PHYSIOLOGY OF *MONASCUS PURPUREUS* IN RELATION TO METABOLITE PRODUCTION AND APPLICATION AS FUNCTIONAL FOOD

THESIS

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FOREWORD

Monascus purpureus during growth on solid medium produces red coloured pigments. Due to this property, the fungus has been used traditionally to colour wine and meat. Chinese folk medicine describes the use of *Monascus* pigments as health tonics and as curents for several metabolite disorders.

The pigments of *M. purpureus* are called as polyketides and also known to produce monacolins. Recent studies have shown antihypertensive, hypocholesterolemic, antioxidant and anticancerous properties of these pigments. Despite the vast literature that have characterized the metabolites, fungal physiology in relation to metabolite production is still scarce.

This thesis describes the role of enzymes in *M. purpureus* pigment production and characterization of two new metabolites for functional food preparations.

INTRODUCTION

The Chinese medical book published in the first Century described Anka (also called as anka-kak) or red mold rice for health benefits. Later literature documented the use of red colour obtained from anka for colouring foods. In 1884, a French Botanist Philippe van Thieghem isolated the fungus, a red mold growing on potato and linseed cakes and called it as *Monascus ruber,* since it produced only one ascus containing a number of spores. Subsequently, Went (1895) isolated *Monascus purpureus* from anka obtained from the markets of Java and Indonesia. The interest for the colour resulted in the characterization of several *Monascus* species isolated from different foods around the world. Presently, there are over 50 patents describing the use of *Monascus* pigments for food. The annual consumption of pigment in Japan alone is approximately 100 tons valued at $1.5 million. Recently, applications of *Monascus* colours for sausage, hams, fish paste, surimi and tomato ketchup have been described (Laurent Dufosse´ *et al.* 2005)

MONASCUS PURPUREUS

Kingdom: Fungi

Class: Ascomycetes

Family: Monascidae

Order: Plectascales

Genus: *Monascus*

Species: *purpureus*

The majority of *Monascus* sp were isolated from several oriental foods. The species thus far characterized are described in **Table 1**.

Table 1. Origin of *Monascus* sp

S. NO	Origin	Strain	Species
1	tofu	*albidus*	*purpureus*
2	tofu	*albidus var. glaber*	*purpureus*
3	anka	*anka*	*purpureus*
4	anka	*anka var. rubellus*	*purpureus*
5	moldy bran	*araneosus*	*purpureus*
6	red koji	*barkeri*	*ruber*
7	moldy bran	*fuliginosus*	*ruber*
8	anka	*kaoliang*	*purpureus*
9	anka	*major*	*purpureus*
10	plant	*paxii*	*ruber*
11	moldy bran	*pilosus*	*pilosus*
12	moldy bran	*pubigerus*	*pilosus*
13	anka	*purpureus*	*purpureus*
14	plant	*ruber*	*ruber*
15	anka	*rubiginosus*	*purpureus*
16	moldy bran	*rubropunctatus*	*pilosus*
17	tofu	*serorubescens*	*pilosus*
18	tofu	*vitreus*	*ruber*

RED RICE PROCESSING

Rice is traditionally processed for fermentation after washing and cooking. Additives like polygonum grass juice and alum water are added to the rice in order to enhance the rate of mold growth. *M. purpureus* is pitched and fermentation is allowed to go on for 9 days at 25°C and pH 5-6 (Ma *et al.* 2000). The fermented rice is air dried, pulverized and encapsulated in gel capsule for use as health tonic (Heber *et al.* 1999). Animal and human subjects fed with these capsules tested for hypolipidemic properties showed a lowered total cholesterol, LDL-cholesterol and triglycerides. Several studies carried out that exemplify the above property have been shown in **Table 2**

Table 2 Health benefits of red rice

Authors	Design	Dose	TC	LDL-C	TG	HDL-C
(Wang *et al.* 1997)	Human, random, sb, positive control n=446	1.2 g/d	P: ↓7% T: ↓22.7%	P: ↓15.3% T: ↓30.9%	P: ↓12.8% T: ↓34.1%	P: ↑8.4% T: ↑19.9%
(Heber *et al.* 1999)	Human, random, db, placebo, n=83	2.4 g/d	P: - T: ↓16.8%	P: - T: ↓22.3%	P: - T: ↓13.3%	-
(Qin *et al.* 1999)	Human, random, db, placebo, n=65	1.2 g/d	P: ↓6.5% T: ↓25.9%	P: ↓7.9% T: ↓32.8%	P: ↑2.3% T: ↓19.9%	N/M
(Rippe *et al.* 1999)	Human, self-controlled ,open label n=187	2.4 g/d	T: ↓16.4%	T: ↓21.0%	T: ↓24.5%	T: ↑14.6%
(Keithley *et al.* 2002)	Human, pilot with HIV + patients, random, db, placebo, n=12	2.4 g/d	P: ↑4.1% T: ↓13.4%	P: ↑25.1% T: ↓25.1%	-	-
(Li *et al.* 1998)	Animal, in vivo: rabbits, quails	0.2-0.8 g/d	↓	↓	↓	-
(Rhyu *et al.* 2000)	Animal, ex vivo: rat aorta and human endothelia cells	0.1-10 g/d	N/M	N/M	N/M	N/M

P: placebo group; T: treatment group (red rice); % express significant percent change after 8 week of treatment; sb: single blind; db: double blind; -: no significant effect observed; N/M: not mentioned; NO: nitric oxide

Several active components have been characterized from red rice extracts. Sixteen types of active components known as monacolins were shown to exhibit cholesterol lowering action by inhibiting HMG Co-A reductase (Heber *et al.* 1999; Endo 2004; Li *et al.* 2004) and antioxidant action for removing free radicals (Dhale *et al.* 2007a & b) and. (Heber *et al.* 1999) also showed that monacolin-K present in rice fermented with *M. purpureus,* was identical to lovastatin. Useful sterols identified from red rice were β-sitosterol, campesterol, stigmasterol, saponin and sapogenin; isoflavones and isoflavone glycoside; selenium and zinc (Wang *et al.* 1997; Heber *et al.* 1999; Ma *et al.* 2000).

REPRODUCTION IN *M. PURPUREUS*

Asexual sporulation is the most common method of reproduction in *Monascus* sp. Sexual reproduction, which produces ascospores, has also been observed during its submerged growth (Carels and Shepherd 1975). An antheridium arises at a hyphal tip by septation. A female organ grows out from the cell beneath the septum as protuberance and a septum separates the female organ into ascogonium and trichogyne. Male nucleus of the antheridium enters the trichogyne through a small opening into the ascogonium, but the nuclei do not fuse. The ascogonium swells and sterile hyphae grow out from the stalk below the ascogonium, rapidly forming a protective rind. Ascogenous hyphae that arise near the base of' ascogonium grows around and lie close to the surface of ascogonium inside the protective rind. The ascogenous hyphae divide into cells, containing male and female nuclei. Asci are formed after the two nuclei fuse. Meiotic division followed by mitosis result in the formation of eight daughter nuclei. Each nucleus becomes the center of a spore by the extension of a wall, which delimits a volume of cytoplasm. The ascus wall disintegrates, releasing the spores into the ascocarpal cavity. The ascocarp, a stalked cleistothecium breaks open to release the ascospores, which germinate to give rise to new hyphae (Young 1931; Carels and Shepherd 1975). The process of sporulation involves many common developmental themes including temporal and spatial regulation of gene expression, cell specialization, and intracellular communication.

PRODUCTION OF SECONDARY METABOLITES

The shift in metabolic pathways of the fungus during its sub-maximal growth phase results in the production of compounds of functional importance called secondary metabolites. These compounds include antibiotics, carotenoids, fatty acids and plant growth hormones (Smith and Berry 1975). Fermentative production of these chemicals is energy efficient and environmentally agreeable (Nagasawa and Yamada 1995). Commercial production of these metabolites is generally carried out by either solid state or submerged fermentation (Castilho *et al.* 2000). Though the mode of fermentation chosen is based on the product, much of these chemicals are produced through

submerged fermentations due to easy maneuverability of the fermentation conditions for yielding a particular compound with high purity (Nagasawa and Yamada 1995).

The physiological role of secondary metabolites in the organism producing them, is still unclear (Challis and Hoopwood 2003) despite their antimicrobial, fungicidal and insecticidal properties (Calvo *et al.* 2002). However, biotechnological industries harness the metabolic activity of compound produced for use in food, pharmaceutical, chemical and health care industries (Parekh *et al.* 2000). High value products obtained from microorganisms has been reviewed by (Demain 1999). In the recent years, production of natural food colourants through microbial fermentation is an extensive area of investigation since they overcome concerns of unfavourable side effects by synthetic colours (Delgado-Vargas *et al.* 2000).

MONASCUS METABOLITES

Main metabolites produced by *Monascus* sp are pigments and monacolins of polyketide origin. *Monascus* pigments as a group are called azaphilones with similar molecular structure and chemical properties. They consist of the pigments, monacolins (Ma *et al.* 2000), and under certain growth conditions mycotoxins like citrinin (Blanc *et al.* 1995). The orange pigments, monascorubrin and rubropunctatin are synthesized after condensation of one acetyl-CoA with one or more malonyl-CoA. This reaction is followed by decarboxylation due to polyketide synthase-I enzyme **(Fig 1)**.

Molecular structures of *Monascus* pigments are shown in **Fig 2.** Reactions of orange pigments with amino acids give rise to water-soluble red pigments, monascorubramine (Hiroi *et al.* 1975) and rubropunctamine (Fowell *et al.* 1956). Reduction reaction of orange derivatives results in yellow pigments, monascin and ankaflavin (Juzlova P. 1996). Substitution of the replaceable oxygen in monascorubrine or rubropunctatine by nitrogen of the amino group of various compounds such as amino acids, peptides and proteins, change the colour from orange to purple. *Monascus* pigments can be reduced or oxidized and react with other products, especially amino acids, to form various derivative products (Kim *et al.* 2006a & b) sometimes called the

complexed pigments. Glutamyl-monascorubrine and glutamyl-rubropunctatine were isolated from the broth of a submerged culture of *Monascus* (Blanc et al., 1995). The above forms exist in two molecular structures differing in the length of the aliphatic chain. The pigments are produced mainly in the cell-bound state. They have low water solubility, are sensitive to heat, unstable in the pH range of 2–10 and fade with light. It was shown that pigments added to sausages or canned meat pie or paste remained stable (ranging from 92 to 98%) for 3 months storage at 4°C (Fabre *et al.* 1993). The main patents described from *Monascus* have focused on solubilization, stability and the extraction of pigments.

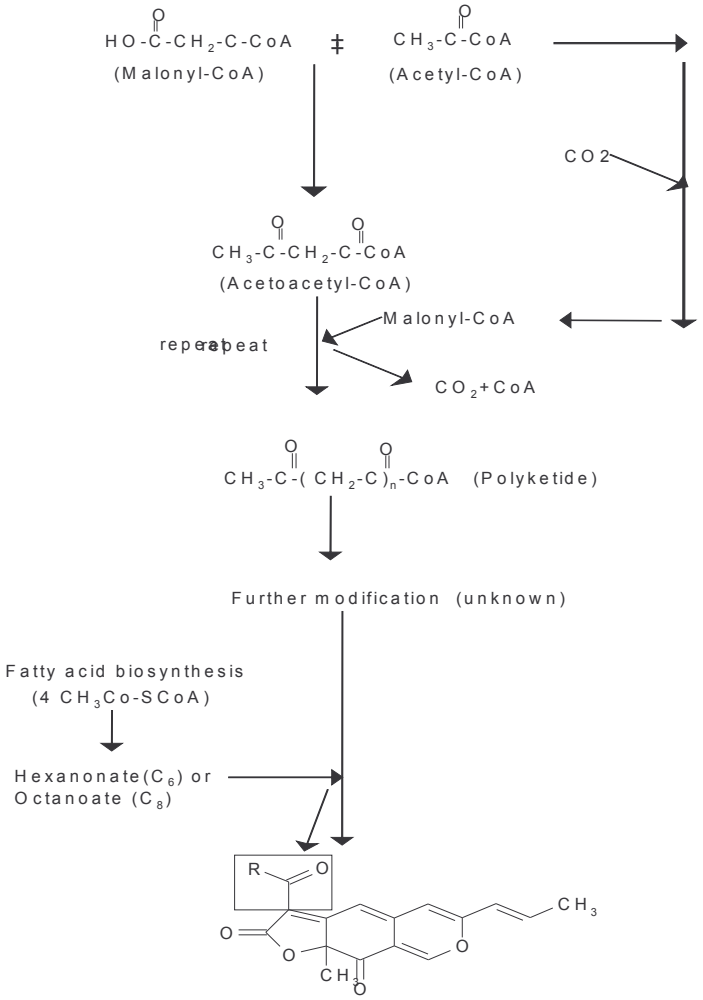


Fig 1. Biosynthesis of orange pigments in *Monascus* (Juzlova *et al.,* 1996b)

SUBMERGED FERMENTATION

Monascus has been successfully cultured under submerged condition for pigment production (Lin 1973; Yoshimura *et al.* 1975; Lee *et al.* 1995). Considerable contradictions exist in the published work as to the best carbon source for red pigment production in liquid cultures. Traditionally, cultures use breads, rice and other amylaceous (starch, dextrins, glucose, maltose and fructose) substrates for high-productivity of pigments which occurs due to glucose and maltose utilization. The nitrogen source seems to have more importance than the carbon source and ammonium, and peptones as nitrogen sources gave superior growth and pigment concentrations compared to nitrate (Hamdi *et al.* 1995). Though *Monascus* sp are aerobic, they utilize glucose at low oxygen concentrations. At this condition, pigment production is hindered due to the production of alcohol and carbon dioxide (Pastrana *et al.* 1995).

The C/N ratio was also shown to be important for pigment production. At a value close to 50 g^{-1}, growth was found to be favored and in the region of 7–9 g^{-1}, pigmentation occurred (Hajjaj *et al.* 1999).

SOLID-STATE FERMENTATION

The solid state fermentation is a traditional method used for centuries. Solid state fermentation has advantages over submerged fermentation for obtaining high pigment yields at low cost (Soccol and Vandenberghe 2003). The classical Chinese methods consist of inoculating steamed rice grains spread on big trays with a strain of *Monascus* sp. The inoculated rice is incubated in an aerated and temperature controlled room for about 20 days. In these types of cultures, moisture content, oxygen and carbon dioxide levels in the gas environment, as well as cereal medium composition, are the most important parameters to control. Red pigments are also produced in plastic bags containing rice grains. It was observed that pigmentation occurred only when initial moisture level was relatively low (26–32%). Initial substrate moisture content regulates pigmentation due to glucoamylase activity increase along with a rise in initial substrate moisture content. Superior yields of pigment were obtained in solid state cultures because of diffusion of intracellular pigments into the surrounding solid matrix and prevented de-

repression of pigment synthesis in solid systems due to the. In submerged culture, the pigments normally remained in the mycelium due to their low solubility in the usually acidic medium (Chen and Johns 1993).

In solid state cultures, oxygen and carbon dioxide influence pigment production significantly while affecting growth to a lesser extent. Oxygen molecules are essential for the polyketide biosynthesis (Turner 1971). Maximum pigment yields were observed at 0.5×10^5 Pa of oxygen partial pressure in closed pressure vessels when *M. purpureus* was cultivated on rice. Low oxygen partial pressure was found to inhibit pigment production in solid state fermentation (Han and Mudgett 1992). High carbon dioxide (Pastrana *et al.* 1995) partial pressures inhibited pigment production and complete inhibition occurred at 10^5 Pa. In packed-bed fermentor, partial oxygen pressures ranging from 0.05 to 0.5×10^5 Pa at constant carbon dioxide partial pressures of 0.02×10^5 Pa resulted in high pigment yields with a maximum at 0.5×10^5 Pa of oxygen. Lower carbon dioxide partial pressures at constant oxygen partial pressures of 0.21×10^5 Pa gave higher pigment yields. Maximum oxygen uptake and carbon dioxide production rates were observed at 70–90 and 60– 80 h respectively, depending on the gas environment. Respiratory quotients were close to 1.0 except at 0.05×10^5 Pa of oxygen and 0.02×10^5 Pa of carbon dioxide partial pressures (Han and Mudgett 1992; Lee *et al.* 1995). *Monascus* sp produces 500 AU g^{-1} dry fermentate under ideal condition like maximum specific growth velocity 0.039 h^{-1} and specific pigment production velocity of 27.5 AU g^{-1} after 12 day fermentation.

MAJOR PIGMENT OF *MONASCUS*

Monascus sp produces six main polyketide pigments **(Fig. 2)** apart from complex pigment bound with protein, amino acids and peptides. The *Monascus* grown culture substrates appear red in colour due to the pigment like, rubropunctamine (Fowell *et al.* 1956) and monascorubramine (Hiroi *et al.* 1975). Red pigments are formed by the chemical modification of orange pigments, rubropunctatin (Haws *et al.* 1959) and monascorubrine. The yellow pigments are monascin and ankaflavin (Juzlova P. 1996b). The content of *Monascus* pigment varies according to culture condition, pH, humidity, oxygen supply and nutrients (Eisenbrand 2006).

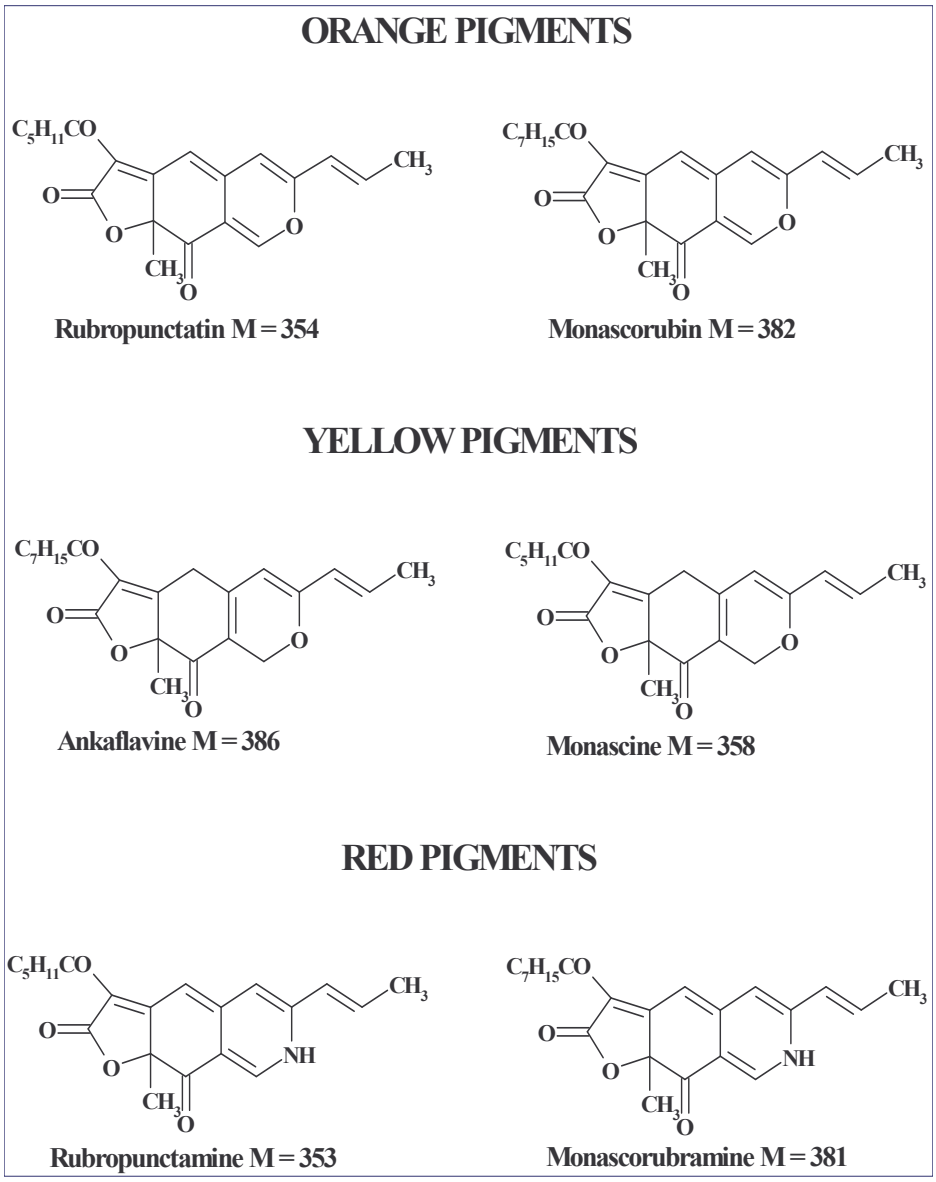


Fig 2. Major pigments produced by *Monascus purpureus*

BIOSYNTHESIS OF RED PIGMENT

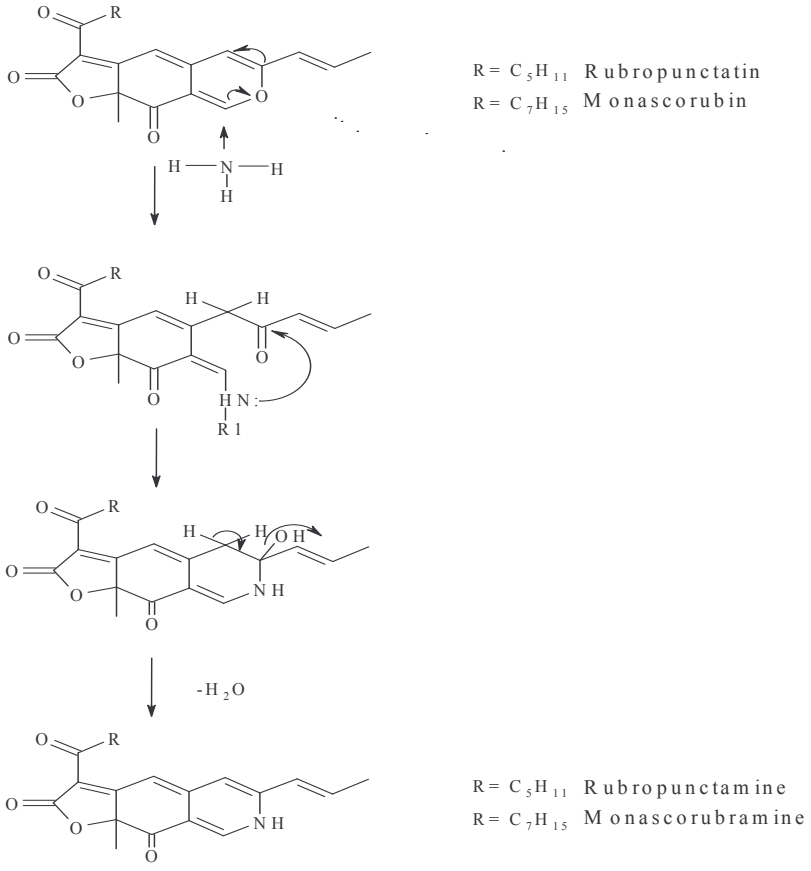


Fig 3. Formation of red pigments by Schiff's base reaction (Juzlova *et al.,* 1996b)

CITRININ OF *M. PURPUREUS*

Monascidin-A produced by *Monascus* sp was identified by Chinese scientists in their papers as a component useful for preservation of food. Later it was called as citrinin (Blanc *et al.* 1995; Hajjaj *et al.* 1999), a nephro and hepato toxin. This discovery led to screening *Monascus* sp available in public collections for identifying toxinogenic strains and species.

Biosynthesis of polyketides occurs by the following reactions. (a) Condensation of 1 mol of acetate with 5 mol of malonate to form a hexaketide chromophore by the polyketide synthase. (b) Binding of a medium-chain fatty acid such as octanoic acid, produced by the fatty acid biosynthetic pathway to the chromophore structure by a

transesterification reaction to generate the orange pigment monascorubrin or rubropunctatin. (c) Reduction of the orange pigment to give rise to the yellow pigment ankaflavin from monascorubrin. (d) Amination of orange pigments with NH_3 units to form red pigments, monascorubramine and rubropunctamine (Kurono *et al.* 1963).

All these pigments remain essentially intracellular because of their high hydrophobicity. The hydrophobic (lipophilic) pigments can react with amine groups (**Fig 3**) contained in culture media components such as proteins, amino acids, and amino sugars leading to the formation of water-soluble pigments (Wong and Koehler 1983; Lin and Demain 1994; Hajjaj *et al.* 1997). They are eventually excreted in the medium after reacting with an NH_2 unit of amino acids (Wong and Koehler 1983; Hajjaj *et al.* 1997). For this reason, glutamate has been the most useful amino acid, since it can serve both as a carbon and as a nitrogen source (Lin and Demain 1995; Pastrana *et al.* 1995).

Several modifications of the culture conditions are possible in order to increase the pigment production and reduce citrinin. Adding fatty acids to the medium was effective in favouring the synthesis of pigment, but citrinin production remained unchanged (Hajjaj *et al.* 2000b). The modification of culture conditions by replacing glutamic acid with other amino acids or culturing in a liquid medium containing glucose and histidine inhibited citrinin production (Hajjaj *et al.* 2000a & b). When the pathway of histidine assimilation was studied, it was found that its catabolism resulted in equimolar concentration of hydrogen peroxide and the peroxide destroyed citrinin due to the activities by peroxidases (Hajjaj *et al.* 2000b).

ENZYMES OF *MONASCUS PURPUREUS*

POLYKETIDE SYNTHASE

Over the past several years it has become clear that polyketides are assembled in a variety of mechanistically complex ways (Hoopwood 1997). Polyketides synthases (PKSs) are structurally and functionally related to the fatty acid synthases (FASs), both catalyzing sequential decarboxylation and condensations between ACP-linked acyl

thioesters. Unlike most FASs, PKSs can omit some or all of the reduction reactions (B-keto reduction, dehydration and enoyl reduction) that takes place after each condensation reaction, there by yielding ketone, alcohol or alkene instead of methylene as functional groups at specific positions along the chain (Hendrickson *et al.* 1999). There are three types of PKSs known (a) modular type I PKSs, (b) iterative type I PKSs and (c) type II PKSs. All described fungal PKSs belong to the iterative type I PKS group. These enzymes contain single copies of several functional domains, a feature that distinguishes this class of PKSs from the modular type I PKS class (Hutchinson *et al.* 2000). The functional domains typically found in the iterative type I PKSs are those for ketosynthase (KS), acyl transferase (AT), ketoreductase (KR), dehydratase (DH), enoyl reductase (ER), methyltransferase (MT), thioesterase (TE), and acyl carrier protein (ACP). Depending on the structural complexity of the polyketides produced, fungal PKSs have been divided into three groups: (i) single-aromatic-ring PKSs, (ii) multi-aromatic-ring PKSs, and (iii) reduced-complex- type PKSs (Hutchinson 1999; Hutchinson *et al.* 2000). Single-aromatic-ring PKSs are the smallest and contain approximately 1,800 amino acid residues. The characteristic positions of the KS and AT domains in multi-aromatic-ring PKSs are shifted closer to the C terminus than in the other two groups. The reduced-complex-type PKSs are the largest, with each protein containing 2,500 amino acid residues. The polyketides produced by this last PKS group contain more reduced and complex chemical structures than those produced by enzymes in the other two PKS classes (Shimizu *et al.* 2005).

AMYLASES

Microorganisms are the major source of amylases used in cosmetics, textiles, soap, and pharmaceuticals. The important application of starch hydrolyzing enzymes (amylases) in the industry is for the production of sugar syrup and alcohol.

Amylases are classified as α-1-4-glucanase and α-1-6-glucanase according to the specific glucosidic bond it cleaves. Endo glucanases act on interior bonds of starch while, exo glucanases cleave the bonds successively from non-reducing ends of starch. Activities of amylases result in smaller molecules called dextrins, disaccharides and

monosaccharides. The number of amylases produced by the microorganisms in culture are many and have been described by Ravi-Kumar and Umesh-Kumar (2006).

PROTEASES

The acid proteases catalyse the hydrolysis of peptide bonds at acidic pH to yield peptides and amino acid. They occur in organisms and are important to many diverse biological functions. Acid protease reported from *M. kaoliang* has a molecular mass of 34 kDa and was stable over the pH range of 3 to 6 (Tsai *et al.* 1978). Recently an extra cellular protease produced by *M. purpureus* growing a in medium made of shrimp and crab shell was reported. The optimal pH, temperature and its stability studies revealed that the protease was stable between pH 5–9 at 40^{o}C, (Liang *et al.* 2006). Carboxypeptidases (an exopeptidase) have also been characterized from *M. purpureus*. It releases free amino acids from the carboxyl termini of peptides or proteins. It was considered to serve as a key enzyme for the production of flavorful amino acids. Carboxypeptidase produced by *M. purpureus* is a heterodimer with a molecular mass of 132 kDa. The two subunits of 64- kDa and 67- kDa have also been characterized. It is an acidic glycoprotein with an isoelectric point at 3.67 and 17.0% carbohydrate content. The optimum pH and temperature for its activity were 4.0 and 40^{o}C. The enzyme was stable for 1 h between pH 2.0 and 8.0 at 37^{o}C (Liu *et al.* 2004).

STATINS AND THEIR APPLICATIONS

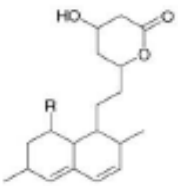
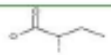
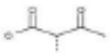
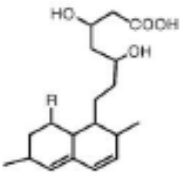
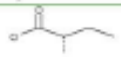
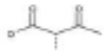
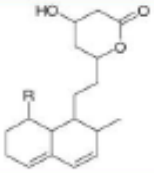
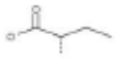
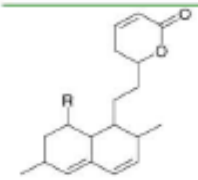
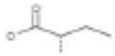
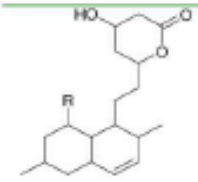
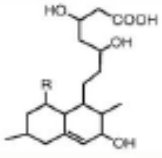
In the early 1970s, Endo (1980) isolated the first statin, mevastatin (formerly called compactin or ML-236B) from *Penicillium citrinum* as a potent inhibitor of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase (Endo 1985a). The enzyme became important since it controlled cholesterol synthetic pathway (Endo *et al.* 1986). By the end of the 1970s the same group demonstrated that mevastatin was highly effective in lowering serum total and low-density lipoprotein (LDL) cholesterol in both experimental animals and patients with primary hypercholesterolemia. The discovery of mevastatin paved the way for the worldwide development of its analogues (statins) and since then statins like lovastatin, simvastatin, pravastatin, fluvastatin and atorvastatin have been approved for human use in many countries (Endo 2004).

Red rice has been used in Chinese cuisine and medicine for centuries as health tonic since it improved blood circulation. The recent research revealed that the red rice forms contain naturally occurring inhibitors of hydroxymethylglutaryl-CoA (HMG-CoA) reductase, and the medicinal properties of fermented red rice have a favorable effect on lipid profiles of hypercholesterolemic patients, by decreasing low-density lipoprotein cholesterol (LDL) and elevating high-density lipoprotein cholesterol (HDL) (Wang *et al.* 1997; Li *et al.* 1998). Clinical trials using red rice on hyperlipidemic elderly patients (Heber *et al.* 1999) as well as HIV-related dyslipidemic patients (Keithley *et al.* 2002) have also demonstrated an improvement in lipid profiles. Recent reports showed that statins effectively functioned as anti-inflammatory compounds with lipid-lowering effects (Rosenson 2004).

Red rice possesses promising activity as a new hypolipidemic drug launched worldwide (Journoud and Jones 2004). The HMG-CoA reductase inhibiting activity of red rice comes from a family of naturally occurring substance named monacolins (Endo 1980; Juzlova P. 1996a & b). Monacolin-K, was characterized as mevinolin or lovastatin and is the major ingredient. The isolation and evaluation of seven monacolins from fermented red rice were described by Ma *et al.* (2000).

Till date, 16 monacolin metabolites have been identified from *Monascus* sp. Fourteen monacolins including monacolin K (mevinolin), J, L, M, X and their corresponding hydroxyl acid forms, as well as dehydromonacolin K, dihydromonacolin L, compactin, 3-hydroxy-3, 5-dihydromonacolin L were identified in red rice, using high-performance liquid chromatography with photodiode array detector (PDA) and tandem mass spectrometry (**Table 3**) (Ma *et al.* 2000; Li *et al.* 2004). Dihydromonacolin-MV and dehydromonacolin-MV2 were isolated from *M. purpureus* and its mutant from this laboratory. They were characterized for antioxidant and antibacterial properties (Dhale *et al.* 2007a & b).

Table 3. Monacolins identified from *Monascus* sp (Li *et al.* 2004)

Structure	Name	R	MW	UV (λ_{max})
	1. Monacolin K (MK)		404	230, 237, 246
	2. Monacolin J (MJ)	OH	320	230, 237, 247
	3. Monacolin L (ML)	H	304	230, 237, 247
	4. Monacolin X (MX)		418	230, 237, 247
	5. Monacolin M (MM)		406	
	1a. MK acid form (MKA)		422	
	2a. MJ acid form (MJA)	OH	338	
	3a. ML acid form (MLA)	H	322	
	4a. MX acid form (MXA)		436	
	5a. MM acid form (MMA)		424	
	6. Compactin (P1)		390	230, 237, 247
	7. Dehydromonacolin K (DMK)		386	
	8. Dihydromonacolin L (DML)	H	306	
	9. 3α-hydroxy-3,5-dihydromonacolin L (HDML)	H	340	

Beneficial effects of statins in preventing cardiovascular disease (Raza *et al.* 2004)

Lipid effects	**Vascular effects**	**Antithrombotic effects**	**Other effects**
Lowers TGL levels	Activates endothelial NO synthesis	Reduces platelet aggregation	Greater reduction in mean blood pressure when used with ACEI
Increases HDL-C and apolipoprotein A-I (apoA-I)	Promotes plaque stabilization	Reduces tissue factor /extrinsic pathway inhibitor (EPI) production	Decreases proteniuria
Decreases oxidation of LDL-C	Blocks the accumulation of cholesterol in macrophages	Decreases plasma fibrinogen concentrations	Anti-oxidant effect
Decreases LDL-C level	Reduces monocyte adhesion to endothelial cells	Improves whole blood and plasma viscosity	Reduces C-reactive protein levels
-	Antiproliferative effects on smooth muscle cells	Reduces plasminogen activator inhibitor-1 (PAI-1) activity	-
-	Suppresses neointimal thickening	Reduces platelet-associated ox-LDL (Pox-LDL) activity	-
-	Increases endothelium-dependent vasodilator response	Decreases platelet-dependent thrombin generation (PDTG)	-
-	-	Reduces thromboxane synthesis	-

LDL-C: low density lipoprotein cholesterol; HDL-C: high-density lipoprotein cholesterol; TGL: triglycerides; NO: nitric oxide; ACEI: angiotensin-converting enzyme inhibitor.

Lovastatin production by *Monascus* sp is dependent on the substrate provided. The carbon and nitrogen sources in the medium affect lovastatin production by *M. pilosus. M. pilosus* require a suitable concentration of organic nitrogen, peptone for high lovastatin production. Although glucose strongly repressed lovastatin production, maltose

increased its productivity. Interestingly, glycerol combined with maltose enhanced lovastatin production, up to 444 mg l^{-1}. Moreover mutant, in which glucose repression was relieved, produced the highest level of lovastatin (725 mg l^{-1}) when grown on glucose-glycerol-peptone medium (Miyake *et al.* 2006b). These observations indicate that lovastatin production by *M. pilosus* is regulated by glucose repression and that an appropriate release from this repression is possible by optimizing medium composition and/or by mutation (Miyake *et al.* 2006a & b).

(Suh and Shin 2000) reported that the novel coculture of *Monascus* J101 with *Saccharomyces cerevisiae* or its culture filtrate significantly enhanced *Monascus* pigment production and cell growth with concomitant morphological changes. A chitinase produced by *S. cerevisiae* was identified as the effective compound causing these changes. Low level hydrolysis of *Monascus* cell wall by the chitinase occurred as an early event in the cocultured *Monascus* cells. Changes in life cycle and intracellular structure of *Monascus* sp. J101 by coculture with *S. cerevisiae* were investigated. Cocultured *Monascus* cells showed accelerated growth and reproduction. Production of asexual and sexual spores was used as an efficient method of cell proliferation. Formation of meiotic (sexual) spores was more frequently observed in the cocultured *Monascus* cells. The interior structure of a cocultured cell was characterized by increased numbers and sizes of vacuoles. The vacuoles probably served as repositories for pigments. Pigments produced by the cocultured *Monascus* cells were more hydrophobic than pigments produced by control cells with no coculture.

MONASCUS PURPUREUS MUTANTS

Characterization of a non-pigment producing mutant of *M. purpureus* was compared with its parental strain *M. purpureus* Went CBS 109.07. The albino mutant exhibited an anamorph life cycle, high conidia forming capability, slower radial growth rate and temperature sensitivity. The assimilation capacity of both strains for mono and disaccharides and some alcohols was in the same range (Y 0.2 – 0.35). Hydrolytic activity towards natural substrates expressed through glucoamylase and protease was approximately 10 fold lower in the non pigment producing strain (0.05 – 0.08 U/mg

protein and 0.01 – 0.07 U/mg protein respectively) compared with the red one. The important qualitative differences between both strains were found in fatty acid composition and in the production of citrinin and monacolin. The mutant strain possessed C, C and C 17, 20, 22 fatty acids and did not produce citrinin (Rasheva *et al.* 2003).

APPLICATIONS OF *MONASCUS* PIGMENTS

Antibacterial properties of *Monascus* were first mentioned by Wong and Bau (1977). Monascidin-A was effective against *Bacillus, Streptococcus* and *Pseudomonas*. It was shown that this molecule was citrinin and its production by various *Monascus* species was studied using different culture media and conditions (Blanc *et al.* 1995).

Recently it has been documented that amino acid derivatives of *Monascus* pigments produced by fermentation showed antimicrobial activities. Thirty-nine L- and D-forms of amino acids were added as a precursor to the fermentation medium for derivation of pigments. Derivatives with L-Phe, D-Phe, L-Tyr, and D-Tyr exhibited high activities against Gram +ve and Gram -ve bacteria (MIC 4-8 μg mL^{-1}). The control red pigment exhibited higher MIC values (32 μg mL^{-1}). Derivatives with L-Asp, D-Asp, L-Tyr, and D-Tyr were effective against the filamentous fungi *Aspergillus niger, Penicillium citrinum, and Candida albicans*. *Monascus* derivatives of amino acids having a phenyl ring like Phe and Tyr derivatives showed high antimicrobial activities. Incubation of the l-Phe derivative with *Bacillus subtilis* caused cells to aggregate as pellets. Easy adsorption of the L-Phe pigment derivative to the surface of *Escherichia coli* cells was observed by SEM and TEM. Addition of *Monascus* pigment derivatives decreased oxygen uptake rate of *E. coli* in culture (Kim *et al.* 2006b).

The proliferation of Caco-2 cells was inhibited by Monacolin K in a dose-dependent manner. Proteomics analysis by two-dimensional gel electrophoresis, matrix-assisted laser desorption ionization time-of-flight/time-of-flight mass spectrometry (MALDI-TOF/TOF MS), tandem mass spectrometry (MS/MS), and database interrogation to separate and identify the proteins of Caco-2 cells treated with monacolin K identified characterization of anti-oxidation enzymes, cytoskeleton proteins, glycolytic

enzymes, and enzymes involved in mediating protein interactions. Furthermore, glutathione S-transferase P 1 and cytoskeleton-8-18, and -19 revealed a down-regulation in a dose-dependent manner upon exposure of Caco-2 cells to monacolin K (Lin *et al.* 2006).

Arbor Acres broiler chicken fed with *Monascus* showed lowered cholesterol content and their meat products contained higher level of unsaturated fatty acids. Triglyceride and cholesterol concentration in serum was also found to be considerably lower in *Monascus* fed groups compared to control group. Ratio of HDL-C/LDL-C and HDL-C/cholesterol were all higher in control group compared to *Monascus* fed group. Thus cholesterol levels could be lowered by adding rice cultured *Monascus* to the diet of chicken (Wang *et al.* 2006).

Ankaflavin was found to be toxic to human cancer cell lines Hep G2 and A549 with a IC_{50} value 15 μg ml^{-1}. Significant toxicity to normal MRC-5 and WI-38 cells at same concentration was not observed. Possible mode of cell death of Hep G2 cells were elucidated by treating cells with ankaflavin for 48 h to examine the morphological change of the chromatin. Chromosomal condensation and fragmentation were found, and a significant sub-G1 peak was found by flow cytometry. Apoptosis was therefore suggested as the possible mechanism. Monascin, an analogue of ankaflavin showed no cytotoxicity and did not induce death of Hep G2 cells (Su *et al.* 2005).

Azaphilone pigments, monascusones A and B, were isolated from chlorofom extract of a yellow mutant of the fungus *M. kaoliang* grown on rice. Monascusone A, was a major metabolite of *M. kaoliang* showed antitubercular activity against *Mycobacterium tuberculosis* H37Ra, and antifungal activity towards *Candida albicans*. Monascusones A exhibited no cytotoxicity against breast cancer and human epidermoid carcinoma cell lines (Jongrungruangchok *et al.* 2004).

Monascin pigment isolated from the extracts of *M. pilosus* fermented rice showed anti-tumor-initiating activity via oral administration on the two-stage carcinogenesis of

mouse skin tumor induced by peroxynitrite ($ONOO^-$) or by ultraviolet light B (UVB). Monascin exhibited marked inhibitory activity on both peroxynitrite and UVB-induced mouse skin carcinogenesis tests. Hence, use of monascin as potential cancer chemopreventive agent in chemical and environmental carcinogenesis (Akihisa *et al.* 2005b) was predicted.

THE PROBLEM

Even though *M. purpureus* has been used in Chinese folk medicine for long, only recently, the pharmaceutical applications of its metabolites were described. While polyketides demonstrated antioxidant, antihypertensive and hypocholesterolemic properties, monacolins were found to compete for the 3-hydroxy-3-methylglutaryl coenzyme A (HMG Co-A) reductase active site and inhibit cholesterol biosynthesis. In spite of these reports, the literature on the basic physiology of the fungus determining metabolite production is still scarce.

Methanol extracts of *M. purpureus* fermented red rice when assayed for antioxidant properties showed the presence of an active metabolite. Purification and characterization of the metabolite identified the production of a bioactive dihydromonacolin-MV by the fungus. The details of the study are described in the **First Chapter**

Induced mutation to obtain strains for dihydromonacolin-MV over production resulted in the isolation of a hyper pigmentation mutant. Characterization of the metabolites produced by it revealed the synthesis of an antibacterial monacolin called dehydromonacolin-MV2. This compound appeared to arise due to aberration in the monacolin biosynthetic pathway. The **Second Chapter** describes the results of these experiments.

Since the above results suggested a relation between polyketide production and monacolin biosynthesis, the mutants were further analyzed in order to characterize their physiology with reference to metabolite production. It appeared that amylases, acid protease and amidases played a significant role in the type of polyketide produced by *M. purpureus*. The details are described in the **Third Chapter** of the thesis.

MATERIALS AND METHODS

THE ORGANISM

General methods used for studies involving microorganisms were routinely followed.

Monascus purpureus (MTCC-410) obtained from Institute of Microbial Technology (IMTECH) Chandhigarh, India and its mutants were used for the study. They were maintained on potato dextrose agar slants at 4℃ by periodical subculturing.

CHEMICALS

The culture medium for cultivation such as, potato dextrose agar (PDA) was purchased from Hi-Media Laboratories Mumbai, India. Glass distilled water was used for the preparation of culture media. Enzyme assays were carried out using double distilled glass water. Solvents for extraction and HPLC grade chemicals were purchased from Ranbaxy Fine Chemicals Limited, New-Delhi, India.

Enzyme substrate like Lintner's soluble starch, casein (Hammerstein grade) and 4-chloro-1-napthol were procured from BDH Laboratories, Poole, England and Sigma Chemicals, St, Louis, USA. L-glutamine and L-asparagine were purchased from Hi-Media Laboratories Mumbai, India. Detection reagents like 3, 5-Dinitrosalicilicacid, Folin-Ciocalteu phenol reagent, silver nitrate, Coomassie Blue G 250, R 250 and Mercuric iodide were obtained from Loba-Chemie, Mumbai, India. Phenol red, Tris-buffer and all other reagents used were of analytical grade, purchased from Qualigens Fine Chemicals, Mumbai, India, and Ranbaxy Chemical, New Delhi, India.

For antioxidant activity, 2, 2, -diphenyl-1-pycrylhydrazyl (DPPH) and BHA were obtained from Sigma Chemicals, USA. L-ascorbic acid, nitroblue tetrazolium (NBT), nicotinamide adenine dinucleotide (reduced form, NADH), phenazin methosluphate (PMS) were obtained from Sisco Research Laboratories, India.

Reagents for electrophoresis, acrylamide, N, N'-methylene-bis-acrylamide, ammonium per sulphate, N, N, N', N'-tetramethylethylenediamine (TEMED) sodium dodecyl sulphate, dithiothreitol and mercaptoethanol were from BDH Laboratories and Sigma. Glucose oxidase peroxidase and glucosidase inhibitor acarbose tablets were from Span diagnostic limited, Surat and Bayer pharmaceuticals, Mumbai, India.

Serogical reagents like goat anti-rabbit IgG tagged biotin and avidin peroxidase were also from Sigma Chemicals. Nitrocellulose membranes were procured from Amersham Pharmacia Biotech, UK.

PREPARATION OF RED RICE

Culture medium was prepared with 10 g rice taken in 500 ml conical flasks. Distilled water (20 ml) was added to the flasks and sterilized for 20 min at 115℃. The flasks were inoculated with 1.0 ml *M. purpureus* spore suspension prepared from slants using 0.85% NaCl. Inoculated flasks were incubated at 30℃ (Adolf Khuner Therm-Lab Switzerland) for 11 days. The flasks were shaken periodically to ensure uniform mixing.

MUTANT SELECTION

Spore suspension of *M. purpureus* (MTCC-410) prepared from one week old slants was spread on plates containing PDA medium and exposed to germicidal UV (Philips 30W 200V) to a time period that resulted in 30-40% kill. After incubating the plates at 4°C in the dark for 16 h to prevent light induced DNA repair, temperature selection was carried out by keeping the plates at 42°C for 24 h. The plates were transferred to 30°C incubator and colonies emerging after 5 day incubation were screened for phenotypic changes by visual observation. The albino and hyper pigment mutants so isolated were used for the study.

ANALYSIS OF CITRININ

After sonicating the *M. purpureus* fermented rice suspended in chloroform: methanol 1:1 (v/v) for 10 min ($Gmb_{H,}$ UP 50H Ultraschallprozessor sonicator), the mixture was kept on rotary shaker at 120 rpm for 60 min for extraction of citrinin. The

presence of citrinin was analyzed in concentrated extract by TLC (alumina silica gel 60 F_{254} plates 20X20 cm Merck) in unsaturated TLC chamber containing chloroform: ethyl acetate: formic acid (4.5:5.5:1 v/v) as a mobile phase. After development, the solvent was air-dried and dipped in 1% $AlCl_3$ for 3 seconds. The $AlCl_3$ untreated plates served as control (Rasheva *et al.* 2003).

EXTRACTION AND QUANTIFICATION OF PIGMENTS

Pigments from rice fermented with *M. purpureus* were extracted using polar and non-polar solvents. The extraction was carried out at 30°C by keeping the flasks for 60 min on rotary shaker (110 rpm). The insoluble debris was removed by filtration and the absorbance of the supernatant was determined spectrophotometrically for quantifying the pigments. Optical density was determined at 375, 475, and 500 nm for yellow, orange and red pigments respectively. Pigment yield was calculated as OD Units using formula.

$$\text{OD Units} = \frac{\text{OD X Total vol of Solvent X Dilution}}{\text{Red Rice (g)}}$$

PHASE CONTRAST AND SCANNING ELECTRON MICROSCOPY

Morphology of *M. purpureus* and its mutants was studied using phase contrast microscope. One week old mycelia cultivated on PDA were mounted in glycerol after staining with cotton blue. The mycelial and spores were observed under Phase Contrast Microscope (Olympus BX40, Japan)

For conventional SEM, the culture grown on PDA slants was gently scarped and fixed in 0.05 M potassium phosphate buffer (pH 7.3) containing 4% glutaraldehyde. The fixed material was rinsed three times with 0.05 M potassium phosphate buffer and distilled water. After dehydration using ethanol (Asensio *et al.* 2005) they were mounted on stub coated with gold and observed under a LEO 435 VP Scanning Electron Microscope.

ISOLATION OF BIOACTIVE COMPOUND

Dried (at 48-50°C) fermented red rice was powdered to 60-80 mesh for extraction with a series of solvents of increasing polarity. After filtration, the pigment containing solvent was flash evaporated and moisture was removed by lyophilization. The extracts obtained were used for preliminary screening of radical scavenging activity.

FRACTIONATION AND PURIFICATION

About 10 g methanol extract was passed through a column prepared using silica gel (60-120 mesh). Stepwise elution was performed with a linear gradient of hexane, chloroform and ethyl acetate (100:0; 100; 25:75 v/v). About 20 fractions, each measuring about 100-150 ml were collected depending upon colour intensity of the eluting fraction. The fractions were concentrated by flash evaporation and pooled together after TLC analysis for purity (silica gel 60 F_{254} plates 20X20 cm Merck, Germany). DPPH radical scavenging activity was assayed and active fractions were pooled, concentrated and again subjected to column chromatography as above. The sample was eluted with linear gradient of chloroform and ethyl acetate (100:0 to 0:100 v/v) and ethyl acetate: methanol (100:0 to 85:15 v/v). About 12 fractions were collected, concentrated by flash evaporation and assayed for DPPH radical scavenging activity. The chloroform: ethyl acetate fractions (75:25) showed maximum activity.

For purification of molecule from chloroform extract, 1 g crude extract was subjected to column chromatography in silica gel (60-120 mesh). Stepwise elution was performed with a linear gradient of hexane, chloroform and ethyl acetate. About 49 fractions measuring approximately 50-150 ml were collected based on colour intensity. The fractions were concentrated by flash evaporation and pooled together after analysis by TLC (silica gel 60 F_{254} plates 20X20 cm Merck, Germany). The fraction showing antibacterial activity was purified further by rechromatography on silica gel columns.

About 6 fractions measuring approximately 30 to 50 ml were collected following elution with linear gradient of hexane, chloroform (100:0 to 0:100 v/v), chloroform: ethyl

acetate (100:0 to 0:100 v/v) and ethyl acetate: methanol (100:0 to 85:15 v/v). The purity of the active fraction was established by thin layer chromatography.

THIN LAYER CHROMATOGRAPHY

The fractions collected were spotted on silica gel TLC plates (alumina silica gel 60 F_{254} plates 20X20 cm Merck, Germany). The plates were developed in ascending direction of 12 to 15 cm height with mobile phase made of dichloromethane and ethyl acetate (7:3, 8:2 and 6:4 v/v) and dichloromethane, ethyl acetate and methanol solvent (6:4:0.5, 9:0.5:0.5 and 8:1:1 v/v). Combination of 8:1:1 v/v of dichloromethane, ethyl acetate and methanol mobile phase showed best separation. The plates were air-dried and exposed to iodine to locate the spots.

HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC)

Purified bioactive compounds were dissolved in acetonitrile or methanol (HPLC grade) to analyze purity by subjecting it to HPLC, using Shimadzu Liquid Chromatograph LC-10A (Shimadzu, Japan) fitted with Waters or Supelco C_{18}, 5 µm, 4.6 X 250 mm analytical column (Waters Corporation, 34 Maples Street, Milford, MA 01757-3696 USA or Supelco Park, 595, North Harrison Road PA. 16823-0048. Ten micro liter compound was injected using 20 µl injection system sample loop.

CHARACTERIZATION OF BIOACTIVE COMPOUND

UV-VISIBLE SPECTROSCOPY

UV-Visible spectrum of the isolated bioactive compound was recorded on a Shimadzu UV-Visible 160A instrument at room temperature. The bioactive compound, dissolved in HPLC grade methanol or acetonitrile was scanned for the absorbance between 200-700 nm.

FOURIER TRANSFORMER INFRA RED SPECTROSCOPY

FTIR spectrum was recorded on a Nicolet 5700 (Thermo Electron Corporation, Madison, WI, US.) spectrometer at room temperature. The bioactive compound was dissolved in DMSO or mixed with KBr pellet and scanned in the range of 4000-400 cm^{-1}.

MASS SPECTROSCOPY

Mass spectrum of the bioactive compounds were obtained using a Q-TOF Waters Ultima instrument (Q-TOF GAA 082, Waters Corporation, Manchester, UK) fitted with an electron spray ionization (ESI) source. A software version 4.0 was used for the data acquisition. The positive ion mode using a spray voltage at 3.5 kV at a source temperature of 80°C was employed for recording the spectra. Mass spectra were recorded under electron impact ionization at 70 eV energy. Sample was prepared in the concentration range of 0.2-0.5 mg ml^{-1} and injected at a flow rate of 10 µl min^{-1}. The recorded mass of samples was in the range of 100-1000.

TWO-DIMENSIONAL HETERONUCLEAR SINGLE QUANTUM COHERENCE TRANSFER SPECTROSCOPY (2D HSQCT) NMR)

The ^{1}H and ^{13}C NMR spectra were recorded on Bruker DRX500 NMR instrument operating at 500 MHz for ^{1}H at room temperature. The region from 0 to 12 parts per million for ^{1}H and 0-200 parts per million for carbon was employed for scanning. Signals were referred to tetramethylsilane within ±0.01 ppm. About 10-20 mg of compound dissolved in 0.5 ml $CDCl_3$ was used for recording the spectra.

DETERMINATION OF ANTIOXIDANT ACTIVITY

Antioxidant activity was determined by measuring DPPH radical scavenging, lipid peroxidation inhibition and scavenging of superoxide radicals. Purified bioactive compounds dissolved in ethanol were used to obtain EC_{50} (IC_{50}) values. All the experiments were carried out in triplicates by maintaining appropriate blanks and controls.

DETERMINATION OF TOTAL PHENOLICS

Concentration of phenols was determined using the method described by Singh *et al.* (2002). The values were expressed as ellagic acid equivalents. Dried *M. purpureus* extracts prepared from fermented rice (1 mg) was dissolved in one ml acetone and water (6:4 v/v) mixture and 0.2 ml sample was used. They were mixed with 1 ml Folin-Ciocalteu reagent (10 fold dilutions) and 0.8 ml sodium carbonate (7.5%) solution. The reaction mixture was incubated for 30 min at room temperature and absorbance was measured at 765 nm with Shimadzu UV-Visible spectrophotometer.

TOTAL ANTIOXIDANT CAPACITY

Total antioxidant capacity and reducing power of *M. purpureus* polyketide pigment extracts was evaluated by the method of Prieto *et al.* (1999)**.** The reaction mixture containing 0.2 ml extract was mixed with 1 ml reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). Following incubation at 95°C for 90 min, absorbance was measured at 695 nm against the reagent blank. The antioxidant capacity was expressed as equivalents of ascorbic acid and α-tocopherol.

DPPH RADICAL SCAVENGING ACTIVITY

The DPPH radical scavenging activity (Blois 1958) was measured according to the method of Moon and Terao (1998). The purified compound dissolved in ethanol (2 ml) was mixed with Tris HCl buffer (100 mM, pH 7.4) and 1 ml DPPH (500 μM in ethanol). The mixture was shaken vigorously and incubated at room temperature for 30 min. The absorbance was measured at 517 nm using spectrophotometer. Blanks and controls contained no DPPH and compound respectively. Antioxidant activity was calculated using the formula.

$$\text{Antioxidant activity}(\%) = \left(1 - \frac{A_{\text{sample}\,(517nm)}}{A_{\text{control}\ (517nm)}}\right) x100$$

LIPID PEROXIDATION INHIBITION ASSAY

Egg lecithin was prepared using egg yolk homogenized in cold acetone and kept in cold for 10 min. Acetone was decanted and the yolk was redissolved in cold acetone till yellow colour disappeared. Acetone was removed by air drying and stored in vacuum sealed pack for future use.

Lipid peroxidation inhibitory activity was measured according to the method of Kulkarni *et al.* (2004). Egg lecithin (3 mg ml^{-1} in phosphate buffer, pH 7.4) was sonicated for 30 min to obtain small membrane liposome vesicles ($Gmb_{H,}$ UP 50H ultraschallprozessor sonicator). Different concentrations of purified compound dissolved in ethanol was added to 0.5 ml liposome mixture. Lipid peroxidation was induced by adding 10 µl 400 mM $FeCl_3$ and 10 µl 200 mM L-ascorbic acid and incubated at 37°C (Buchi Heating-bath B-490, Switzerland) for 60 min. The reaction was inhibited by adding 1 ml 0.25 N HCl containing 15% TCA and 0.375% TBA and the reaction mixture was kept in a boiling water bath for 15 min, cooled and centrifuged at 10,000 rpm for 5 min. The absorbance of the supernatant was measured at 532 nm. Blank and control were maintained without liposome and compound respectively.

$$\text{Lipid peroxidation inhibitory activity (\%)} = \left(1 - \frac{A_{\text{sample}\,(532\,nm)}}{A_{\text{control}\,(532\,nm)}}\right) x100$$

SUPER OXIDE RADICAL SCAVENGING ACTIVITY

Super oxide radicals were generated in 1 ml 0.02M Tris-HCl buffer (pH 8.3) containing 0.1 mM NADH, 0.1mM NBT, 10 µM PMS and different concentrations of purified compound. The colour reaction due to super oxide radical and NBT was detected at 560 nm (Liu *et al.* 1997). The reaction kinetic was scanned for 2 min.

$$\text{Superoxide radical scavenging activity } (\%) = \left(1 - \frac{A_{\text{sample}\,(560\,nm)}}{A_{\text{control}\,(560\,nm)}}\right) x100$$

HYDROXYL RADICAL SCAVENGING ACTIVITY

The hydroxyl radical scavenging activity (Klein *et al.* 1981) was determined according to the method of Singh *et al.* (2002). Various concentrations of purified compound, dissolved in ethanol and taken in different test tubes were evaporated to dryness. To this, 250 µl iron-EDTA solution (0.13% ferrous ammonium sulfate and 0.26% of EDTA), 125 µl EDTA (0.018%) and 250 µl DMSO (0.85% v/v in 0.1M phosphate buffer, pH 7.4) were added. The reaction was initiated by the addition of 125 µl 0.22% of ascorbic acid. After 15 min incubation at 85°C, the reaction was inhibited by adding 250 µl ice cold TCA (17.5%) and 750 µl Nash reagent (75.0 g ammonium acetate, 3 ml glacial acetic acid and 2 ml acetyl acetone and the final volume made to 1 L with distilled water). The absorbance was measured at 412 nm after 30 min incubation at room temperature.

ANTIBACTERIAL ACTIVITY

The antibacterial activity was tested against *Bacillus subtilus, B. cereus Enterobacter aerogenes, Escherichia coli, Klebsiella pneumoniae, Listeria monocytogenes, Micrococcus luteus, Proteus mirabilis, Pseudomonas aeruginosa, Salmonella typhi, Staphylococcus aureus, Streptococcus fecalis* and *Yersinia enterocolitica.* The above bacterial strains were obtained from the stock cultures of Microbiology laboratory, Department of Microbiology, Mysore Medical College, Mysore, India. The test organisms were maintained on nutrient agar slants.

ASSAY

In vitro antibacterial activity of crude extracts and purified compound was determined by disc diffusion or agar well method Vardar-Unlu *et al.* (2003). Solvent extracts and purified compound dissolved in ethanol were used for assay. About 75 µl compound placed on Whatman filter paper disc was allowed to diffuse into agar gels containing the growing colonies of bacteria. The antibacterial activity was measured as inhibition zones. Discs containing ethanol were used as control. Discs made with penicillin constituted positive control.

α-AMYLASE ACTIVITY

Amylase activity was assayed using starch as substrate by determining reducing sugars formed with dinitrosalicylic acid (Miller 1959).

Dinitrosalicylic acid reagent contained the following constituents (Bernfeld 1955)

Constituents	**g L^{-1}**
3, 5- dinitrosalicylic acid	10.0
Sodium hydroxide (anhydrous)	16.0
Sodium-potassium tartarate	300.0

Dinitrosalicylic acid and Sodium hydroxide (anhydrous) were dissolved in 400 to 500 ml of water. To this, 300 g sodium-potassium tartarate was added slowly and the volume was made to 1 L with water. Reducing sugars were estimated by adding 1 ml appropriately diluted sample to 1 ml dinitrosalicylic acid reagent taken in test tubes. The mixture was boiled for 5 min, cooled and the volume was made to 12 ml with water. The colour intensity was measured at 540 nm. The glucose equivalents formed was determined using a standard graph prepared with glucose standard.

For determining enzyme activity, 2% soluble starch prepared in 0.1 M acetate buffer (pH 4.3) was used. Three ml substrate solution was treated with 1 ml appropriately diluted enzyme in buffer (0.1 M acetate buffer, pH 4.3), incubated at 40°C for 30 min. The reaction was stopped by adding 1 ml NaOH (4M) and the reducing sugars in the reaction mixture were determined using 3, 5-dinitrosalicylic acid (Kavitha 2000a). One unit enzyme activity corresponded to 1 μM of glucose released per min.

STABILITY OF ENZYME

pH:

The enzyme extracted using 0.1 M sodium acetate and sodium phosphate buffer of different pH was used to determine the activity using 2% starch prepared in corresponding buffer.

TEMPERATURE

The enzyme extracted in 0.1 M sodium acetate buffer (pH 4.5) was incubated in water bath at 30, 40, 50, 60, 70 and 80°C for 30 min. The residual enzyme activity was determined after comparing with the activity determining of the untreated control.

PRODUCT ANALYSIS

The products after enzymatic hydrolysis of starch were analyzed in Shimadzu LC 10A HPLC equipped with an RID 10A detector (Suresh *et al.* 1999). The products were separated in a Supelco LC-NH_2 coloumn (25 × 4.6 mm, 5μ) using acetonitrile and water (80:20) solvent at a flow rate of 1 ml min^{-1}.

ACID PROTEASE ACTIVITY

Protease activity was assayed by determining tyrosine equivalents due to protease action on casein. Released tyrosine equivalent was estimated using Folin-Ciocalteau Phenol reagent (Ichishima 1970). For measuring tyrosine, 2.5 ml sodium carbonate and 0.5 ml Folin-Ciocalteau Phenol reagent (1:5 diluted with water) were added to 0.5 ml test. After incubation at 30°C for 30 min, the colour intensity was measured at 660 nm against blank. Standard graph prepared with tyrosine (Sigma 0.02 mg ml^{-1}) was used to measure activity.

Casein (2%) prepared in 0.1 M sodium acetate–HCl buffer (pH 2.7) was used as substrate for protease activity. A uniform suspension of casein was made in 10 ml buffer by stirring the suspension for 15 min in a boiling water bath. After cooling to room

temperature, the volume was made to 100 ml using 0.1 M sodium acetate-HCl buffer (pH 7.2).

Acid protease activity was determined using 2% casein (Hammerstein) prepared in 0.1 M sodium acetate-HCl buffer (pH 7.2) as substrate. The reaction was carried out at 30°C for 60 min and stopped by the addition of 0.4 M trichloroacetic acid. The clear filtrate from the reaction mixture obtained after centrifugation (4000 g for 5 min) was analyzed for tyrosine released using Folin-Ciocalteau Phenol reagent. The activity corresponded to μmoles of tyrosine released min^{-1} at 30°C.

RAPID PLATE ASSAY FOR L-ASPARAGINASE AND L-GLUTAMINASE

L-asparaginase and L-glutaminase production by *M. purpureus* and its mutants were tested as described by Gulati *et al.* (1997). The spores were inoculated in the growth medium containing phenol red indicator and pink zone was measured after six to seven day of incubation. Medium contained the following constituents:

Constituents	**g L^{-1}**
Glucose	2.0
L-asparagine/L-glutamine	10.0
KH_2PO_4	1.52
KCl	0.52
$MgSO_4.7H_2O$	0.52
$CuNO_3.3H_2O$	trace
$ZnSO_4.7H_2O$	trace
$FeSO_4.7H_2O$	trace
Agar-agar	20.0

Control plates were prepared with $NaNO_3$, replacing L-asparagine and L-glutamine as nitrogen source.

ENZYME ASSAY

L-glutaminase and L-asparaginase activities were determined in the fermented rice extracts by direct nesslerization of NH_3 produced in the reactions (Imada *et al.* 1973). Extract of fermented rice was prepared by shaking in 0.1 M phosphate buffer (pH 8) on rotary shaker for 30 min at 4°C. Substrate was prepared using the same buffer. The reaction mixture contained 0.5 ml 0.04 M L-glutamine or L-asparagine, 0.5 ml phosphate buffer (0.1 M, pH 8), 0.5 ml enzyme and 0.5 ml distilled water. After incubation at 37°C for 30 min, reaction was stopped by the addition of 0.5 ml TCA (1.5 M). To 0.250 ml reaction mixture, 6.750 ml distilled water and 0.5 ml Nessler's reagent were added. The mixture kept at 18°C for 20 min was used to measure absorbance at 480 nm. The blanks contained the enzyme added after the addition of TCA.

NESSLER'S REAGENT

Nessler's reagent contained the following constituents:

Constituents	**g L^{-1}**
Mercuric iodide ($Hg I_2$)	100.0
Potassium iodide (KI)	70.0
Sodium hydroxide (NaOH)	100.0

Mercuric iodide and potassium iodide were dissolved in 400 ml distilled water. To this, sodium hydroxide (cooled) prepared in 500 ml water was added by constant stirring and the volume was made to 1 L. The supernatant was used as the reagent.

TOTAL PROTEIN DETERMINATION

Protein concentrations were routinely determined by the dye binding method using Coomassie Brilliant Blue G250 (Spector 1978). The reagent for this assay consisted of Coomassie Brilliant Blue G250 prepared in 3% perchloric acid (optical density of the prepared reagent at 465 nm was adjusted between 1.3-1.5). Protein was quantitated by adding appropriately diluted sample to 1.5 ml reagent and volume made to 3.0 ml with water. Optical density was measured at 595 nm. Bovine serum albumin was used as

standard. Specific activity of enzymes corresponded to activity estimated for one milligram protein.

ELECTROPHORESIS

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed in 10% acrylamide gels (Laemmli 1970). The protein samples for loading the gel were prepared in sample buffer containing dithiothreitol (DTT) and the proteins were visualized by silver staining (Morrissey 1981). Molecular mass of the protein was determined by running the following standards alongside the protein sample in SDS-PAGE. Molecular mass standards were obtained from Bangalore Genei, India.

Molecular mass standard	**kDa**
Phosphorylase b	97.4
Bovine Serum Albumin	66.2
Ovalbumine	42.7
Carbonic Anhydrase	30.0
Soyabean Trypsin Inhibitor	20.1
Lysozyme	14.3

Based on the migration of standards, the molecular mass of the samples was determined using Bioprofile Image Analysis System (Vilber Lourmat, France).

ENZYME ZYMOGRAMS

Amylase activity was detected as enzyme zymograms after separating the proteins by SDS-PAGE in 10% acrylamide gel containing 0.1% Lintner's soluble starch. The SDS sample buffer used contained no reducing agents. The samples diluted in the buffer were directly used for electrophoresis without boiling. After electrophoresis, the amylase proteins were renatured by incubating the gel in 0.1 M acetate buffer (pH 4.3). The gel was incubated at 30°C for 90 min and amylase activity was detected as zones of clearance after staining with 0.6 g I_2 and 6 g KI L^{-1} solution (Ravi-Kumar *et al.* 2004).

IMMUNOLOGICAL METHODS

For western blot analysis, proteins were separated in 10% SDS-polyacrylamide gels and electro blotted on nitrocellulose membranes (Towbin *et al.* 1979). The membranes were blocked with 10% skim milk solution and amylase protein identified using antibody raised against pure *A. niger* amylase (Dubey *et al.* 2000) at a dilution of 10:20000. Anti-rabbit goat IgG (15:20000 dilution) and peroxidase conjugate (15:20000 dilution) in 10mM Tris-HCl buffer saline, pH 8.0.(Sigma St. Louis, MO, USA) were used as detecting reagents. Tris-HCl buffer (50 mM, pH 7.6) containing hydrogen peroxide (0.025%) and 4-chloro-1-napthol (0.04%) was used as substrate for enzyme reaction.

PRODUCTION OF POLYKETIDES BY *MONASCUS PURPUREUS* DURING SOLID STATE GROWTH ON RICE

INTRODUCTION

M. purpureus is a red koji fungus known for its natural pigments used to colour food, wine and processed meats (Fabre *et al.* 1993). The pharmaceutical applications of polyketides (azophilones) produced by *Monascus* were evidenced by their anti-inflammatory properties and ability to reduce body cholesterol (Akihisa *et al.* 2005a & b). Red koji rice fed to rats, induced for oxidative damage with carbon tetrachloride showed, protected liver function, even though they were suppressed for glutathione S-transferase (Aniya *et al.* 1999). Amino acid derivatives of azaphilones were found to be antibacterial and induced apoptosis in human cancer cell lines, HepG2 and A549 (Su *et al.* 2005). Monacolins isolated from *Monascus* sp were found to protect body organs against oxidation of low density lipoproteins and inhibit cholesterol biosynthesis by competing for the HMG- CoA reductase active site (Istvan and Deisenhofer 2001).

M. purpureus produces the polyketides when it is grown in solid state cultures made of rice (Soccol and Vandenberghe 2003). The pigments from cultures are extracted with alcohol for use after macerating the fermented rice (Johns and Stuart 1991; Duliu *et al.* 2000). Though the polyketides are also produced during growth in shake flasks ((Lin 1973; Yoshimura *et al.* 1975) their yield is significantly low (Pastrana *et al.* 1995).

Solid state growth of *M. purpureus* (MTCC-410) on rice and studies on characterization of metabolites secreted by it during the course of fermentation revealed the production of newer bioactive molecule apart from polyketides. The details are described in this Chapter.

EXPERIEMENT 1: TAXONOMIC CONFIRMATION OF THE MTCC CULTURE

M. purpureus is characterized by pigmented growth on potato dextrose agar (Hawksworth and Pitt 1983). The septate mycelia are generally coloured and the fungus produces cleistothecium enclosing ascospores (Young 1931; Carels and Shepherd 1975 and 1977).

Growth characteristics of the MTCC-410 culture on PDA (**Fig 4**) and its ability to produce cleistothesia were similar to that reported of *M. purpureus* in literature. Hence the taxonomic status of the culture was confirmed as *M. purpureus*.

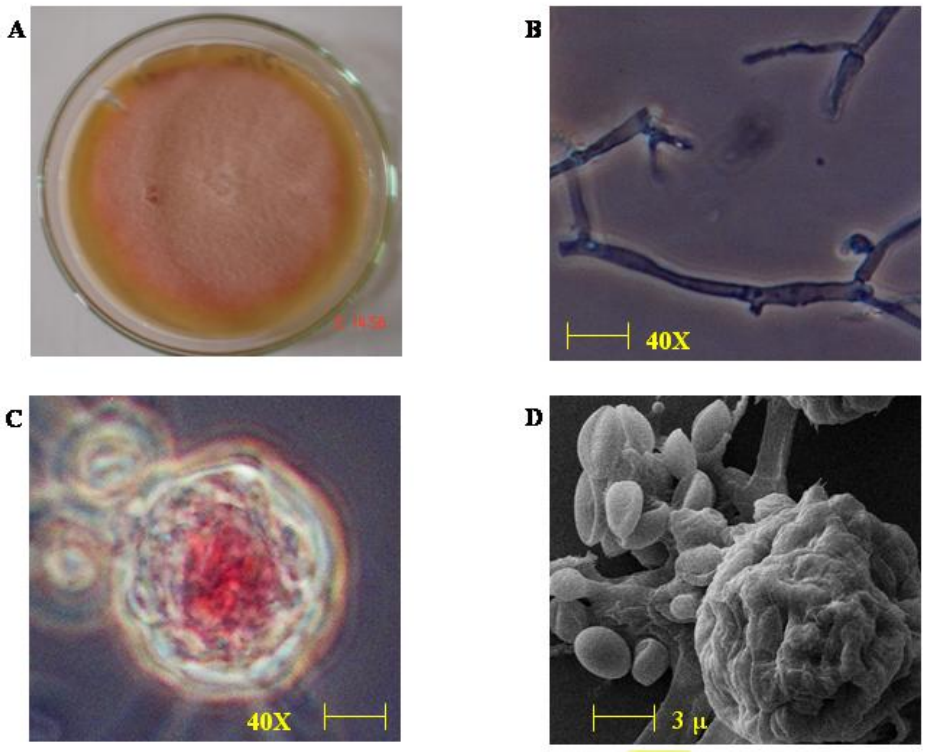


Fig 4. A. PDA grown *M. purpureus.* B. Showing the typically coloured mycelium (phase contrast microscope) C. pigment accumulation in cleistocthecium (Phase contrast) D. Oval shape spore released due to ascocarp rupture (SEM picture)

EXPERIEMENT 2: PIGMENT PRODUCTION BY *M. PURPUREUS* ON RICE

M. purpureus pigments are a group of metabolites called polyketides (azophilones). They are similar in molecular structure and chemical properties. Monascorubrine, rubropunctatin, ankaflavin, monascin, monascorubramine and rubropuntamine produced by the fungus are synthesized by the condensation reaction of acetyl Co-A with malonyl Co-A. Chemical modifications in the lactone rings of the primary product formed due to condensation reaction gives rise to typically coloured polyketides like monascorubrine and rubropunctatin (orange), ankaflavin and monascin (yellow) and monascorubramine and rubropuntamine (red).

Solid state cultures prepared with rice were inoculated with *M. purpureus* and the cultures were incubated for 11 days at 30°C. The polyketides were extracted using different solvents as detailed in Materials and Methods.

Rice upon fermentation with *M. purpureus* turned red in colour **(Fig 5).** Extraction of polyketides from the cultures using hexane resulted in a fraction that showed maximum absorption at 375 nm **(Fig 6A).** After hexane extraction, red rice was treated with chloroform resulted in a filtrate that showed highest absorption at 375 and 475 nm **(Fig 6A and C).** The absorbance of ethyl acetate extracts was like that of hexane **(Fig 6A, C and E).** The similar OD values at 375, 475 and 500 nm obtained upon extraction of red rice with acetone and water **(Fig 6B, D and F)** showed that the pigments were distributed. Polar methanol extracts of red rice showed a characteristic peak of maximum absorption at 500 nm **(Fig 6F).**

The variation in the absorbance optima obtained with solvent extracts of red rice showed production of different pigments by *M. purpureus* during solid state growth and their differently solubility in particular solvents **(Fig 7).**

Fig 5. Solid state grown *M. purpureus.* Substrate: rice

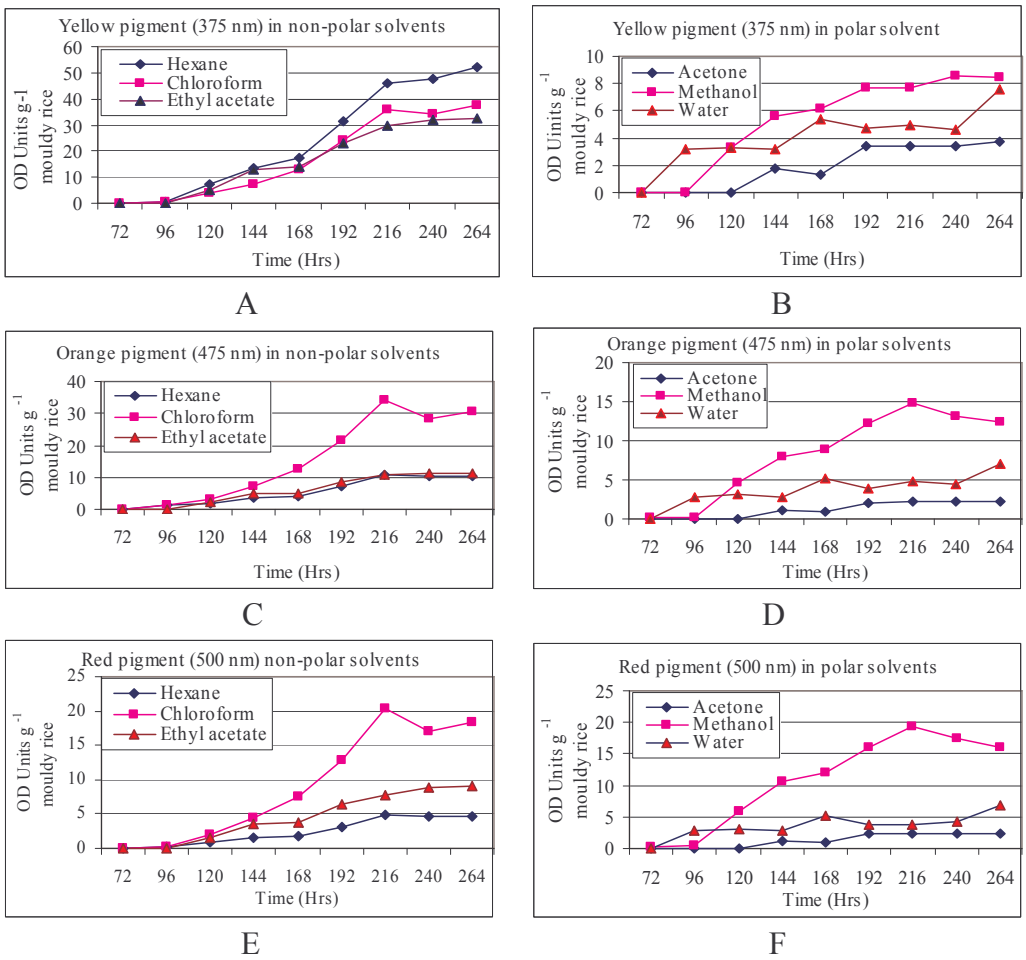


Fig 6. Polyketide pigments extracted from *M. purpureus* with non-polar and polar solvents

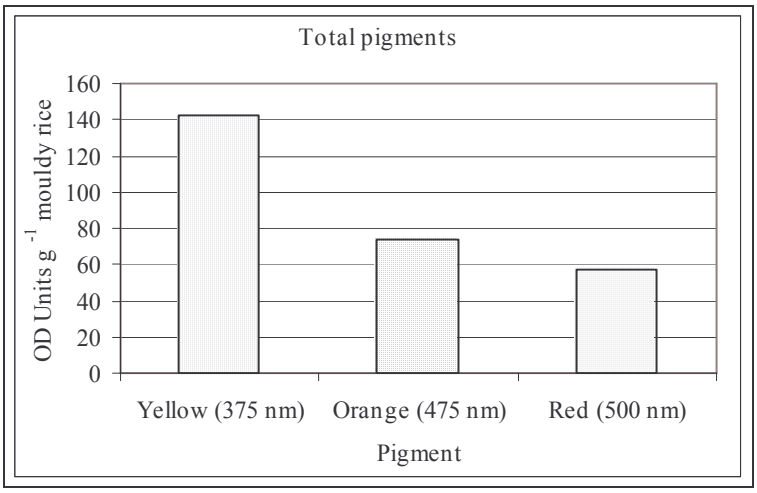


Fig 7. Total pigments produced by *M. purpureus*

EXPERIEMENT 3: FREE RADICAL SCAVENGING ACTIVITY OF *M. PURPUREUS* POLYKETIDE

The observation that the polyketides of *M. purpureus* can be differently fractioned using solvents, suggested the partial purification. In order to identify the bioactive molecule in each solvent fraction, DPPH free radical scavenging activity was determined.

Hexane, chloroform, ethyl acetate and methanol extracts containing polyketides (**Expt 2**) were used for the study. Since acetone or water extracts of red rice showed only little polyketides, they were used as controls for DPPH free radical scavenging assays. The procedure followed for activity determination is described in Materials and Methods.

Highest activity of DPPH free radical scavenging activity was estimated with methanol extracts (**Table 4**). The activity of 59% was 3 and 4 folds higher compared to that estimated of ethyl acetate and hexane extracts respectively (**Table 4**). DPPH free radical scavenging activity of chloroform extract was similar to that of control (acetone extract). That the phenolics present in the extracts did not contribute for the DPPH free radical scavenging was evidenced by low DPPH free radical scavenging activity in acetone (control) and chloroform extracts they contained more phenols(**Table 4**).

Table 4. DPPH free radical scavenging activity of solvent extracts of *M. purpureus* fermented rice

Solvents used for extraction	Phenolics[a] (mg g^{-1})	DPPH radical scavenging activity[a] (%)
Hexane	0.10 ±0.00	13.91±0.33
Chloroform	2.34±0.03	9.26±1.22
Ethyl acetate	1.22 ±0.01	19.17±0.20
Acetone	2.46 ±0.00	8.67±0.63
Methanol	2.19±0.02	59.78±1.66*
Water	0.39±0.00	-
Total yield	-	-

[a]Values expressed are mean ± S D of three extracts

*Significant when compared to other extracts

EXPERIEMENT 4: ANTIOXIDANT PROPERTIES OF *M. PURPUREUS* POLYKETIDES

The DPPH radical scavenging activity of methanol extracts suggested the occurrence of unique compound in it (**Previous Expt**). In order to further augment the above observation, other antioxidant activities were determined using these extracts.

The polyketides obtained from fermented rice by solvent extraction were evaporated to dryness by flash evaporation and lyophilization. After solubilaztion in ethanol, they were used for the assay. Inhibition of lipid peroxidation was studied using phospholipid liposomes. Hydroxyl radical scavenging activity was estimated by generating the free radical using ascorbic acid and iron-EDTA. Total antioxidant capacity was also determined by estimating the reduction of molybdenum. The procedural details are described in Materials and Methods.

All the extracts and the standard butylated hydroxy anisole, inhibited lipid peroxidation induced by Fe^{+3} and ascorbic acid (**Fig 8**) However, ethyl acetate and chloroform extracts showed higher activity compared to other extracts. Similar results

were obtained in measurement of total antioxidant activity (**Fig 9**) and also assays for the ability to scavenge hydroxyl radicals (**Fig 10**). The results showed that the properties were not unique for any particular solvent extracts.

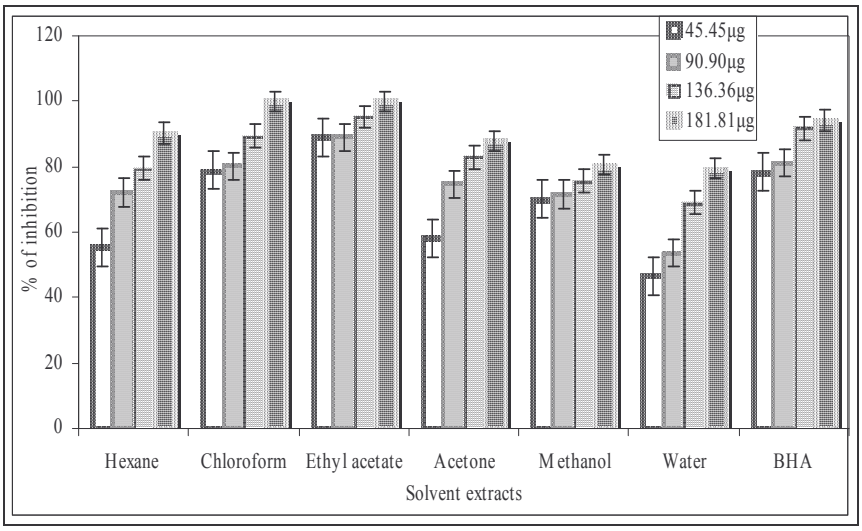
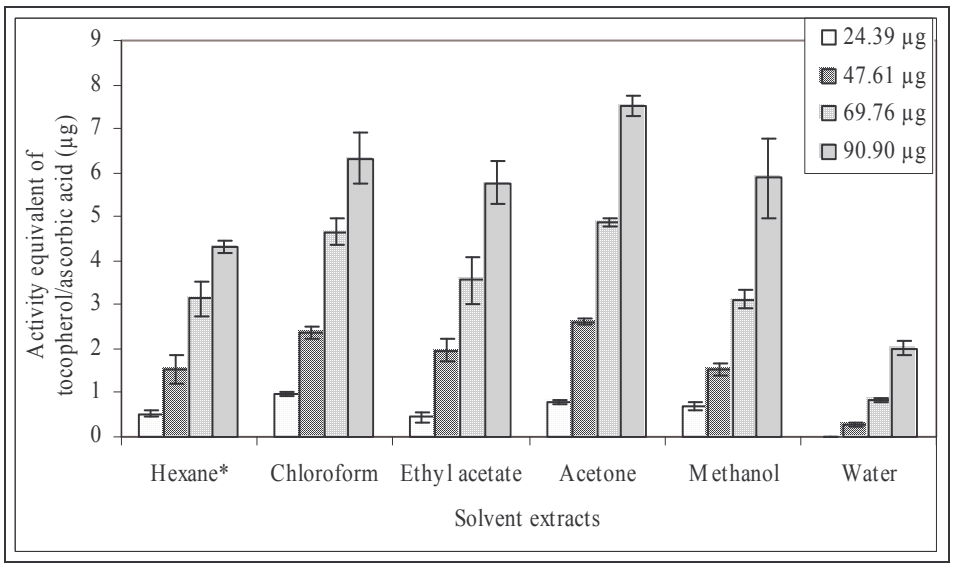


Fig 8. Inhibition of lipid peroxidation by solvent extracts of *M. purpureus* fermented rice



*equivalent of tocopherol

Fig 9. Total antioxidant activity of solvent extracts of *M. purpureus* fermented rice

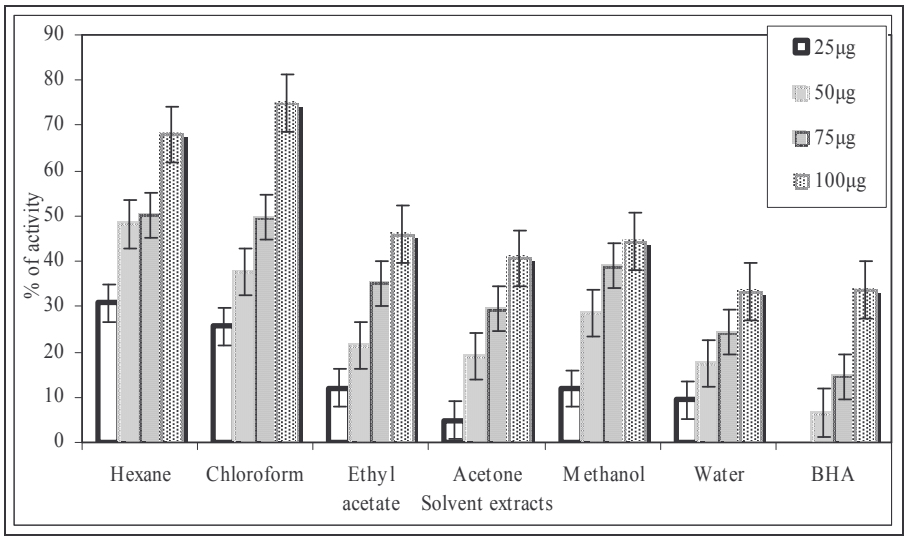


Fig 10. Hydroxyl radical scavenging activity by solvent extracts of *M. purpureus* fermented rice

EXPERIEMENT 5: ACTIVITY GUIDE PURIFICATION FOR THE BIOACTIVE COMPOUND IDENTIFIED IN THE METHANOL EXTRACT

Free radical quenching activity of methanol extracts assayed in terms of DPPH scavenging suggested the production of an yet unidentified bioactive compound by *M. purpureus* during rice fermentation (**Expt 3**). This observation is important since there is wide interest for such compounds for application in cardiovascular diseases (Endo 1980) and cancer (Su *et al.* 2005). Rice fermented by *M. purpureus* being a common food to Chinese population, it appears that they have properties to prevent digestive and vascular dysfunction (Fabre *et al.* 1993; Blanc *et al.* 1995; Ma *et al.* 2000) In the light of above, purification of bioactive compound from methanol extract was found necessary for the characterization of the bioactive compound.

Eleven day old solid state cultures of *M. purpureus* grown on rice were used. It was powdered to 60-80 mesh and the polyketides were extracted using methanol. After filtration, the solvent was removed by flash evaporation and lyophilization. The polyketides solublised in ethanol constituted crude extract. Ten gram crude extract was subjected to silica gel column chromatography followed by step-wise elution in a linear

gradient of hexane, chloroform and ethyl acetate (100:0; 100 and 25:75 v/v). About 20 fractions, approximately measuring 100-150 ml, were collected based on the colour intensity. The DPPH active fractions were pooled and concentrated by flash evaporation. This fraction was subjected to thin layer chromatography on silica gel plates, following development with dichloromethane and ethyl acetate (7:3, 8:2 and 6:4 v/v) and dichloromethane, ethyl acetate and methanol (9:0.5:0.5 and 8:1:1). The spots were identified by exposing to iodine. They were eluted and assayed for DPPH scavenging activity. The fraction showing the activity was taken in methanol and HPLC was performed. The procedural details are described in Materials and Methods.

Fractionation of the crude extract by column chromatography on silica gel column characteristically separated the coloured compounds from a mixture (**Fig 11**). Since the highest DPPH activity was identified in the third and fourth fractions, these were pooled after thin layer chromatography on silica gel. One of the compounds eluted, showed the DPPH free radical scavenging activity. When subjected to HPLC, only single compound was eluted (**Fig 12**). The homogeneity of the compound showed its purity for structure elucidation.

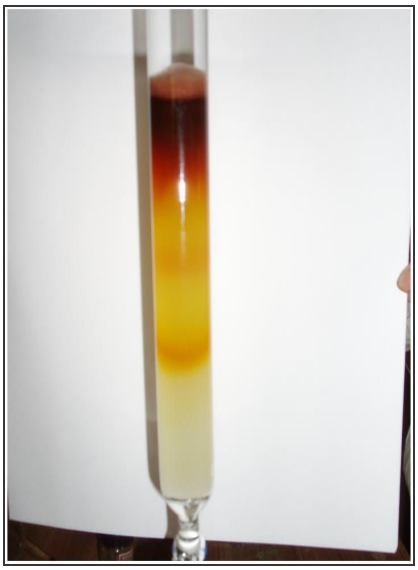


Fig 11. Column chromatography showing purification of *M. purpureus* metabolite

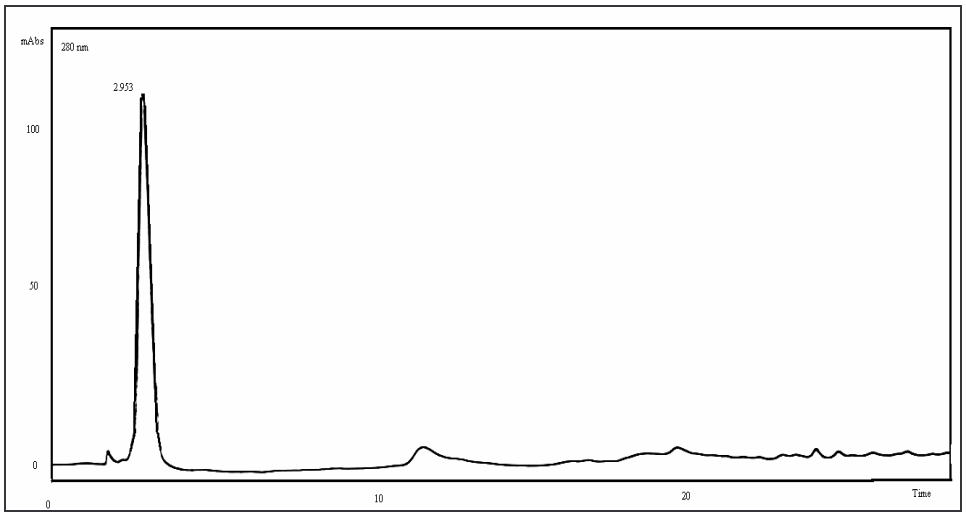


Fig 12. HPLC profile of the pure compound

EXPERIEMENT 6: CHARACTERIZATION OF BIOACTIVE COMPOUND BY NUCLEAR MAGNETIC RESONANCE (NMR) SPECTROSCOPY

H^1 and C^{13} NMR were recorded with the purified sample (Materials and Methods) H1 NMR spectrum was assigned to the hydrogen atoms in the molecule. The assignment strategies involved the analysis of 1-D H^1 NMR (**Fig 13**). The assignments of proton signals are shown in **Table 5**. Likewise the data from C^{13} 1-D NMR (**Fig 14**) and C^{13}- H^1 NMR HSQCT spectra (**Fig 15**) were simultaneously analyzed in order to assign individual carbons to the molecule (**Table 5**).

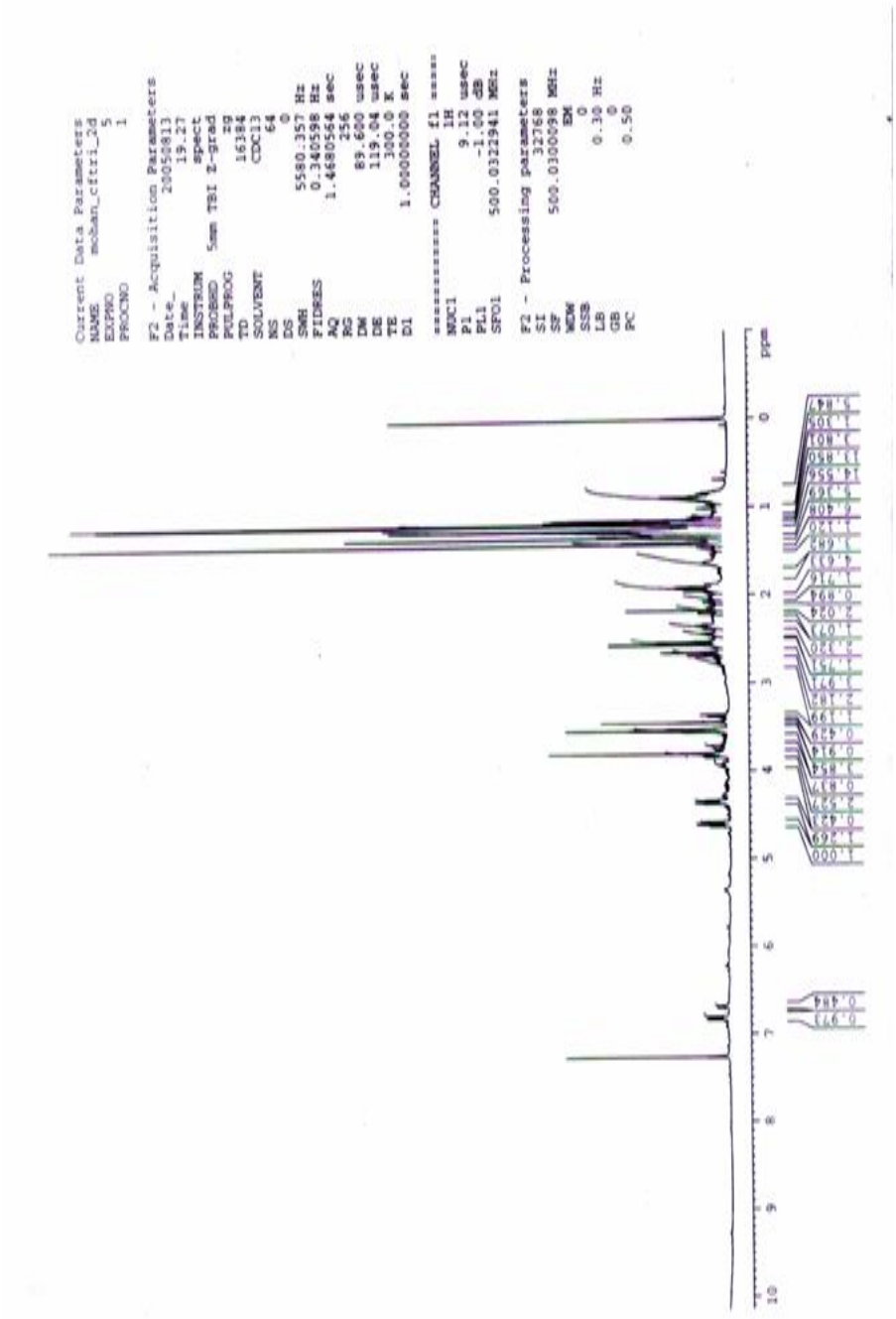


Fig 13. Proton Nuclear Magnetic Resonance (^{1}H NMR) Spectroscopy of the pure compound

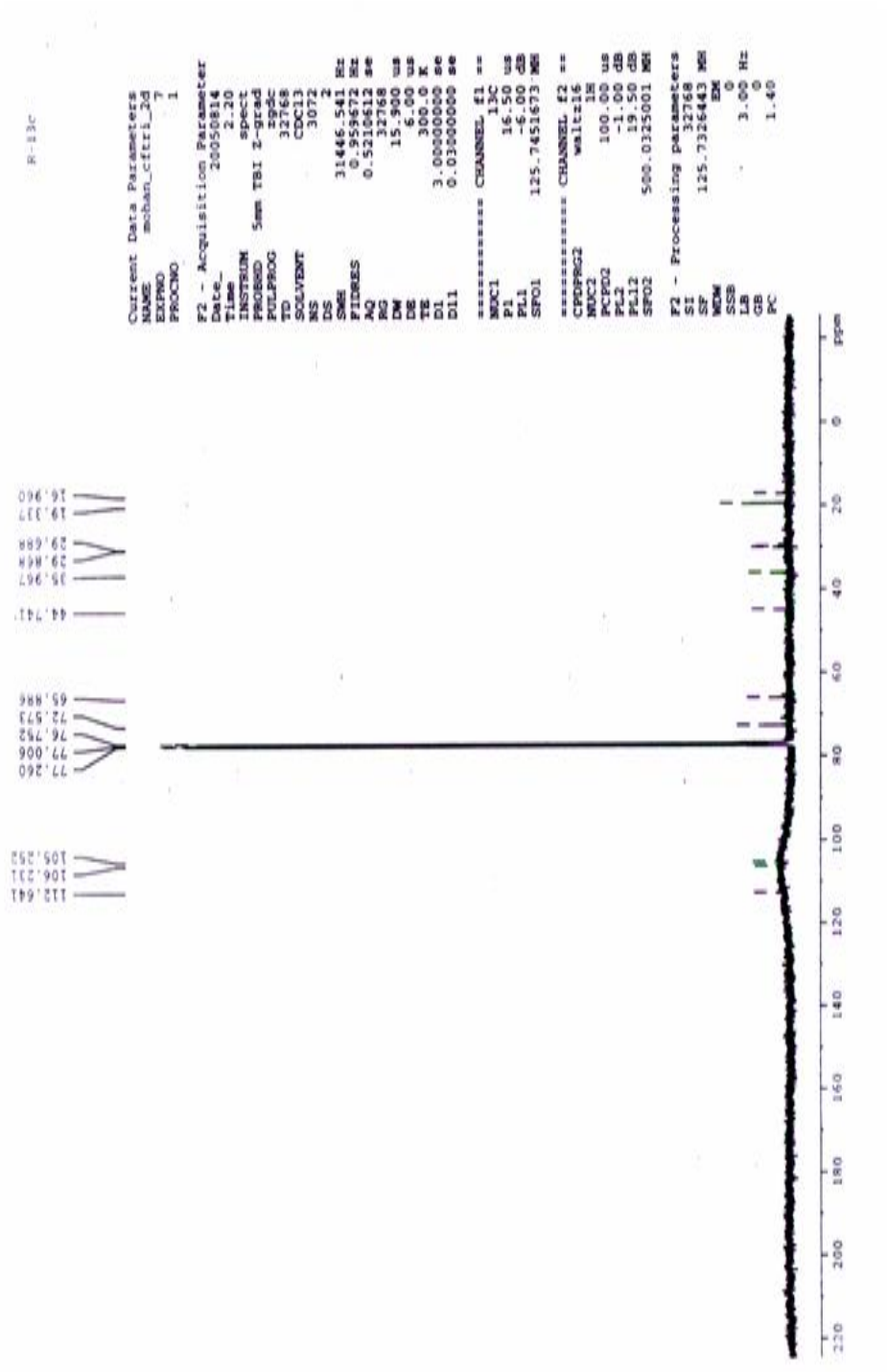


Fig 14. Carbon-13 Nuclear Magnetic Resonance (^{13}C NMR) Spectroscopy of the pure compound

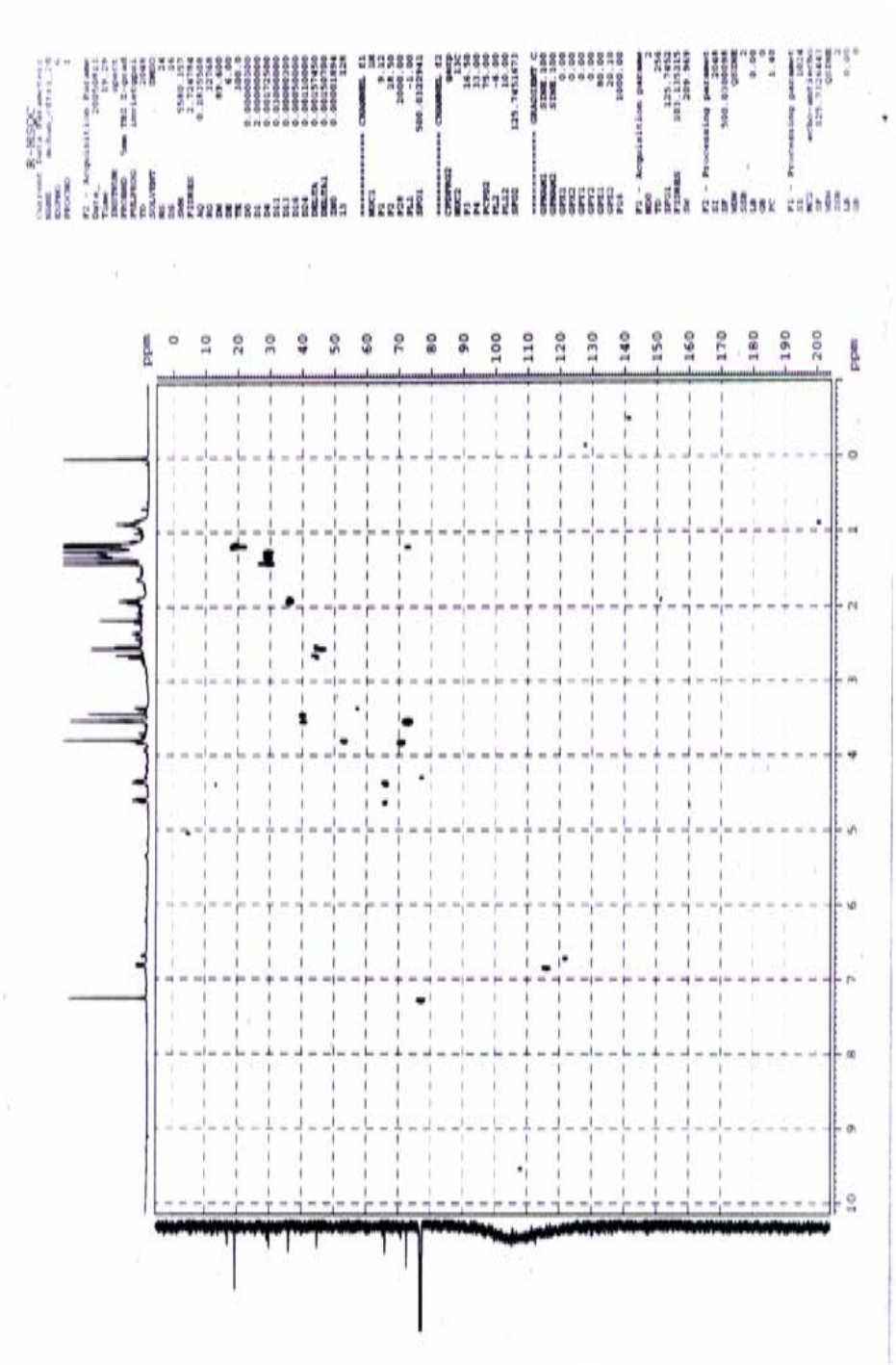


Fig 15. Two Dimensional Heteronuclear Single Quantum Coherence Spectroscopy (2D-HSQCT) NMR Spectrum of the pure compound

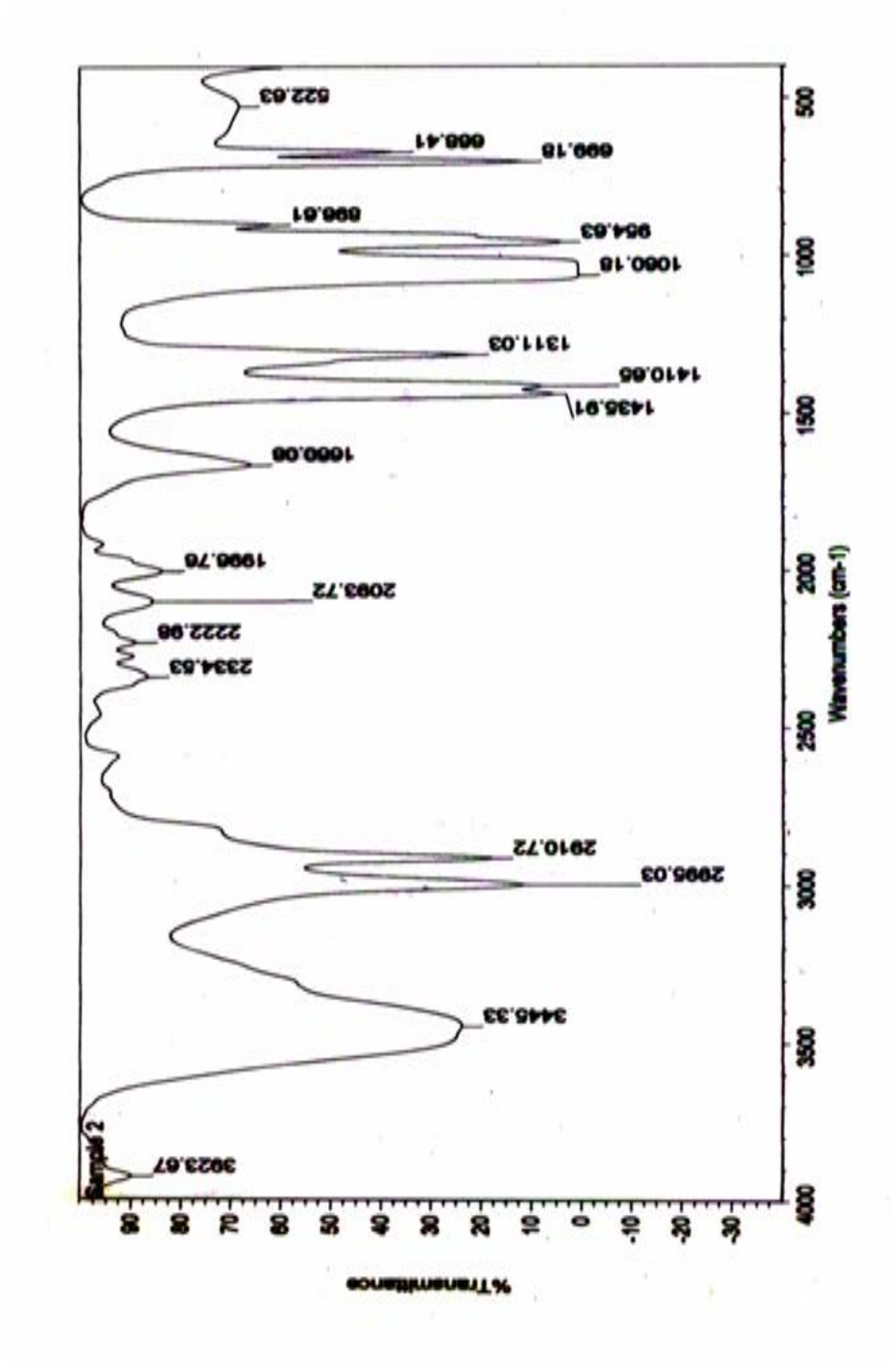


Fig 16. IR spectrum of the pure compound

Table 5. 2D-HSQCT NMR Spectral data of the pure compound

Assignments	Chemical shift	
	Carbon-13	Proton[a]
11-CH_3	18.9	1.21(s)
12-CH_3	19.1	1.25 (s)
23-CH_3, 24-CH_3	22.5	1.18 (d, 6.5Hz), 1.15 (d, 6.5Hz)
20-CH_3	23.1	1.27 (d, J=6.3Hz)
13-CH_2	27.2	1.26, 1.43 (m)
22-CH	29.8	1.3 (J=3.3Hz, septet)
6-CH	30.9	1.33 (m)
14-CH_2	35.2	1.92 (m)
4-CH_2	36.3	1.89 (m)
5-CH_2	36.1	1.85 (m)
10-CH	42.3	2.68 (m)
18-CH_2	40.9	2.65, 2.51 (dd, J=3.8, 6.1Hz)
1C	42.3	-
2-CH	43.4	2.57(t_1)
9-CH	44.3	2.55 (m)
16-CH_2	46.9	2.52, 2.68 (m)
17-CH	65.8	4.35 (m)
3-CH	65.4	4.63 (m)
15-CH	70.9	3.8 (m)
8-CH	117.2	6.75 (d, 10.5)
7-CH	121.5	6.55 (m, 10.5)
19-CO	171.5	-
21-CO	176.1	-

[a]Some of the assignments are interchangeable

The presence of 5-CH_3 group was detected in the proton NMR and the respective carbon-13 correlation (**Table 5**). Two olefinic protons were detected at 6.75 and 6.55 ppm respectively with characteristic cis coupling constant of 10.5 Hz. The ^{13}C signal at 176.15 ppm indicated the presence of a carbonyl group. The region between 1.2 to 2.7 ppm pointed to the presence of several -CH and magnetically non-equivalent -CH_2 groups. Three signals at 65.8 ppm (17-CH), 65.4 ppm (3-CH) and 70.9 ppm (15-CH) suggested the presence of carbon next to oxygen atom in the form of esters, lactone and OH groups.

Thus a derivative of dihydromonacolin-K was identified as the structure of the bioactive compound. Presence of 2-methyl propionate as the ester group at position 3 instead of 2-methyl butyrate found in dihydromonacolin-K was also observed.

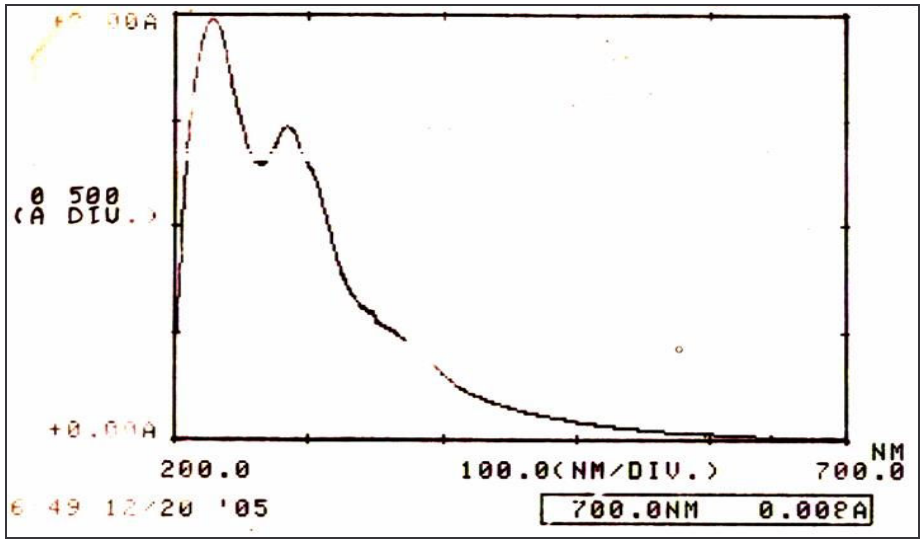


Fig17. UV-Visible spectrum of the pure compound

UV spectrum (**Fig 17**) of the compound as absorption at 228 nm indicated π-π* transition of the olefinic group. IR data (**Fig 16**) showed OH- stretching at 3, 445 cm^{-1}, carbonyl stretching at 1,660 cm^{-1} and C=C stretching at 1,435 cm^{-1}. Direct mass spectrum of the sample showed the parent ion (M^+) at 406.

Based on the above data, the compound was characterized as dihydromonacolin-MV the structure of which is shown in **Fig 18.**

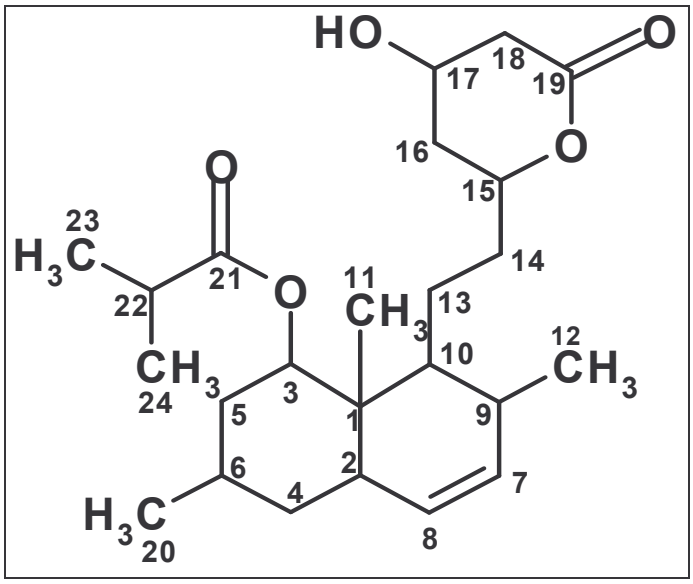


Fig 18. Structure of dihydromonacolin-MV

EXPERIEMENT 7: FREE RADICAL SCAVENGING ACTIVITY OF DIHYDROMONACOLIN-MV

Dihydromonacolin-MV isolated from the solid-state cultures of *M. pupureus* was a potent DPPH radical scavenger known to abstract the liable hydrogen atom. It also strongly inhibited the peroxidation of lipids. The IC_{50} value to scavenge DPPH radical was found to be 20±1 μg ml^{-1}. The synthetic antioxidant BHA (control) showed an IC_{50} of 15.21±0.78 μg ml^{-1}. The lipid peroxidation inhibitory activities of methanol extract and dihydromonacolin-MV are shown in **Table 6.** The IC_{50} value for dihydromonacolin-MV, methanol extract and BHA were found to be 5.71, 36.16 and 32.41 μg ml^{-1} respectively.

Superoxide, the most important source of initiating radicals *in vivo,* is produced in mitochondria during electron chain transfer and it regularly leaks outside of the

mitochondria. The superoxide radical scavenging activity of dihydromonacolin-MV (IC_{50} = 163.97±2.68 μg ml^{-1}) was significant.

Table 6. Antioxidant activities of crude and purified dihydromonacolin-MV

Sample	IC_{50} value (μg ml^{-1})		
	DPPH radical scavenging activity	Lipid peroxidation inhibition activity	Super oxide radical scavenging activity
Methanol extract (Crude)	100.78±2.66	36.16±1.32	-
Dihydromonacolin-MV	20±1	5.71±0.38	163.97±2.68
BHA (Standard)	15.21±0.78	32.41±1.49	264±1.6

Thus dihydromonacolin-MV characterized in this study was a bioactive molecule.

MONACOLINS OF *MONASCUS PURPUREUS*

INTRODUCTION

Free radicals are highly reactive, short-lived, toxic molecules that have one or more unpaired electrons. They damage DNA, proteins, lipids and carbohydrates (Baskar *et al.* 2004) within tissues. The main cause of mortality and morbidity in the Western world is atherosclerosis caused due to the accumulation of oxysterol, cholesterol and peroxide lipids in arteries, generated by free radicals. Since this leads to heart attack, there has been an increased interest in the application of antioxidants for medical treatments based on the information that oxidative stress leads to generation of free radicals (Vaya and Aviram 2001). The effect of dietary antioxidants on the development of human atherosclerosis is also controversial and a number of contradictory examples have been published. Most of the research on the role of antioxidants in cardiovascular diseases has focused on testing pure compounds to prevent lipid peroxidation by examining its ability to scavenge free radicals. However, antioxidant contribution *in vivo* goes far beyond scavenging free radicals. Moreover, single antioxidants are usually not present in biological systems but act in combination with other antioxidants. Hence the protective effect of a diet is not equivalent to the effect of antioxidants in it (Helliwell 2000).

Korantzopoulos (2004) reported that antioxidant effects of statins extend beyond atherosclerosis, atrial fibrillation and heart failure. Red rice obtained by fermentation with *M. purpureus* produces various secondary metabolites and is a common food item found in China. It has been in use for many Centuries to enhance the colour and flavour of foods, as well as a traditional medicine for digestive and vascular dysfunctions (Ma *et al.* 2000). The monacolins from *Monascus* sp (Heber *et al.* 1999) and *Aspergillus terreus* dihydromevinolin (Albers-Schonberg *et al.* 1981) are reported to exhibit a cholesterol lowering action by inhibiting the HMG-CoA reductase similar to the commercial statins. Till date, fourteen different types of monacolins have been identified in *Monascus* sp (Li *et al.* 2004).

Since a new monacolin called dihydromonacolin-MV has been identified in this study, attempts were made to improve its productivity by mutating the fungus. The details are described in this Chapter.

EXPERIMENT 8: MUTATIONAL IMPROVEMENT OF *M. PURPUREUS* FOR DIHYDROMONACOLIN-MV

Industries that use microorganisms generally depend on the process economics defined by the titre of the metabolite. Hence, generation of microbial genotypes that produce high titre metabolite is a fundamental requirement for defining a fermentation system. There are several reports on obtaining novel genotype fungi through induced mutation from this laboratory (Dubey *et al.* 2000; Kavitha 2000b; Kavitha and Umesh-Kumar 2000a; Ravi-Kumar *et al.* 2004; Venkatesh 2004; Kumaresan 2007) and elsewhere (Meyrath *et al.* 1971; Nevalainen and Palva 1979).

Mutations are generally divided into two classes, *viz.,* physical (radiation) and chemical. It is now generally accepted that induced mutations do not depend directly on the type of DNA damage caused. Rather, it is the result of the cellular DNA repair processes acting on the damage to convert it into a fixed alteration in the base sequence. The best understood repair pathways are those involved in the repair of damages caused by Ultraviolet rays and chemicals where, errors in excision repair and recombination repair are responsible for the majority of mutations. The molecular basis of mutagen specificity is not well understood. The results with *A. nidulans* system using UV and ethylmethylsulfonate as mutagens appeared attractive since, it promised to mutate all of the genome with equal frequency. This enabled a successful screening as there was no failure due to lack of mutation at a particular site (Drake 1969).

In the light of the above literature, strain improvement of *M. purpureus* (MTCC-410) by mutation was programmed in order to obtain strains producing high titre dihydromonacolin-MV. The mutation protocol by using UV rays and selection of strain based on temperature tolerance has been described in Materials and Methods.

The earlier observations that monacolin biosynthesis is related to polyketide production (**Expt 2**) suggested, isolation of mutants that over produced polyketides. Tolerance to temperature at 42°C resulted in the selection of hyper pigment producing mutant of *M. purpureus* (**Fig 19 B**). An albino mutant also arose as a temperature tolerant mutant (**Fig 19 C**).

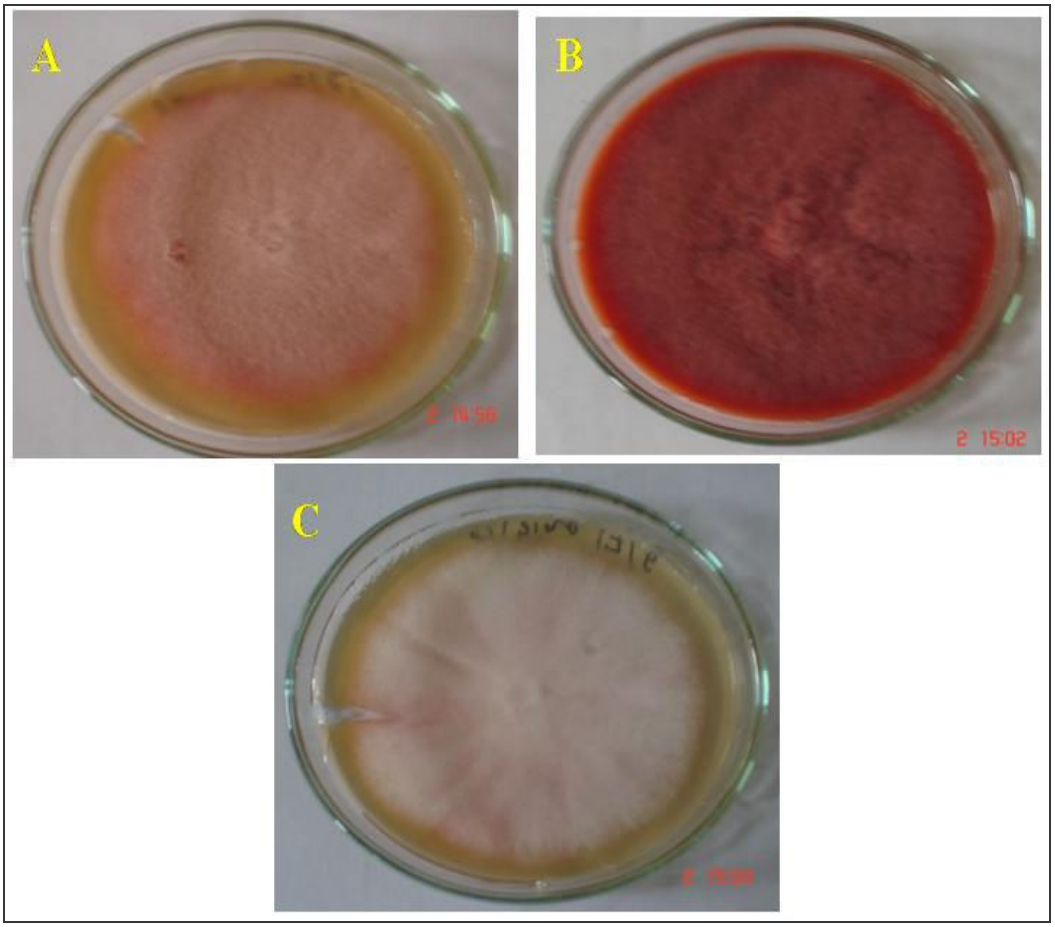


Fig 19. Mutants of *M. purpureus* isolated after temperature selection of UV mutants. A: Wild type, B: Hyper pigment producing mutant, C: Albino mutant

Induced mutation and temperature selection resulted in strains with affected cell wall structure (Gordon *et al.* 2000). Isolation of albino and hyper pigment mutant by this procedure suggested a relationship between polyketide production and cell wall synthesis.

EXPERIMENT 9: THE UV MUTANTS OF *M. PURPUREUS*

The mutants isolated as described above was grown on PDA medium to study their morphology. Microscopic (Scanning Electron Microscopy and Phase Contrast Microscopy) observations were made of the growing fungi as described in Materials and Methods.

Microscopic studies showed the accumulation of polyketides (red pigment) in the mycelia and the cleistothecium of the hyper pigment mutant (**Fig 20 A and B**). Scanning Electron Microscopic studies revealed typical oval shaped spores characteristic of *M. purpureus* (**Fig 20 C**) Morphology of albino mutant (**Fig 21**) was also similar to that described of *M. purpureus*.

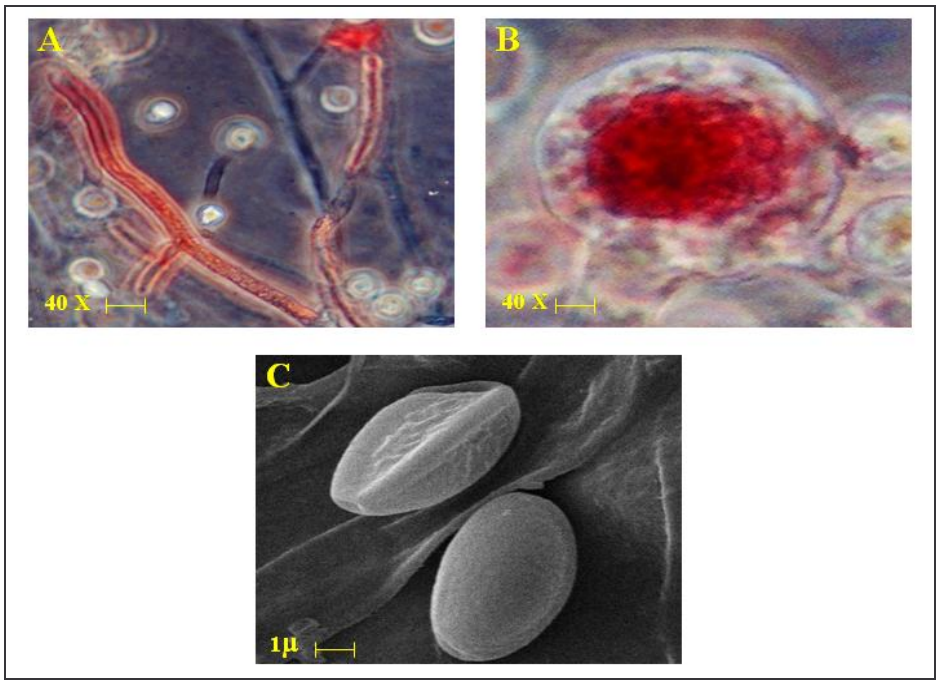


Fig 20. Morphology of hyper pigment mutant A: Red mycelia of *M. purpureus;* B: Polyketide accumulation in cleistothesium; C: Oval shape spore (SEM picture)

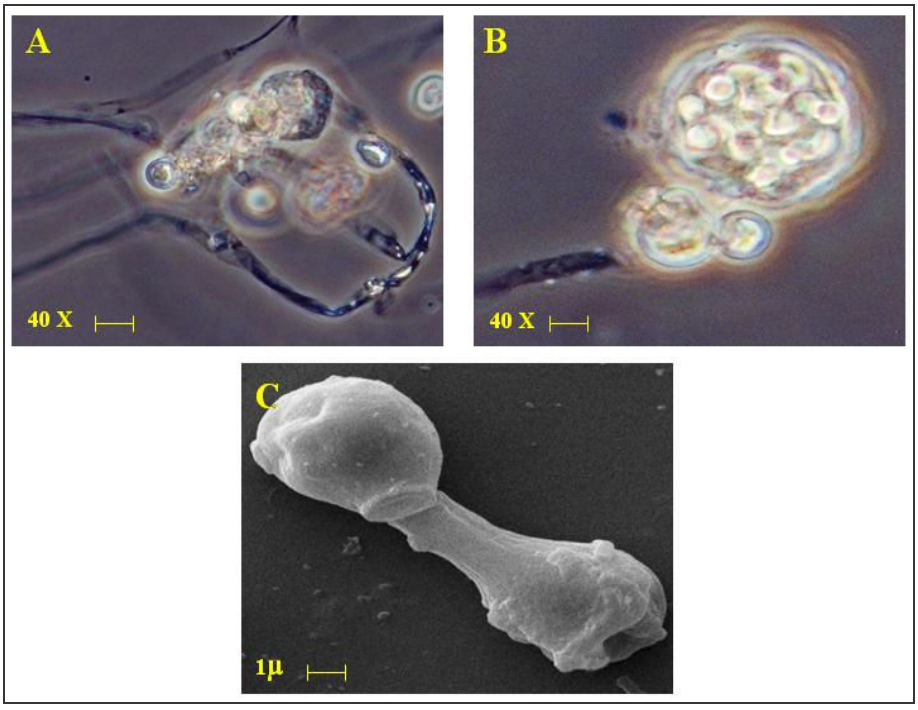


Fig 21. Morphology of albino mutant A: Mycelia; B: Cleistothesium; C: Spore (SEM picture)

Since the morphology of the mutants conformed to *M. purpureus*, they were used for further studies.

EXPERIMENT 10: CHARACTERIZATION OF THE MUTANT IN RELATION TO DIHYDROMONACOLIN-MV OVER PRODUCTION

Since the mutants were isolated for dihydromonacolin-MV over production, they were grown on rice by solid state and methanol extracts obtained were assayed for DPPH radical scavenging activity.

The procedural details of the growth of the mutants, extraction of polyketides and activity estimation are described in Materials and Methods.

Hyper pigment mutants over produced polyketides compared to the Wild type. Though albino on PDA plates, the mutant during rice fermentation produced low quantity of polyketides (**Table 7**). Despite more polyketides production, the methanol extract of the rice fermented with hyper pigment mutant fungus did not scavenge DPPH free radicals. Similar results were also obtained with extracts of rice fermented with albino mutant.

Table 7. Pigment production by *M. purpureus* and its mutants

Strain	Yellow (375 nm)	Orange (475 nm)	Red (500nm)	Total pigment
Wild type	77.43	84.00	83.85	245.28
Albino	64.65	37.45	45.15	147.25
Hyper	95.75	83.87	99.50	279.12

Analysis of the mutants for the production of dihydromonacolin-MV in relation to hyper and less pigmentation revealed no correlation.

EXPERIMENT 11: ANALYSIS OF *M. PURPUREUS* HYPER PIGMENT MUTANT FOR THE PRODUCTION OF NEWER MONACOLINS

The hyper pigment mutant of *M. purpureus* arose due to random mutation induced by UV rays. Hence it is possible that, several mutations could have affected the synthesis of dihydromonacolin-MV. In order to identify the production of modified monacolins by this mutant, the primary chloroform extracts were screened for lipid peroxidation inhibition, hydroxyl radical scavenging and antibacterial activities.

Preparation of chloroform extracts of rice fermented with wild type *M. purpureus* and mutants and assay procedures have been described in Materials and Methods.

The chloroform extracts prepared from rice fermented with the mutant showed reduced ability to inhibit lipid peroxidation and scavenge hydroxyl ions, compared to the wild type extracts (**Table 8**). Importantly, it showed an antibacterial activity (**Table 9**) not found in the extracts prepared from the wild type cultures (**Table 8**).

Table 8. Antioxidant activity of chloroform extracts

Strain	Hydroxyl radical scavenging activity	Inhibition of lipid peroxidation
	IC_{50} Value	
Wild type	69.0±2.0	28.0±2.0
Hyper	75.0±2.0	32.0±2.0

IC_{50} expressed as 50% inhibition concentration

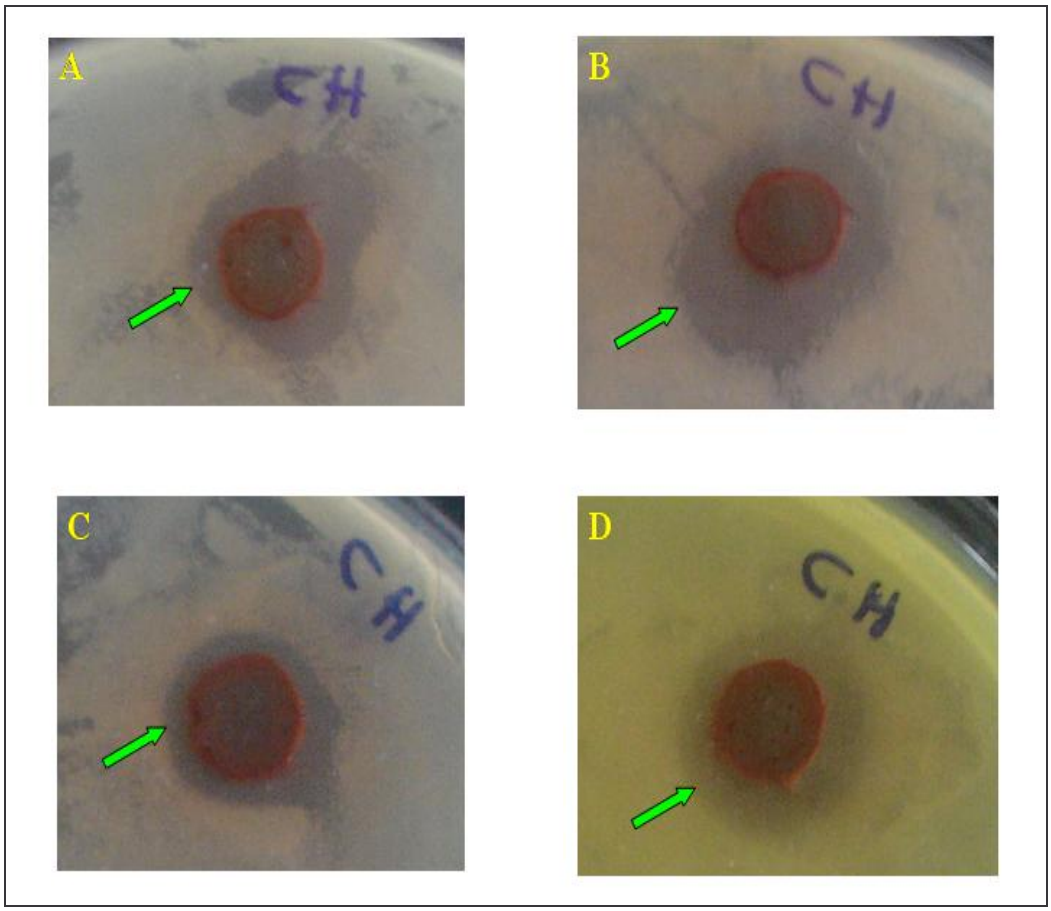


Fig 22. Antibacterial activity of chloroform extracts. Activity shown as zones of inhibition (arrows) on growing A. *Bacillus cereus* B. *B. subtilis* C. *Pseudomonas aerogenosa* D. *Klebsiella pneumonia*

Since the antimicrobial activity was found in the chloroform extracts (**Fig 22**) prepared from rice fermented with hyper pigment strain of *M. purpureus*, the compound responsible was purified for further characterization.

Table 9. Antibacterial activity of chloroform extracts of rice fermented with *M. purpureus* hyper pigment mutant

Bacteria	Chloroform* extract	Penicillin# standard
	Inhibition zone (mm)@	
Bacillus cereus	10.0	nd
B. subtilis	18.0	8.0
Enterobacter aerogenes	nd	15.0
Eshcheria coli	2.0	16.0
Klebsiella pneumonia	6.0	nd
Listeria monocytogen	2.0	26.0
Micrococcus luteus	nd	29.0
Proteus vulgarius	nd	27.0
Pseudomonas aerogenosa	6.0	12.0
Salmonella typhi	4.0	15.0
Staphylococcus aureus	2.0	nd
Streptococcus fecalis	nd	9.0
Yersinia entirolytica	10.0	nd

*Lyophilized extract dissolved in ethanol (5 mg ml^{-1})

@Inhibition zone excluding well or disc

Penicillin disc (6 mm)

nd- not detected

EXPERIMENT 12: ANTIBACTEIAL ACTIVITY GUIDED PURIFICATION OF BIOACTIVE COMPOUND

Eleven day old solid state cultures of rice fermented with *M. purpureus* hyper pigment mutant was used for the study. The fermented rice was powdered to 60-80 mesh and the polyketides were extracted using chloroform. After filtration, the solvent containing the bioactive compound was flash evaporated to remove the solvent. The active molecules were obtained as a powder after lyophilization. One gram chloroform

extract was subjected to silica gel column chromatography followed by step-wise elution using a linear gradient of hexane, chloroform and ethyl acetate (100:0; 100 and 25:75 v/v) partially purify the active compound. About 49 fractions (approximately measuring 50 ml) collected based on the colour intensity were tested for antibacterial activity. The active fractions were concentrated by flash evaporation and pooled after thin layer chromatography analysis on silica gel plates. The TLC plates were developed with dichloromethane and ethyl acetate (7:3, 8:2 and 6:4 v/v) and dichloromethane, ethyl acetate and methanol (9:0.5:0.5 and 8:1:1). The fractioned compounds were identified by exposing to iodine. Each of these were eluted and assayed for antibacterial activity. The fraction showing the activity was taken in acetonitrile and HPLC was performed. The procedures are described in Materials and Methods.

Fractionation of the crude extract by column chromatography on silica gel column characteristically separated the coloured compounds from the mixture. Since the antibacterial activity was identified in the first fraction, it was subjected to thin layer chromatography on silica gel. One of the compounds eluted showed the presence of active compound in it. HPLC analysis identified it as a pure compound (**Fig 23)**. Hence it was used for characterization for elucidation of its structure.

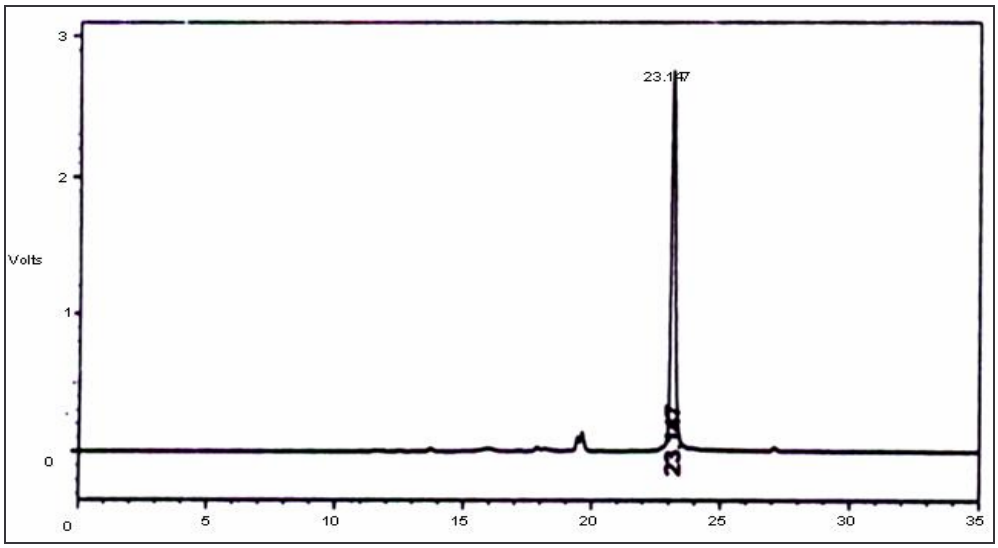


Fig 23. HPLC profile of the pure compound

Table 10. Antibacterial activity of the purified compound

Bacteria	Purified compound * Inhibition zone (mm)@
Bacillus subtilis	6.0
Pseudomonas aerogenosa	7.0
Streptococcus fecalis	6.0
Micrococcus luteus	7.0
Proteus vulgarius	6.0

*The purified compound was dissolved in ethanol ad a 15 mg ml^{-1} solution was used

@Inhibition zone excluding disc

In agar gel diffusion assays, the purified compound at 15 mg ml^{-1} concentration inhibited the growth of *B. subtilis*, *P. aerogenosa, S. fecalis, M. luteus* and *P. vulgarius* (**Table 10**).

EXPERIMENT 14: CHARACTERIZATION OF BIOACTIVE COMPOUND

H^1 and C^{13} NMR were recorded with the purified sample (Materials and Methods). H^1 NMR spectrum was assigned to the hydrogen atoms in the molecule. The assignment strategies involved the analysis of 1-D ^1H NMR (**Fig 24**). The assignments of proton signals were shown in **Table 11**. Likewise, the data from ^{13}C 1-D NMR (**Fig 25**) and ^{13}C- ^1H NMR 2D-HSQCT spectra (**Fig 26**) were simultaneously analyzed to assign individual carbons to the molecule (**Table 11**).

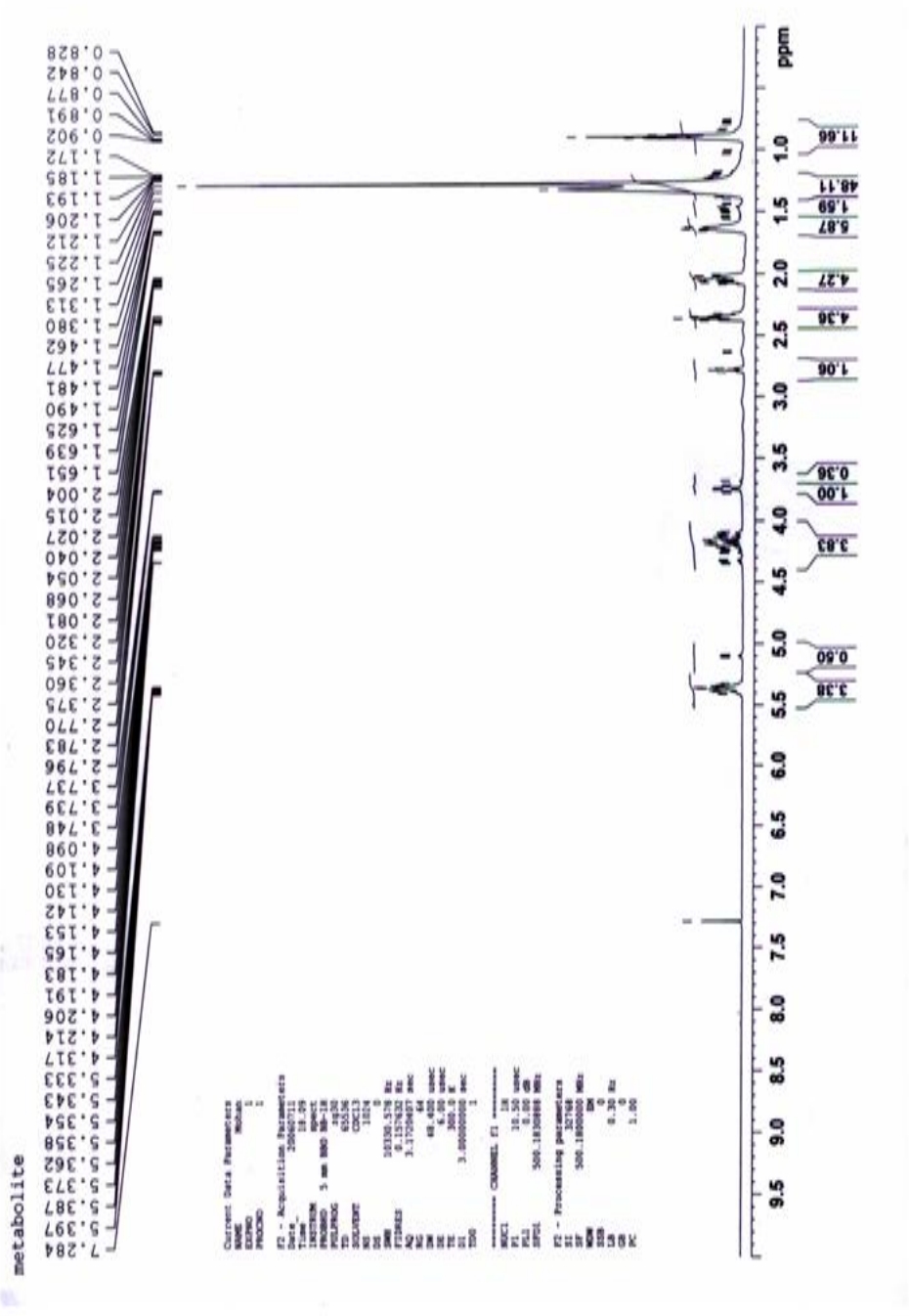


Fig 24. Proton Nuclear Magnetic Resonance (^{1}H NMR) Spectroscopy of the pure compound

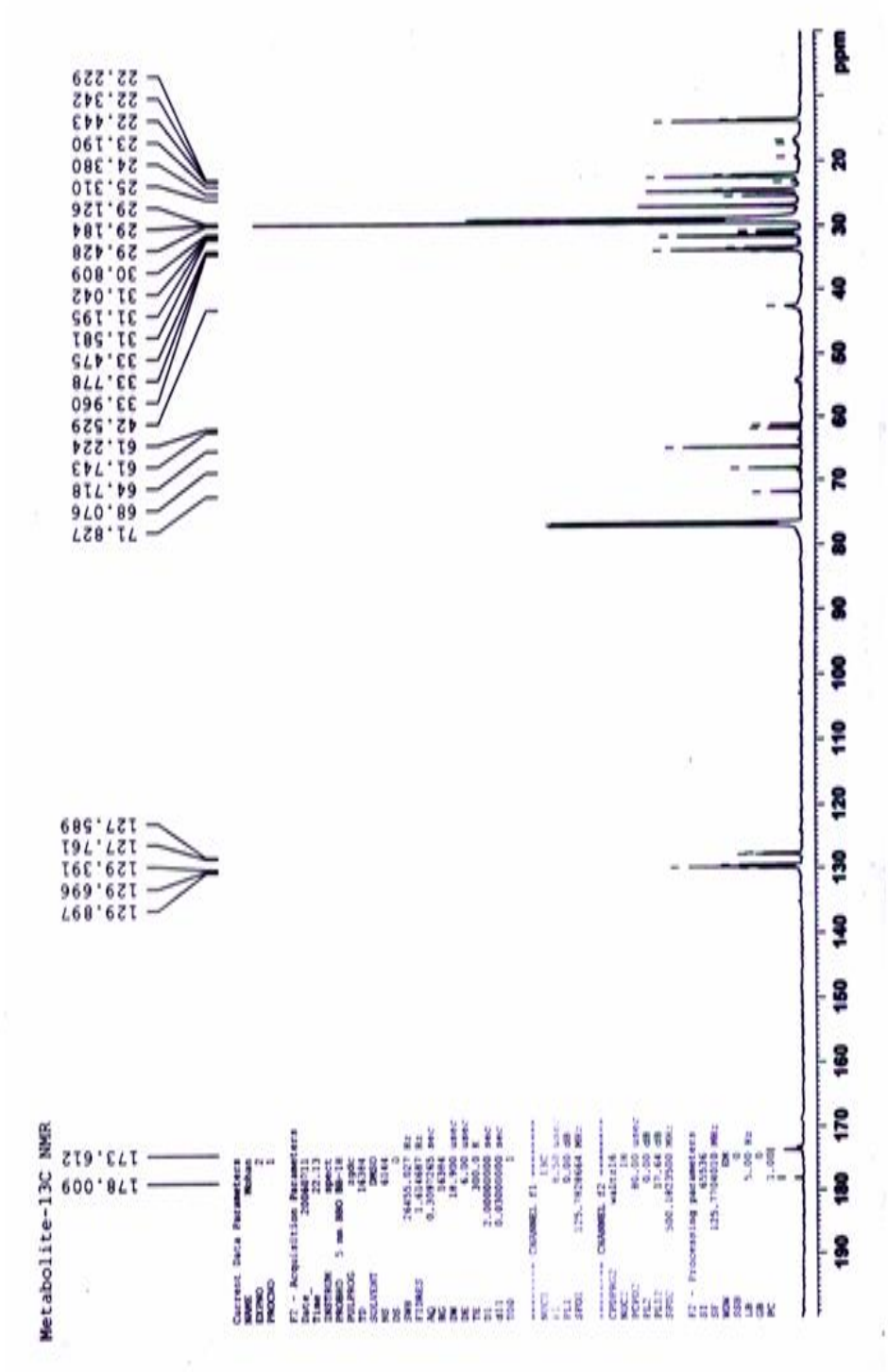


Fig 25. Carbon Nuclear Magnetic Resonance (^{13}C NMR) Spectroscopy of the pure compound

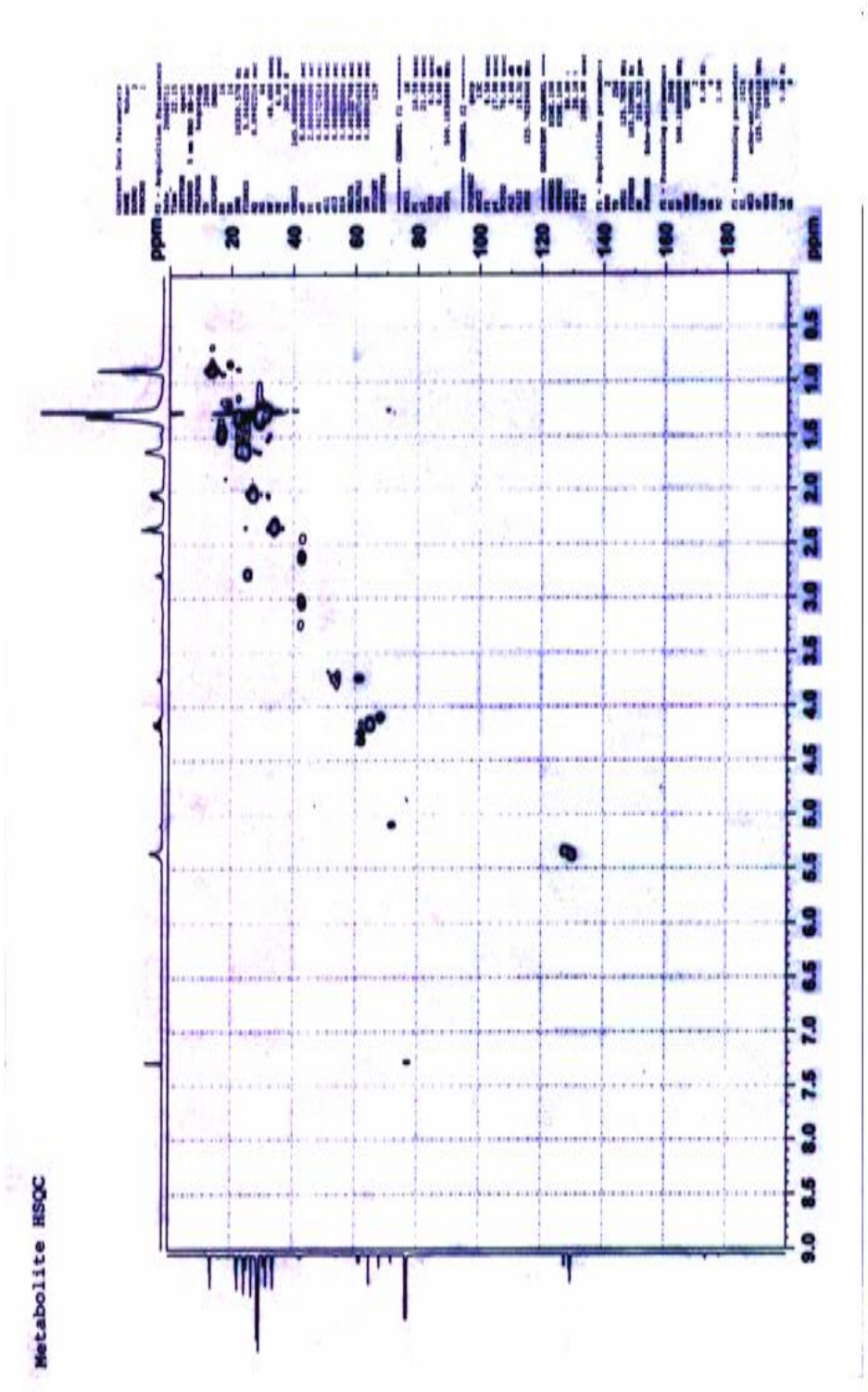


Fig 26. Two Dimensional Heteronuclear Single Quantum Coherence Transfer (2D-HSQCT) Spectroscopy of the pure compound

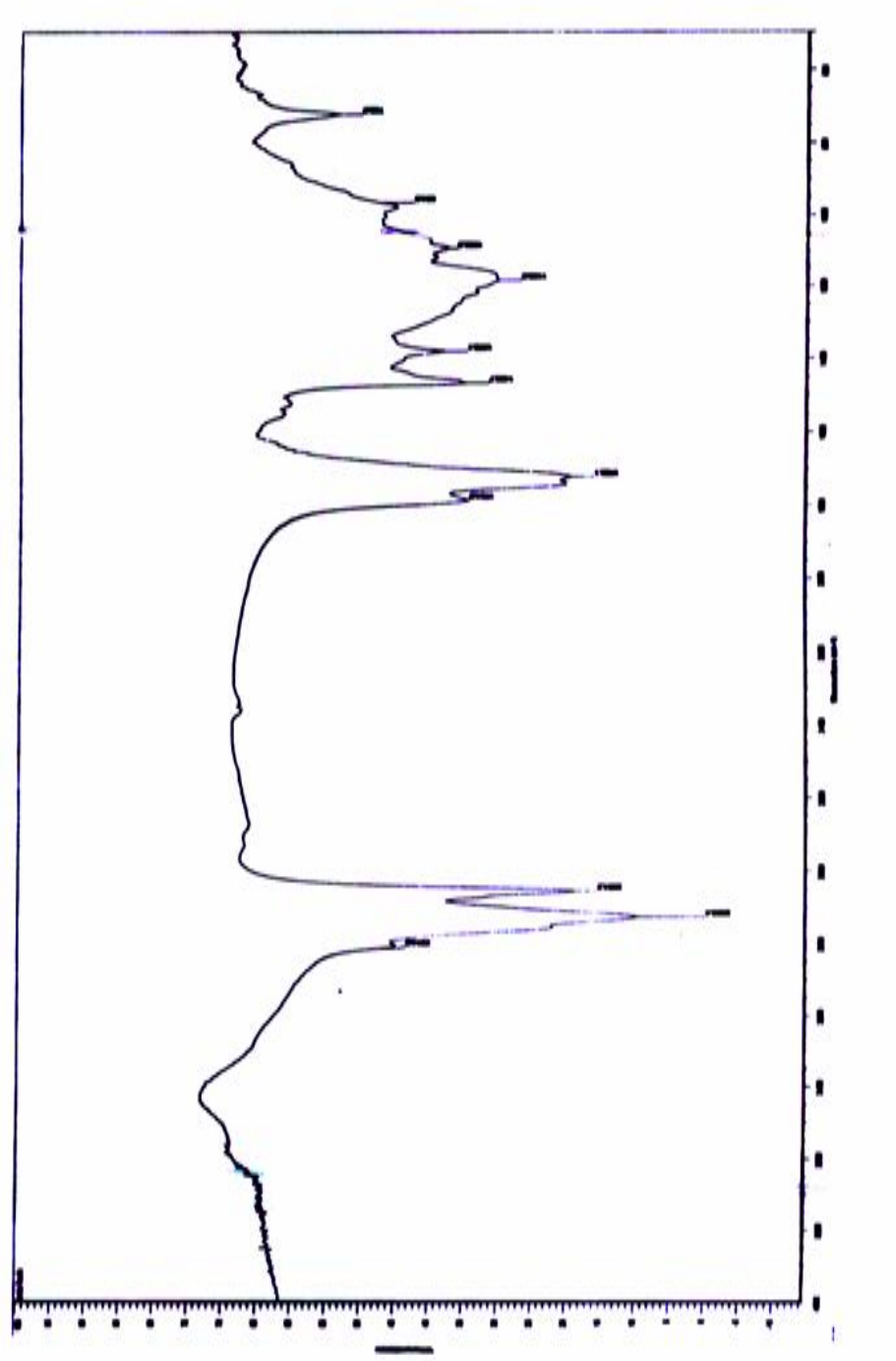


Fig 27. IR spectrum of the pure compound

Table 11. 2D-HSQCT NMR data of the pure compound[a]

Carbon-13		Proton	
Chemical shift	Assignment	Chemical shift (J in Hz)	Assignment
14.1	C-16	1.27 (d, 6.0)	CH_3-7
22.2	C-7	1.29 (d, 5.8)	CH_3-16
24.4	C-14	1.64 (6.0, 6.5)	H-13 (a and b)
25.3	C-4	2.02, 2.09 (6.0, 5.5)	H-1 (a and b)
29.1	C-1	2.04, 2.07 (6.0, 5.5)	H-4 (a and b)
29.2	C-6	2.36 (m, 7.5)	H-14
30.8	C-13	2.33 (7.3)	H-18 (a and b)
33.8	C-18	2.79 (m, 6.5)	H-6
61.2	C-17	3.74 (m)	H-3
64.7	C-12	4.12 (m)	H-2
68.1	C-3	4.18 (m)	H-17
71.8	C-2	4.32 (m)	H-12
127.6	C-10	5.1	OH
127.8	C-15	5.36 (d, 11.5)	H-10
129.4	C-11	5.40 (m)	H-11
129.7	C-5	5.42 (d, 11.2)	H-15
129.9	C-9	-	-
130.1	C-8	-	-
173.6	C-19 (CO)	-	-

[a]Some assignments are interchangeable

One dimensional ^{1}H and ^{13}C NMR (**Fig 24 and 25**) and 2D HSQCT (**Fig 26**) spectroscopy predicted the presence of six olefinic carbons between 127.6 and 130.1 ppm and three double bonds in the structure. Corresponding proton chemical shift values were detected between 5.36 and 5.42 ppm. The compound showed the presence of quite a few secondary OH groups. The CH carbons at 61.2, 64.7 and 68.1 ppm indicated the presence of 3-CH carbons attached to OH groups. Analogous proton chemical shift values were observed between 3.74 and 4.32 ppm with characteristic multiple couplings of the adjacent CH or CH_2 groups. CH ring attached to lactone oxygen showed up as a signal at 71.8 ppm. Two methyl groups at 14.1 and 22.2 ppm, proton doublets at 1.27 and 1.29 ppm and quite a few CH_2 groups next to asymmetric CH-carbons were identified by the splitting pattern of the two magnetically non-equivalent geminal protons. A carbonyl (C=O) signal was observed at 173.6 ppm.

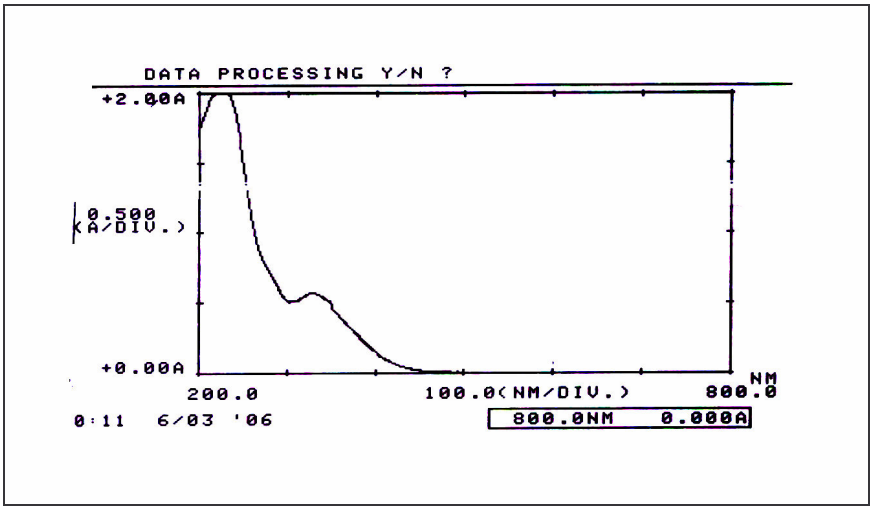


Fig 28. UV-Visible spectrum of the pure compound

The UV spectrum of pure compound showed λ max at 329 nm (π-π* transition) and 225 nm (σ-σ* transition) in methanol (HPLC grade) **(Fig 28)** indicating that the molecule possessed double bonds in extended conjugation **(Fig 30)**. IR spectrum showed a broad band corresponding to OH stretching at 3400 cm^{-1} and CH stretching between 3014 cm^{-1} and 2864 cm^{-1}. Prominent carbonyl stretching was observed at 1720 cm^{-1} **(Fig 27)**. Direct mass spectrum (**Fig 29**) of the sample showed the parent ion (M^{+}) at 335.

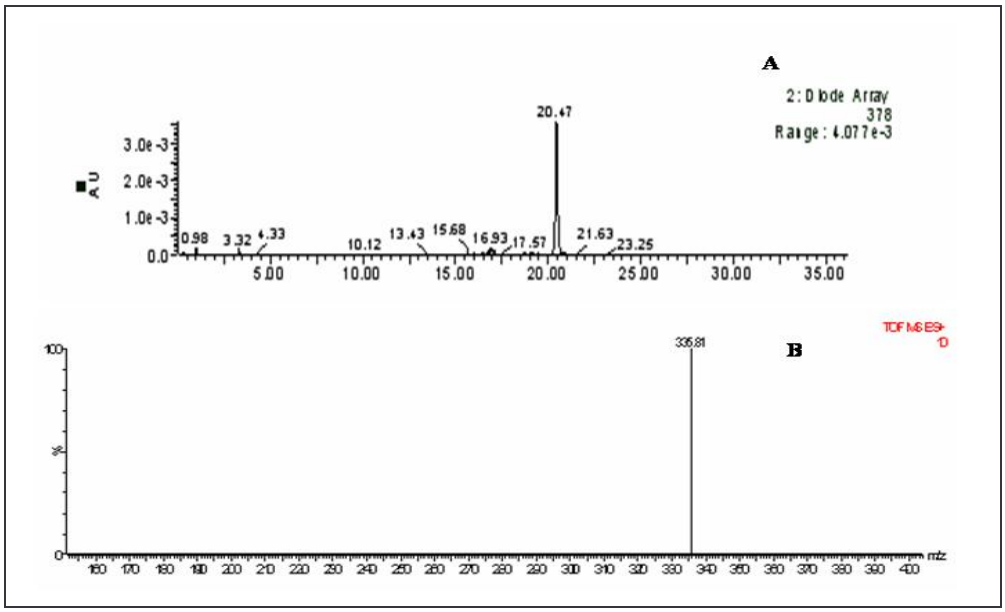


Fig 29. Liquid chromatography (A) and mass spectrum (B) of the pure compound

Based on the above data, the compound that showed antibacterial activity was characterized as dehydromonacolin-MV2 of the structure shown in **Fig 30.**

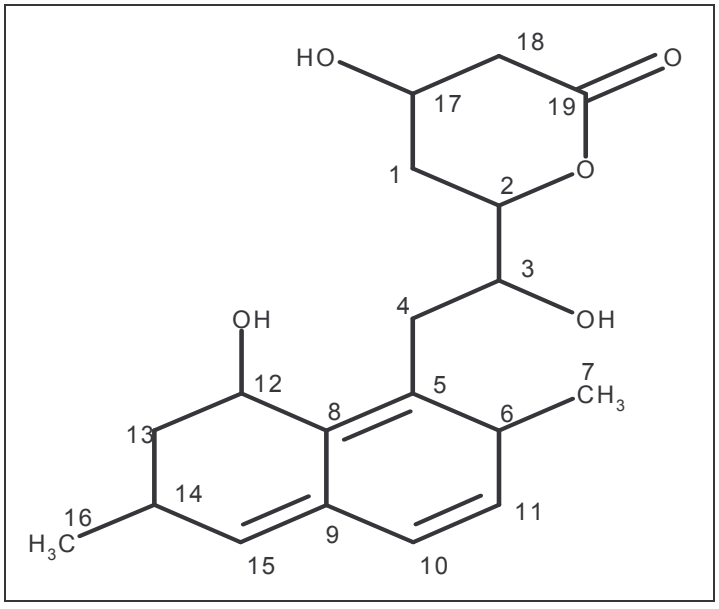


Fig 30. Structure of dehydromonacolin-MV2

EXPERIMENT 13: OTHER BIOACTIVE PROPERTIES OF DEHYDROMONACOLIN-MV2

Chloroform extracts of the solid state grown *M. purpureus* hyper pigmentation mutant inhibited lipid peroxidation (EC_{50}=32.0±2.0 µg ml^{-1}) and scavenged hydroxyl radicals (EC_{50}=75.0±2.0 µg ml^{-1}). Though the crude extract did not scavenge DPPH free radicals, the dehydromonacolin-MV2 purified from the extract was found to posses a low DPPH radical scavenging activity of EC_{50}=443.0±20.0 µg ml^{-1}. Its EC_{50} for hydroxyl radical scavenging and lipid peroxidation inhibition were 286.0±2.0 µg ml^{-1} and 72.0±7.0 µg ml^{-1} respectively (**Table 12**).

Table 12. Antioxidant activities of dehydromonacolin-MV2

Sample	EC_{50} (µg ml^{-1})		
	DPPH radical scavenging activity	Lipid peroxidation inhibition activity	Hydroxyl radical scavenging activity
Chloroform extract	-	32.0±2.0	75.0±2.0
Dehydromonacolin-MV2	443.0±20.0	172.0±7.0	286.0±2.0
BHA*	15.0±1.0	33.0±2.	196.0±4.0

*Control

Thus dehydromonacolin-MV2 apart from possessing antibacterial activity also showed antioxidant properties.

ENZYMES OF *MONASCUS PURPUREUS*

INTRODUCTION

Monascus species are well known for the production of water insoluble pigments and monacolins (Dhale *et al.* 2007a & b). Water soluble pigments from *M. purpureus* arise when they are grown in media containing amino acids (Lin and Demain 1991; Jung *et al.* 2003 and. 2005). Lin and Demain (1994) showed that soluble red pigment formation was regulated by the type of nitrogen source and presence of glutamate as a sole nitrogen source induced their production. There are also reports on the ammonium salts and ammonium nitrate determining the production of a particular pigment during *M. purpureus* fermentation (Lin 1973; Su 1978; Lin and Demain 1995).

Acid proteases catalyse the hydrolysis of peptide bonds at acidic pH. In Tofuyo making, *M. purpureus* proteolytic enzymes specifically serine carboxy peptidase was found essential since, it released free glutamic acid and aspartic acid (Liu *et al.* 2004). Thus the type of substrate available for fermentation determines the nature of pigment produced by the *M. purpureus* and rice and bread were normal substrates for pigment biosynthesis. It is also believed that the enzymes secreted define the type of pigment produced during fermentation (Birch *et al.* 1962; Manchand *et al.* 1973).

Growth of *M. purpureus* on rice produced a new monacolin called dihydromonacolin-MV (Dhale *et al.* 2007a). Under the identical growth conditions the hyper pigment mutant secreted dehydromonacolin-MV2 (Dhale *et al.* 2007b). This study describes the role of enzymes on the pigment production by *M. purpureus* and its mutants.

EXPERIEMENT 15: AMYLASE PRODUCTION DURING GROWTH OF *M. PURPUREUS*

Growth of *M. purpureus* in solid state cultures made of rice suggested production of amylase during fermentation. In order to study the role of this enzyme in pigment production, their activities were determined during growth of the fungus and its mutants in solid state.

Enzyme preparation from the solid state cultures of *M. purpureus* fermented rice for amylase activity determination is described in Materials and Methods.

Six days cultures of the wild type recorded more amylase activity. In the hyper pigment mutant, the activity determined of three day old culture reduced during the next six days and increased partially. Though the amylase production by albino mutant was comparable to the wild type, there was a sudden spurt in activity during growth between ninth and twelfth day (**Fig 31**).

The results are discussed in the later section of the thesis.

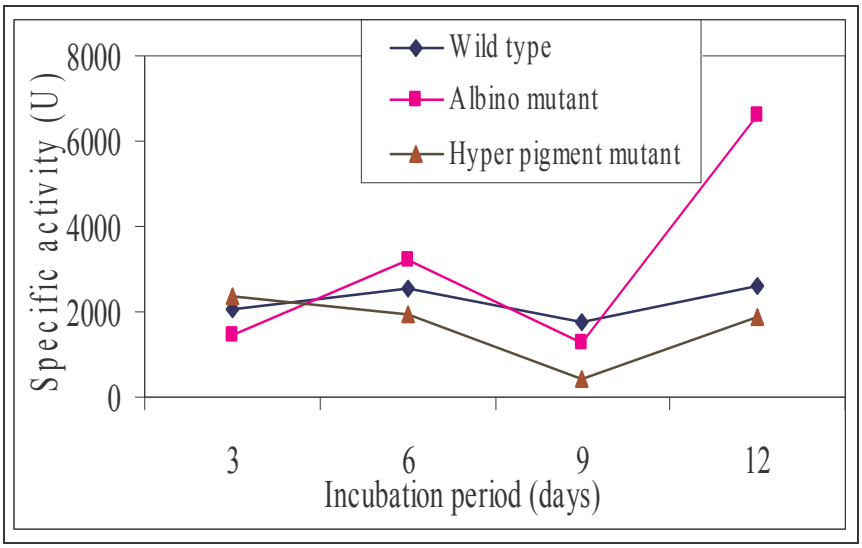


Fig 31. Amylase production by *M. purpureus* during solid state growth

EXPERIEMENT 16: CHARACTERIZATION OF *M. PURPUREUS* AMYLASE

The observation that *M. purpureus* and its mutants vary in their ability to produce amylase is new to literature. Since the enzyme of the organism has not been characterized, studies were carried out to identify the nature of the enzyme.

Extracts of *M. purpureus* fermented rice were used for the study. Amylase activities were visualized as zymogram reaction after electrophoresis (Ravi-Kumar *et al.* 2004). For enzyme characterization, reactivity of the protein to antibodies raised against purified glucoamylase of *A. niger* (Dubey *et al.* 2000) was studied by western blot reactions. The details of the procedure are described in Materials and Methods.

Zymogram reactions in acrylamide gels under non-reducing conditions identified the production of a single amylase corresponding to molecular mass 71 kDa (**Fig 32**). *A. niger* glucoamylase antibodies reacting with the *M. purpureus* amylase in the western blot reaction after PAGE under reducing conditions identified the similarity of the two proteins (**Fig 33).** In these reactions, the *M. purpureus* amylase migrated as 90 kDa protein.

Secretion of more enzymes by the hyper pigment mutant (**Fig 33 C**) suggested a role for this enzyme in pigment secretion. The results are discussed in the later section of thesis.

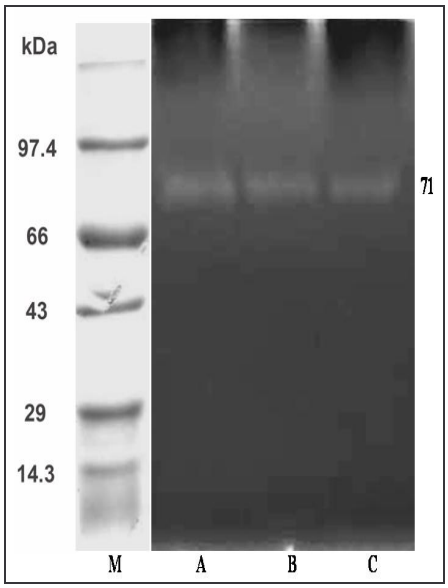


Fig 32. Zymogram reactions identifying the amylase of *M. purpureus*. Ten microgram protein was used for electrophoresis

Lane A: Wild type; B: Albino; C: Hyper pigment mutant

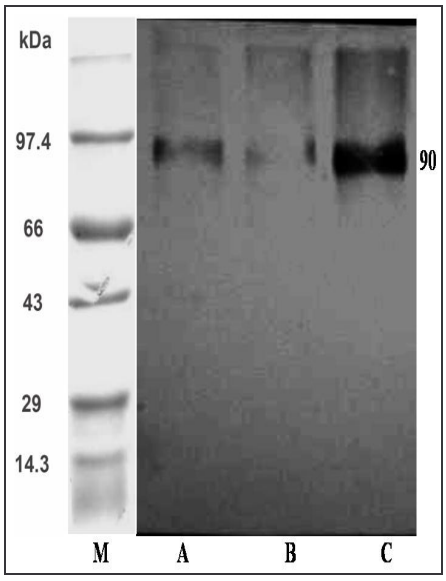


Fig 33. Western blot reactions identifying amylases in the crude extract of *M. purpureus*. Ten microgram protein was used for electrophoresis. Antibodies raised against the purified *A. niger* glucoamylase were used

Lane A: Wild type; B: Albino; C: Hyper pigment mutant

EXPERIEMENT 17: PROPERTIES OF *M. PURPUREUS* AMYLASE

The observations that the amylase of *M. purpureus* was similar to *A. niger* glucoamylase suggested studies to characterize the enzyme. The crude extracts prepared from rice fermented with *M. purpureus* and its mutants were assayed for activity by DNS procedure. For this assay, protease inhibitor cocktail obtained from Boehringer Mannheim GmbH Germany was added in order to avoid interference by proteases. The details are described in Materials and Methods.

The enzyme of the wild type and hyper pigment producing mutant showed optimum activity at 40°C. However, the amylase of the albino mutant was optimally active at 50°C (**Fig 34**). Despite the above variations, all the enzymes showed activity at pH 5 (**Fig 35**). HPLC analysis of the products formed from starch due to enzyme activity revealed the formation of glucose (**Fig 36**).

Thus the production of exo-acting glucoamylase by *M. purpureus* was identified in the study.

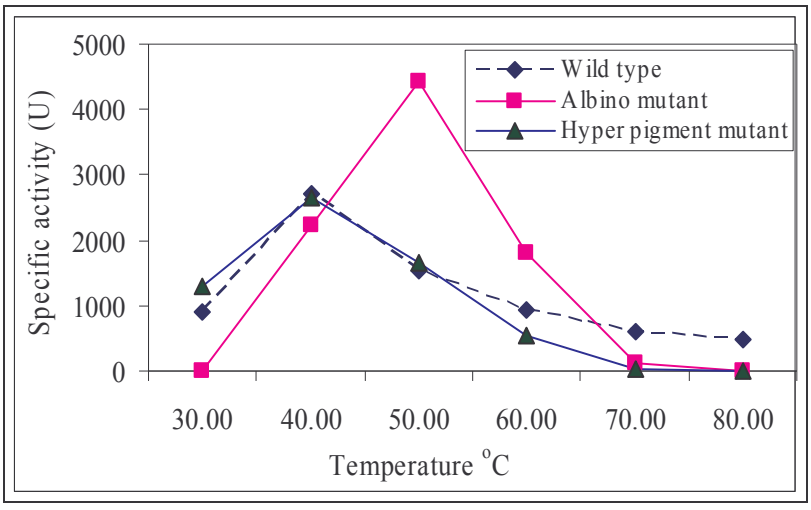


Fig 34. Effect of temperature on *M. purpureus* amylase activity

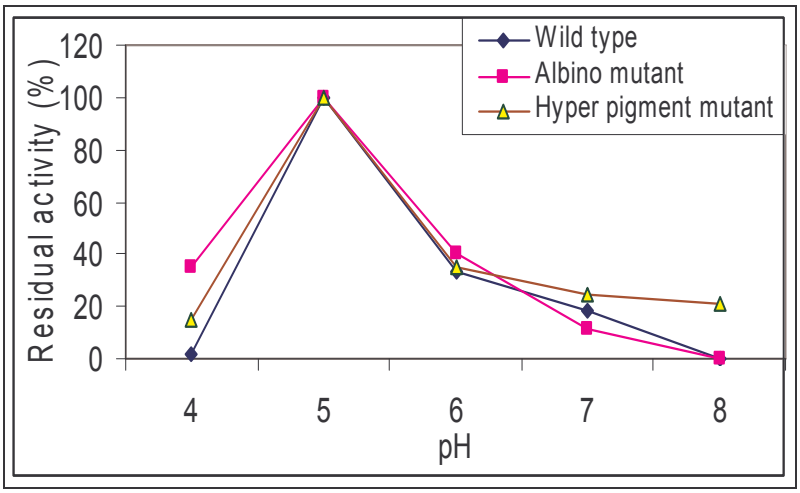


Fig 35. Effect of pH on *M. purpureus* amylase activity

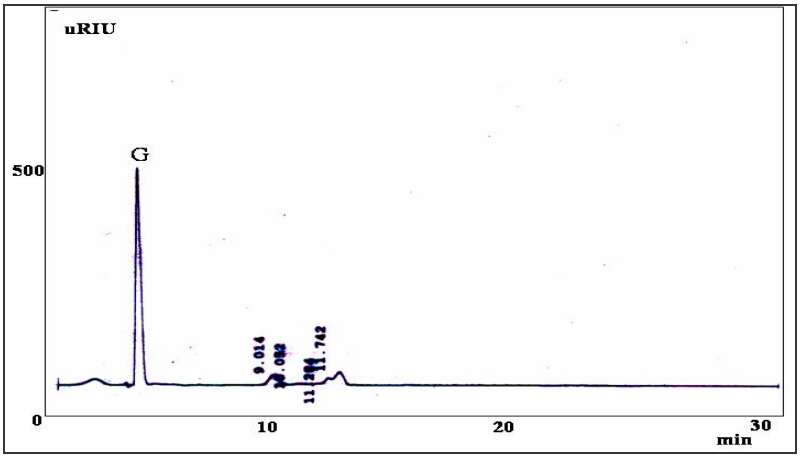


Fig 36. HPLC analysis of enzyme hydrolyzed starch (G: glucose)

EXPERIMENT 18: ACID PROTEASE OF *M. PURPUREUS*

Acid proteases have several biological functions. The organism produces the enzyme for utilizing proteins as nitrogen source during fermentation. Two extracellular proteases, an acid protease and a carboxy peptidase have been characterized from *M. purpureus* (Tsai *et al.* 1978; Yasuda *et al.* 1984; Liu *et al.* 2004). Since these enzymes have a role in the growth physiology of the organism, production of acid protease by *M. purpureus* and its mutants were studied.

The growth conditions for solid state fermentation on rice and methods to determine enzyme activity have been described in Materials and Methods.

The albino mutant produced large quantities of acid protease compared to the wild type. Least acid protease production was observed in the growing cultures of hyper pigment mutant (**Fig 37**)

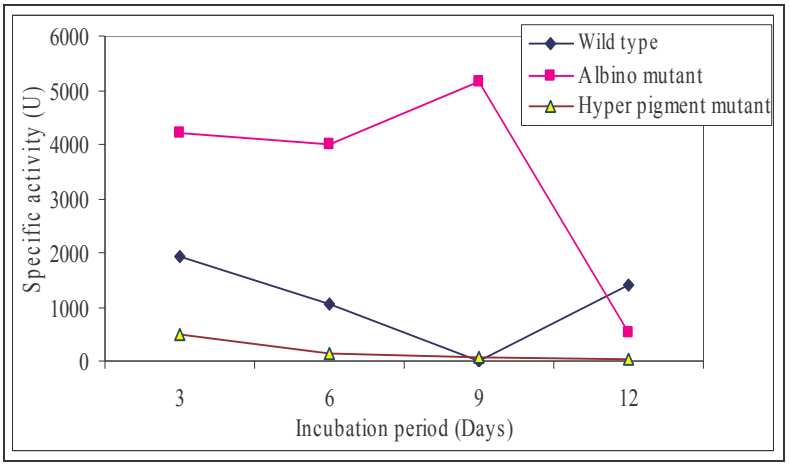


Fig 37. Acid protease production by *M. purpureus* during solid state fermentation on rice

The results suggested a relation between acid protease production and pigmentation in *M. purpureus*

EXPERIMENT 19: AMIDASES OF *M. PURPUREUS*

Schiff's base reaction caused by ammonia has been related to red pigment synthesis by *M. purpureus* (Juzlova P. 1996b). Since L-asparaginase and L-glutaminase can cause the release of ammonia from the corresponding amino acids, the activities of these enzymes were determined in the study.

L-asparaginase and L-glutaminase assays were carried out on plates and enzyme production was visualized as changes in pH of the culture medium identified as change in colour using phenol red indicator (Gulati *et al.* 1997). Enzyme activities were also

assayed using extracts of rice prepared from *Monascus* grown cultures. The details are described in Materials and Methods.

Rapid plate assays showed more L-asparaginase production by hyper pigmentation mutant compared to the Wild type. Very little L-asparaginase activity was visualized in albino mutant during its growth (**Fig 38 C**). Though assays confirmed the above results, the 12 day cultures of albino mutant showed the production of high quantities L-asparaginase. Hyper pigmentation mutant produced the enzyme between third and sixth day of growth unlike the wild type and the albino mutant (**Table 13**). L-glutaminase activity when assayed, it was found that the enzyme was secreted during the early growth phases of the fungi and its production was highest in hyper pigment mutant (**Table 13**).

The results showed that more pigment synthesis occurred in hyper pigmentation mutant due to amidase production during early growth phase.

Table 13. L-asparginase and L-glutaminase activities during solid state fermentation (U)

Strain	L-asparginase				L-glutaminase			
	Time (Days)				Time (Days)			
	3	6	9	12	3	6	9	12
Wild type	0.00	0.00	0.00	85.02	371.60	0.00	0.00	70.85
Albino	0.00	0.00	50.86	777.40	371.60	0.00	0.00	70.85
Hyper	0.00	78.63	0.00	0.00	862.89	78.63	0.00	0.00

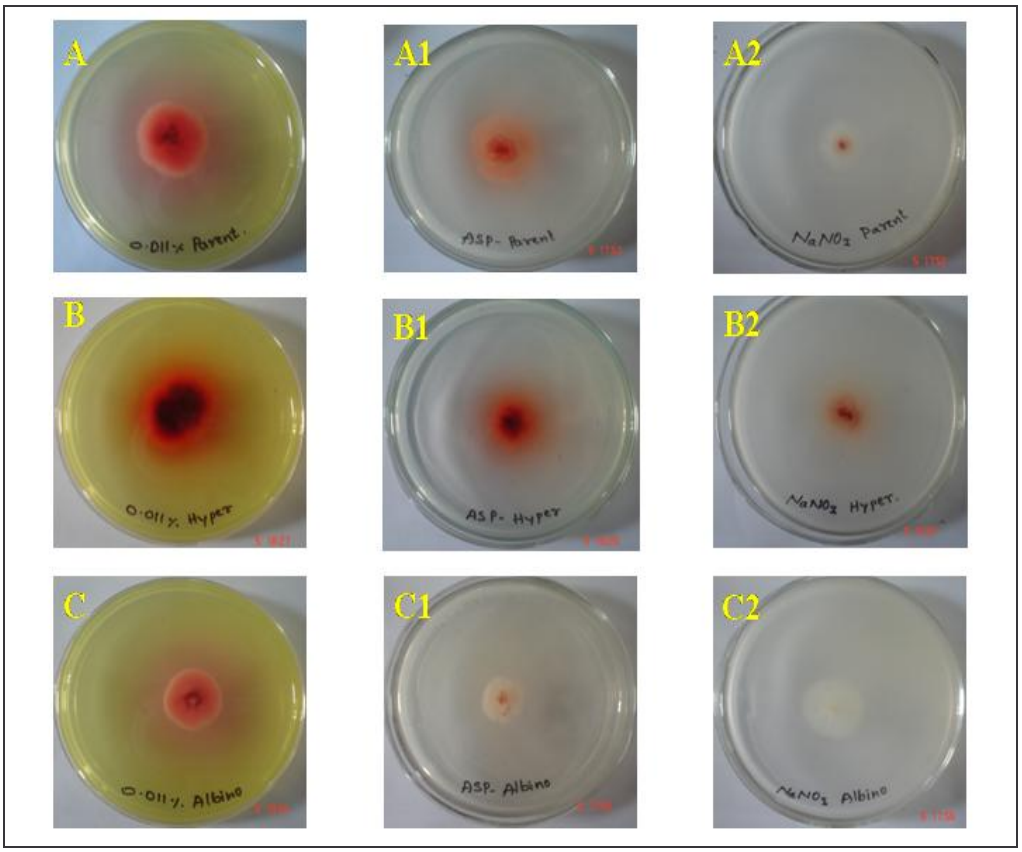


Fig 38. Rapid plate assay to determine L-asparaginase activity in *M. purpureus.* A. wild type B. albino mutant and C. hyper pigmentation mutant. The cultures were grown on L-asparagine medium containing phenol red (0.011%)

A, B and C test plate

A1, B1 and C1 medium containing phenol red (Control)

A2, B2 and C2 grown on media containing $NaNO_3$ (Control)

EXPERIMENT 20: CITRININ PRODUCTION BY *M. PURPUREUS*

Citrinin is a nephro and hepato toxin produced by several *Monascus* sp (Blanc *et al.* 1995; Monica *et al.* 1999). Hence food use of *M. purpureus* requires strains that do not produce the toxin. Even though strain characterized for production of bioactive molecules like dihydromonacolin-MV and dehydromonacolin-MV2 by *M. purpureus*, the evaluation for toxin production is important for its use in the preparation of red rice.

Studies were carried out to identify the presence of toxin in the rice fermented by *M. purpureus*.

Tests were carried out to identify citrinin in the polar and non-polar solvent extracts of *M. purpureus* fermented rice. The detection procedure is described in Materials and Methods.

Citrine could not be detected in any of the extracts by thin layer chromatography. Hence the *M. purpureus* used in the study was found to be safe for food use.

DISCUSSION

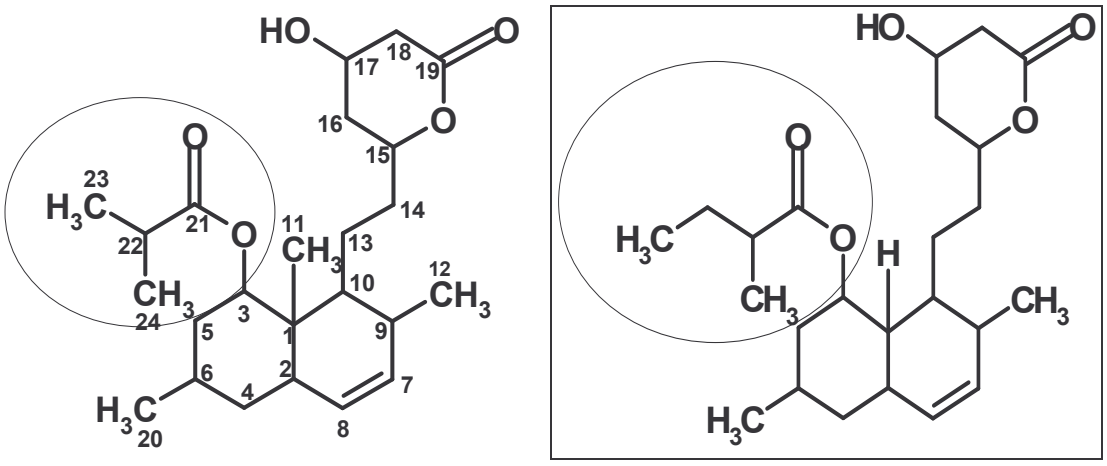
Extensive biochemical, physiological and taxonomical studies of *Monascus* sp revealed that the fungus produces pigments (polyketides) ranging from bright yellow to deep red (Carels and Shepherd 1975 and 1977; Wong and Bau 1977; Shin *et al.* 1998). The results of these studied showed that the fungus is safe (Lee *et al.* 2005) for food application and the pigments had several bioactive properties (Martinkova *et al.* 1999; Manzoni and Rollini 2002; Su *et al.* 2003; Li *et al.* 2005; Lin *et al.* 2006). Fermentation studies for pigment production resulted in the observation that more polyketides are produced by the fungus in solid state cultures (rice grains) than in shake flasks (Chen and Johns 1993; Soccol and Vandenberghe 2003). Intracellular retention of the pigment, inhibiting their further synthesis (Lin 1973; Lee *et al.* 1995) was reasoned for the cause for low pigment yields during submerged growth. Lin and Demain (1991) showed that the attachment of the fungus to rice grains is important for higher productivity in solid state cultures (Johns and Stuart 1991).

Over the last two decades, the interest for new natural pigments has been growing due to their enormous use in the food industry. Thus *Monascus* pigments became a choice to replace traditional additives such as ammonium nitrites, potassium nitrates and potassium nitrites (Lin and Demain 1991 and 1995; Fabre *et al.* 1993; Hajjaj *et al.* 1997 and 2000b). The growing interest also resulted in the identification of newer metabolites from *Monascus purpureus* for application as drugs (Manzoni and Rollini 2002; Lee *et al.* 2005; Li *et al.* 2005 and 2006; Lin *et al.* 2005).

Monascus purpureus (MTCC-410) when cultured on rice produced polyketides characteristic of the fungus (**Fig 5**). Cleistothesia and ascospores identified in the colony of the growing fungus (**Fig 4**) confirmed its taxonomical status. The characteristic feature of this strain was its ability to produce a monacolin not identified previously (**Experiment 6**). The monacolin or statin characterized from *M. purpureus* and *Aspergillus terreus* have been shown to inhibit the enzyme HMG Co-A reductase (Endo 1980 and 1985a; Albers-Schonberg *et al.* 1981; Istvan and Deisenhofer 2000 and 2001),

that has several implication in atherosclerosis (Li *et al.* 1998; Rosenson and Tangney 1998; Wang *et al.* 2000; Hsieh and Tai 2003; Journoud and Jones 2004; Rosenson 2004; Lee *et al.* 2006). Dihydromonacolin-MV identified as a new metabolite in this study (Dhale *et al.* 2007a) caused lipid peroxidation inhibition and scavenged super oxide radicals (**Table 6**). This bioactive substance was identified as DPPH radical scavenger in the crude methanol (**Table 4 and 6**) extracts prepared from *M. purpureus* fermented rice.

Proton, carbon and 2D-HSQCT assignments (**Table 5**) based on NMR spectra (**Fig 13 to 17**) obtained from purified active compound, identified dihydromonacolin-MV (**Fig 18**) as a derivative of dihydromonacolin-K. The two were differentiated based on the presence of functional groups. The 2-methyl-propionate as ester group is present at position 3 in dihydromonacolin-MV unlike dihydromonacolin-K which contained 2-methyl-butyrate at this position (**Fig 39).**



A B

Fig 39 Schematic representation of dihydromonacolin-MV (A) and dihydromonacolin-K (B). Circled region showing the variation in the two compounds

BIOACTIVE PROPERTIES OF DIHYDROMONACOLIN-MV

The compound is a potent DPPH radical scavenger (**Table 6**) with a capacity to abstract the liable hydrogen atoms. It strongly inhibited the peroxidation of lipids and inhibited the formation of super-oxide, the most important causative agent that initiates free radicals *in-vivo* during electron chain transfer. Ability of dihydromonacolin-MV to

attenuate these radicals, gave it an antioxidant property. Atherosclerosis is characterized by the accumulation of cholesterol, lipid peroxide and oxysterols in the arterial wall and is the main cause of heart attack and stroke (Vaya and Aviram 2001). The property of dihydromonacolin-MV to chelate metal ion, inhibited oxidation of lipid by breaking the chain reaction due to a Fe^{+3}. The activity of lipid peroxidation inhibition along with its ability to scavenge free radicals showed *M. purpureus* as an excellent source of compounds for use to prevent atherosclerosis.

ANOTHER BIOACTIVE MOLECULE DEHYDROMONACOLIN-MV2 PRODUCED BY *M. PURPUREUS*

Though our effort to obtain a strain of *M. purpureus* for increased production of dihydromonacolin-MV by mutation was futile, a mutant producing an antibacterial metabolite called dehydromonacolin-MV2 (**Fig 30**) was isolated (**Experimental Results Chapter II**). Spectroscopic characterization (**Fig 24 to 29**) of this compound suggested that it arose as a variant of monacolin-J. Since this metabolite was not identified in the rice fermented with the wild type culture, apparently the compound arose due to an aberration (mutation) in the enzyme that synthesized monacolins. The pathway hypothesized (**Fig 40**) for its evolution is described below.

Monacolins like the *Monascus* polyketides are produced by polyketide synthase (PKS) catalysis. PKS determines the size of the polyketide carbon chains and sequence steps. In lovastatin/ dihydromonacolin L pathway, the nonaketide carbon chain derived from one acetate and eight malonate molecules under go electrocyclic cyclization (Diels-Alder cyclization) catalyzed by lovastatin nonaketide synthase to form dihydromonacolin L (Auclair *et al.* 2000). The *lovC* protein, an accessory enzyme that complexes to *lovB* protein (lovastatin nonaketide synthase), imparts enoyl reductase activity necessary for successful assembly of the normal PKS product an established intermediate of lovastatin biosynthesis (Hutchinson *et al.* 2000) called dihydromonacolin L (Kennedy *et al.* 1999). Endo *et al* (1985a & b and 1986) reported that dihydromonacolin L spontaneously gives rise to 3α-hydroxy-3, 5-dihydromonacolin L and its dehydrated form monacolin L. Hydroxylation of monacolin L at C8 by molecular oxygen results in the formation of

monacolin J. Oxidation at position C8 probably resulted in the formation of double bond between C5 and C8 in monacolin-J as shown by UV λ max determined at 329 nm (**Fig 27**). This suggested that a hydroxylation and oxidation at C3 and C8 in monacolin J resulted in the formation of dehydromonacolin-MV2 (**Fig 30**).

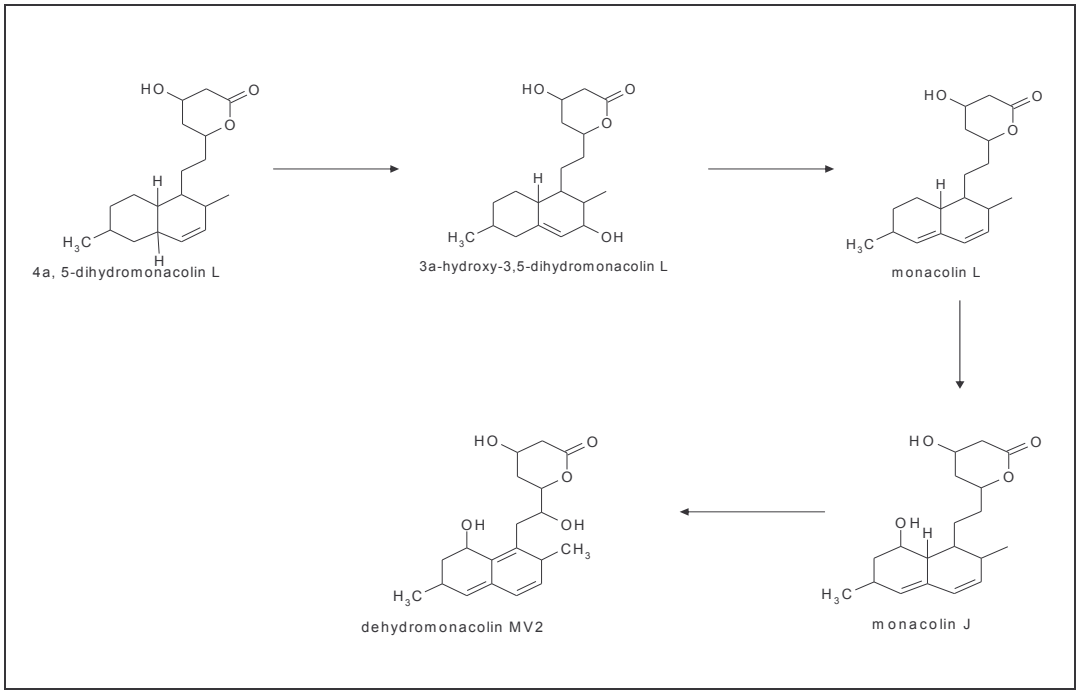


Fig 40. Hypothetical pathway of dehydromonacolin-MV2 biosynthesis in *M. purpureus* mutant

PHYSILOGY OF *M. PURPUREUS* IN RELATION TO PIGMENT PRODUCTION

Though pigmentation is a characteristic property of *M. purpureus,* many strains characterized have shown varied pigment production (Broder and Koehler 1980; Chen and Johns 1993; Hamdi *et al.* 1997; Blanc *et al.* 1999; Krairak *et al.* 2000; Campoy *et al.* 2006). It is believed that the complexity in the biosynthesis of *M. purpureus* pigments is dependent on the physiology of the fungus reflected by growth conditions (Lin and Demain 1991; Suh and Shin 2000; Tseng *et al.* 2000; Schneweis *et al.* 2001). A mutant of *M. purpureus* isolated by temperature selection after UV treatment demonstrated a characteristic albino phenotypy on PDA plates (**Fig 19 C and 21**). However, when fermenting rice, this mutant produced a low quantity of the pigment (**Table 7**). Such a

variation was not obtained in the case of hyper pigmentation mutant (**Fig 19 B, 20 and Table 7**). Hence the above variation could be attributed to the growth conditions, specifically extracellular enzymes likely amylase, acid protease and amidases that were produced for substrate utilization.

Alcohol production by *M. purpureus* and its non-assimilation has been reasoned as a factor responsible for the inhibition of the pigment (Yongsmith *et al.* 1993 and 1994; Blanc *et al.* 1999; Rasheva *et al.* 2003). Thus the absence of pigmentation during the growth of albino mutant on PDA (**Fig 19 C**) can be reasoned to alcohol production from glucose, a constituent of the medium. Since no glucose is present in solid state substrate made of rice, low pigment yields occurred during initial growth until amylase secretion followed. The activity that converted the starch to glucose inhibited subsequent pigment synthesis due to the production of alcohol from glucose (**Fig 34**). Thus the mutation which altered the growth physiology of albino mutant, indirectly contributed to reduced polyketide synthesis and made the inhibitory effects of alcohol obvious. On the other hand, the hyper pigmentation strain (**Fig 19 B and 20**) produced more polyketides (**Table 7**) due to a mutation that affected the ability of the fungus to utilize the alcohol.

Identification of L-asparaginase and L-glutaminase activities in the extracellular fluids of *M. pupureus* suggested their role in the production of red pigment (**Table 13**). These amidase upon synthesis caused a Schiff's base reaction of orange pigment (precursor) in order to change it to red coloured polyketide. Ammonia released due to the reactivity of the amidase on the amino acids, L-asparaine and L-glutamine seems to have caused a Schiff's base reaction in the primary orange coloured monascorubrine and rubropunctatin (Haws *et al.* 1959) converting them to red coloured monascorubramine (Hiroi *et al.* 1975) and rubropucntamine (Fowell *et al.* 1956).

In relation to amidase activity described above, protease activity is important in the purview of pigment production by *M. purpureus*, since free aspartate and glutamate are needed for Schiff's base reaction. As expected, the organism and its mutants excreted protease that released free amino acids from casein in the assay (Materials and Methods).

An interesting observation made in the study on enzymes, is the similarity of amylase proteins of *M. purpureus* and *A. niger*. In the western blot reaction (**Fig 33**), antibodies raised to the *A. niger* amylase identified the *M. purpureus* enzyme as a 90 kDa protein. Since the protein is synthesized during solid state growth, higher molecular mass amylase in the case of *M. purpureus* may have arisen due to increased glycosylation. Western blot reactions (**Fig 33**) also showed reduced reactivity of *A. niger* antibody to the enzyme produced by the albino mutant. Probably the mutation affected amylase genome, enhancing its productivity to over come reduced substrate affinity as evidenced by variation in pH optimum as compared to that of the enzyme produced by the wild type (**Fig 35**).

APPLICATION OF *M. PURPUREUS* AS FUNCTIONAL FOOD

M. purpureus is a fungus traditionally used in food items in South China, Taiwan, Japan, Indonesia and other Oriental Countries. The red mold rice, red bean curd and red rice wine consumed directly, have been shown to contain several health benefits (Hsieh and Tai 2003; Cicero *et al.* 2005; Lee *et al.* 2005; Lin *et al.* 2005). Since monacolins, dihydromonacolin-MV and dehydromonacolin-MV2 with bioactive properties are produced by the wild type and the mutant during growth on rice, they can be directly consumed as health food. This showed their application as functional food. Red rice prepared with *M. purpureus* when consumed can prevent cancer due to the inherant antioxidant properties associated with the new monacolins. L-asparaginase and L-glutaminase have received significant attention owing to their potential as anticancer agents (Roberts *et al.* 1972; Fu *et al.* 1998; Medina 2001). Identification of the production of these enzymes during rice fermentation apart from increasing the functional food value, also makes it safe, since the fungus and its mutants did not produced citrinin (**Expt 20**), a nephro and hepatotoxic metabolite known of *M. purpureus*. An additional aspect of foods prepared with the *M. purpureus* is the improvement of the quality by imparting a desired flavour due to amidases. Similarity of the amylase with *A. niger* enzyme showed that the food is also easily digestible upon consumption.

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