8614

Appendix II f. Correlation coefficients for the effect of light on growth and pigment production in fungi.

Parameter	<i>M. purpureus</i>	<i>M. ruber</i>	<i>P. purpureogenum</i>	<i>Verticillium sp</i>	<i>Humicola sp</i>
Intracellular × Extracellular Biomass	0.9501	0.8564	0.9252	0.7953	0.7374
× intracellular pigment	0.8814	0.7745	0.4614	0.3731	0.3505
× extracellular pigment	0.7495	0.7472	0.5638	0.3442	0.0527

List of Publications of The Author

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