DECLARATION

I hereby declare that, the thesis entitled **"*Monascus purpureus* in relation to statin and sterol production and mutational analysis"** submitted to the University of Mysore for the award of Doctor of Philosophy in Biotechnology is the result of research work carried out by me in the Department of Food Microbiology, Central Food Technological Research Institute, Mysore, under the guidance of **Dr. G. Vijayalakshmi,** during the period of June 2004 to June 2009.

I further declare that the results of this work have not been previously submitted for any degree or fellowship.

Place: Mysore

Date: 21st Dec 2009

(H.P. Mohan Kumari)

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Mohan Kumari. H.P.

DEDICATED TO MY PARENTS AND

FAMILY MEMBERS

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ABSTRACT

Fermented products of *Monascus purpureus* are used as traditional natural dietary supplement in China. It contains monacolins, sterols and unsaturated fatty acids and believed to decrease serum lipids such as triglycerides and cholesterol.

M. purpureus and mutants isolated in this study were found to produce various bioactive secondary metabolites like statins, sterols and fatty acids during growth. The metabolites were characterized after preparatory TLC and HPLC. The fermentation conditions were optimised for metabolite production. In solid-state cultures, more quantities of the metabolites were quantitated. The fungi accumulate sterols mainly in shake flask growth.

Cultural conditions have a significant influence on the yield of statins. The fungus grown at pH 5.0 at 28º C in a medium made of maltose and peptone as carbon and nitrogen sources resulted in maximum statin production. Among the two statins produced by *M. purpureus* and mutants lovastatin (Monacolin K) was produced in significant amount. The antioxidant molecule ankaflavin was also characterized by ^{1}H, ^{13}C, 2D HSQC and HMQC NMR, FTIR, MS and UV-visible spectroscopic studies.

L-asparaginase and L-glutaminase activities were comparatively more in the mutant CFR 410-11 compared to the wild type MTCC 410 and the mutant CFR 410-22. CFR 410-11 secreted more red pigment when cultured on rice and in broth. Significant difference in L-asparaginase and L-glutaminase activities in MTCC 410, CFR 410-11 and CFR 410-22 revealed the importance of these enzymes in pigment production.

Characterization of compounds that showed applications as functional food resulted in the safety evaluation of *M. purpureus.* Only low levels of the nephrotocin citrinin were estimated in rice fermented by *Monascus* (RMR). Feeding acute doses of RMR to rats did not cause any symptoms of toxicity or mortality. Similarly, dietary

feeding of RMR to rats did not produce any significant changes in food intake or gain in body weight of the experimental animals and they were comparable to the rats fed with normal diet (control). No significant differences in the relative weight of vital organs, hematological parameters, macroscopic and microscopic changes in vital organs and serum clinical enzyme levels between the experimental and control groups were determined.

RMR containing lovastatin fed at a concentration of 11.51g/ kg fermented rice reduced total cholesterol (TC), triglyceride (TG), lipoprotein cholesterol (LDL) in high fat diet (HFD) fed rats. The results were similar with reference to serum and liver. There was no significant difference with regard to the food intake, gain in body weight and organ weights of rats in different dietary groups. In addition, histological examinations of liver of hyperlipidemic rats showed decreased lipid accumulation in red mould rice powder fed rats.

RMR effectively scavenged 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radicals (IC_{50}=100μg/ml). RMR affected oxidative stress in male albino rats fed on high fat diet (HFD) by increasing activities of glutathione reductase, glutathione peroxidase, superoxide dismutase and Catalase. It also increases total thiols, glutathione and ascorbic acid (antioxidants) in rats. Lipid peroxidation was significantly inhibited in rats fed with RMR compared to rats fed with HFD. Consumption of RMR by rats induced antioxidant enzymes and molecules to scavenge the reactive oxygen species (ROS) released due to oxidative stress in rats fed on HFD.

FOREWORD

Monascus purpureus during growth on rice medium produces red coloured pigments. Due to this property, it has been called as Red mould rice (RMR). RMR has been used in food and folk medicine for thousands of years in China and Asian nations. Its special effects and application on food have been recorded in ancient Chinese records.

The types of secondary metabolites produced from the *Monascus* species include a group of pigments, antihypercholesterolemic molecules monacolins, hypotensive agent, γ-aminobutyric acid (GABA), antioxidant compounds like, dimerumic acid and dihydromonacolin-MV and antibacterial molecules. Citrinin content of *Monascus* is a predicament to complete application of its use. Monacolin K competitively affects the early critical enzyme of the mevalonate pathway, thus inhibiting the synthesis of cholesterol and other sterol end products. Despite, the considerable literature that has characterized the metabolites, a comparision fungal metabolite production in relation to amidase enzymes and application of metabolites is still scarce.

This thesis describes the secondary metabolites produced by *Monascus purpureus* MTCC 410 and its muatnts, safety and hypolipidemic evaluation of RMR and effect of RMR extracts on beneficial organism like probiotics.

INTRODUCTION

The health benefits of Anka (also called as anka-kak) or red mould rice were published in first Century in Chinese medical book. Red mould rice (RMR) prepared from *Monascus* (also known as red fermented rice [RFR] or red yeast rice [RYR]), is used in many Chinese processed foods and is used for red color enhancement and nutraceutical supplements at least for more than thousands of years. However, the formal written records were not unveiled until two pharmacopoeias were published in the Post-Han and Yuan Dynasties, which first described the medicinal functions of RMR (Bau, 1996; Su, 2001). Later literature documented the use of red colour obtained from anka for colouring foods.

A microbiological study of red molds was first conducted in 1884 by a French microbiologist, van Tieghem and was categorized as the genus *Monascus* (Huang, 1985; Su *et al.*, 1970). Many species of filamentous fungi were isolated with similar morphological and physiological characteristics and since then, named after different kinds of products. The most widely used red mold species in Taiwan was first named as *Monascus anka* in 1931 by two Japanese Misawa and Sato (Su *et al.*, 1970).

CLASSIFICATION AND HABITAT

Kingdom: Fungi

Phylum: Ascomycota

Class: Ascomycetes

Order: Eurotiales

Family: Monascaceae

Genus: *Monascus*

Species: *purpureus*

M. purpureus exist widely in soil, fresh graze, grain, rubber, dried fish and surface sediments of river and root organ of pine tree.

REPRODUCTION IN *MONASCUS PURPUREUS*

M. purpureus, a homothallic fungus is able to produce spores asexually as well as sexually. It was clearly recognizable that meiotic (sexual) spore formation was accompanied by the asexual reproduction process.

Like other Ascomycete's, the genus *Monascus* also reproduces sexually. After the isolation of first species by (Van Tieghem, 1884), a long debate followed concerning the nomenclature and sequence of the different stages of the sexual cycle. The principle investigators (Went, 1895; Barker, 1903; Kuyper, 1905) have depicted different interpretations for the sequence of sexual cycle. The interpretation proposed by (Young, 1931) has virtually remained unchallenged.

An antheridium arises at a hyphal tip by septation. A protuberance grows out from the cell beneath the septum and becomes the female organ. The two organs grow towards each other, the female organ pushing the antheridium to one side, becoming the main growing point. A septum separates the female organ into ascogonium and trichogyne. Male nuclei migrate from the antheridium via 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 and rapidly form a protective rind. Ascogenous hyphae arise near the base of' the ascogonium; they grow round and lie close to the surface of the ascogonium inside the protective rind. The ascogenous hyphae become divided into cells, each containing a male and female nucleus. Asci are formed, then the two nuclei fuse, and a meiotic, followed by a mitotic division results 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. A stalked cleistothecium, ascocarp, breaks open, releasing the ascospores, which germinate to give a new hypha **(Figure 1)**.

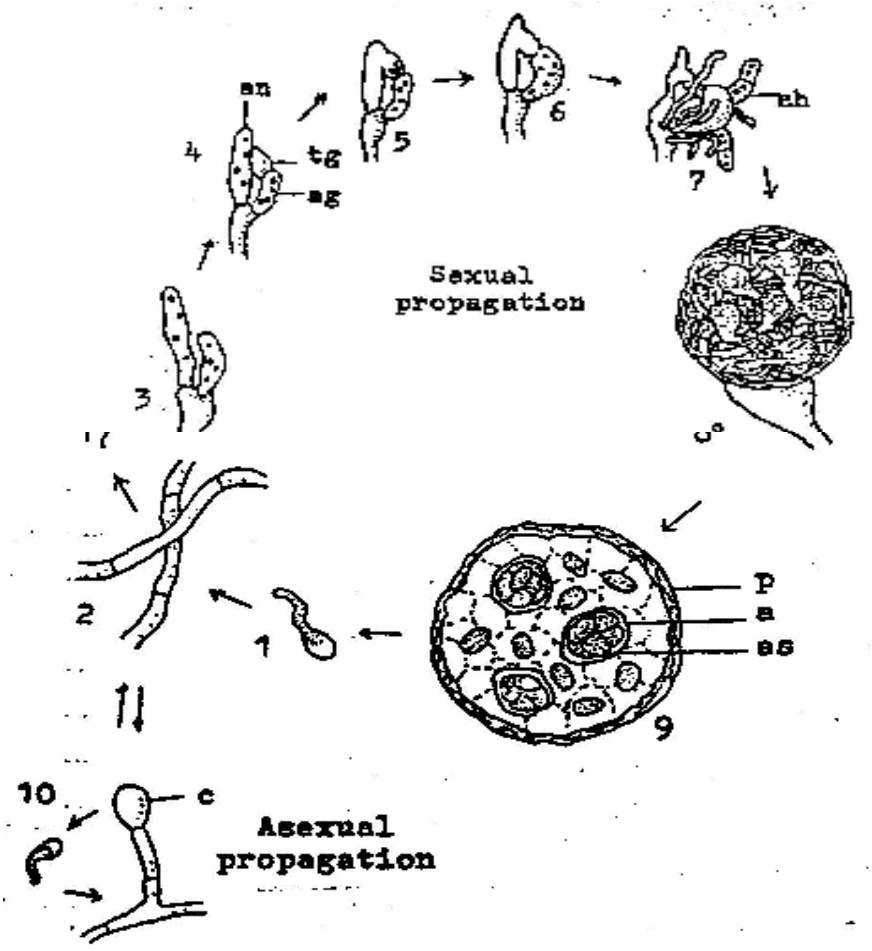


Figure 1. Asexual and sexual reproduction in *M. purpureus.* (P; perithecium, a; ascus, as; ascospores, ag; ascogonium and tg; trichogyne, ah; ascogenous hyphae, an; antheridium c; cleistothecium).

STRAINS OF *MONASCUS*

The majority of *Monascus* sp were isolated from several oriental foods. The species thus far characterized are mentioned in **Table 1**. Among these species, *M.*

purpureus, *M. anka* and *M. ruber* are most often used for research and industrial production of RMR. Monacolins, GABA, flavonoids, citrinin and pigments (red, orange and yellow), etc., are produced by this genus as secondary metabolites and production capacities of these metabolites varies from strain to strain. However, their capacities to produce monacolin K, GABA, and red pigments are the major concerns (Su, 2001) of research.

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*

PRIMARY AND SECONDARY METABOLIC PRODUCTS

Natural products continue to serve the pharmaceutical sciences as an abundant source of new drug leads. The ingenuity of natural product assembly and their structural complexity inspires chemists, the bioactivities of natural products fascinate pharmacists, and their relevance in ecosystems, if known at all, intrigues biologists, thus making natural product research truly and intrinsically interdisciplinary. In past, much effort was directed toward optimizing the production of those drugs which have already been introduced into clinical practice. Researchers primarily relied on classical approaches like chemical or UV-mutagenesis or varied fermentation parameters.

Many microorganisms produce a wide range of natural products as primary and 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 & Berry, 1975). Biosynthesis of secondary metabolites is usually associated with cell differentiation or development and most of the secondary metabolites are produced by organisms that exhibit filamentous growth with relatively complex morphology (Bu'Lock, 1961).

Fermentative production of these metabolites is energy efficient and environmentally agreeable (Nagasawa & 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 & Yamada, 1995).

The genus *Monascus* grown in glucose containing medium as source of carbon produces succinic acid, citric acid, gluconic acid, oxalic acid and ethanol as a primary metabolites and pigments, statins, citrinin as secondary metabolites (Su *et al.*, 1970).

SECONDARY METABOLITES OF *MONASCUS PURPUREUS*

The genus *Monascus* is known to produce various secondary metabolites of polyketide origin. RMR produced from solid-state fermentation of cooked rice with *Monascus* sp, also contains many high value secondary metabolite, such as monacolin K (Ma *et al.*, 2000), citrinin (Lee *et al.*, 2007), γ-aminobutyric acid (GABA), natural red pigment, angiotensin I-converting enzyme inhibitory peptides (Kuba *et al.*, 2009), antioxidant compound (Dhale *et al.*, 2007a) and other unidentified active components (Aniya *et al.*, 2000; Blanc *et al.*, 1995b; Su *et al.*, 2003). RMR also contains useful sterols like β-sitosterol, campesterol, stigmasterol, saponin and sapogenin; isoflavones and isoflavone glycoside; selenium and zinc; (Heber *et al.*, 1999; Ma *et al.*, 2000; Wang *et al.*, 1997). The pigments produced by *Monascus* have traditionally been used in the processing of alcoholic beverages, red soybean curd, meat and vegetables in Asian countries. The molecular structures of six major pigments of *Monascus* have been reported (**Figure 2)** in literature.

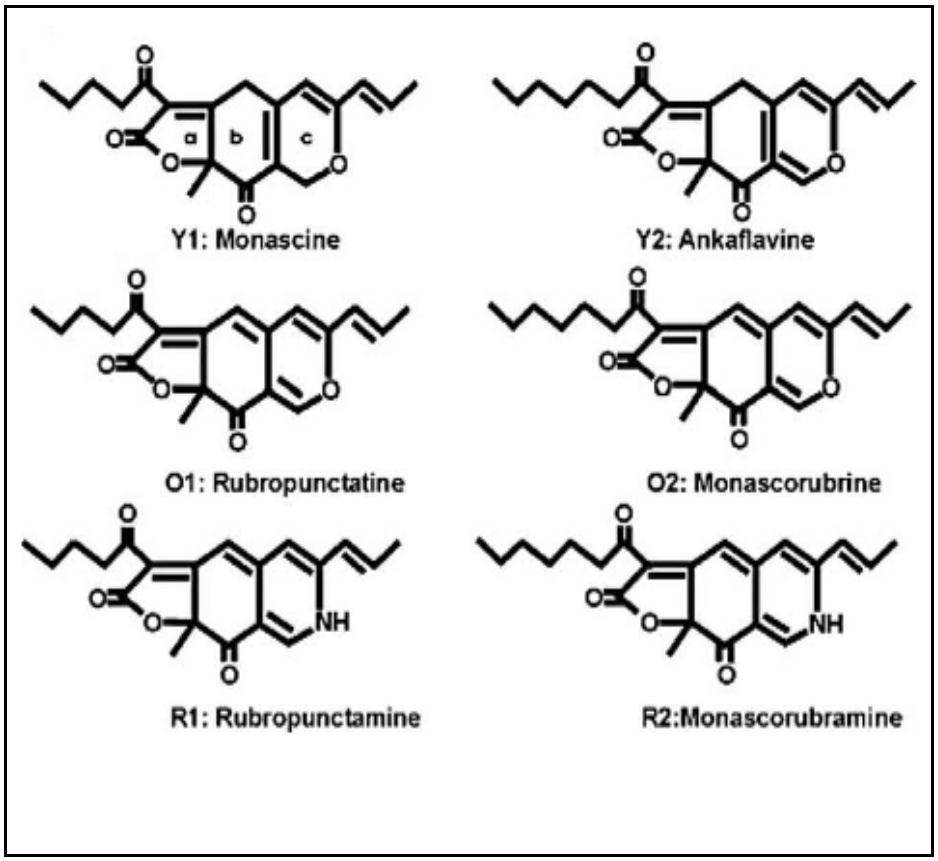


Figure 2. Major pigments of *Monascus* sp. Yellow: Monascine (Y1, M-358) and Ankaflavin (Y2, M-386), Orange: Rubropunctatine (O1, M-354) and Monascorubrine (O2, M-382), Red: Rubropunctamine (R1 M-353) and Monascorubramine (R2, M-381) (Campoy et al. 2006)

These secondary metabolites of *Monascus* are medically proven to possess anticholesterol, anticarcinogenic activities (Endo 1979, 1980; Hawksworth and Pitt 1983; Lee et al. 2005), and antifatigue activities (Wang et al. 2005). Fermentation of *Monascus* sp supplemented with diascoria, ginger and curcumin have shown increased antihypertensive, antioxidant, antihyperlipidemic activities (Kuo *et al.*, 2009b; Lee *et al.*, 2008). The Chinese ancient pharmacopoeia, Ben Tsao Gum Mu, indicates the use of RMR to promote the health of the cardiovascular systems (Su, 2001).

The orange pigments, monascorubrin and rubropunctatin, are synthesized from acetyl coenzyme A by polyketide synthase-I in the cytosol (**Figure 3**). Reactions with amino acids give rise to the water-soluble red pigments, monascorubramine (Fowell *et al.*, 1956) and rubropunctamine (Hadfield *et al.*, 1967). Adlay angkak a newly developed product from an adlay substrate fermented by *Monascus* can be used as a natural coloring and a dietary supplement. It was not only produced useful secondary metabolites such as mevinolin and pigments but also produced citrinin (Pattanagul *et al.*, 2008).

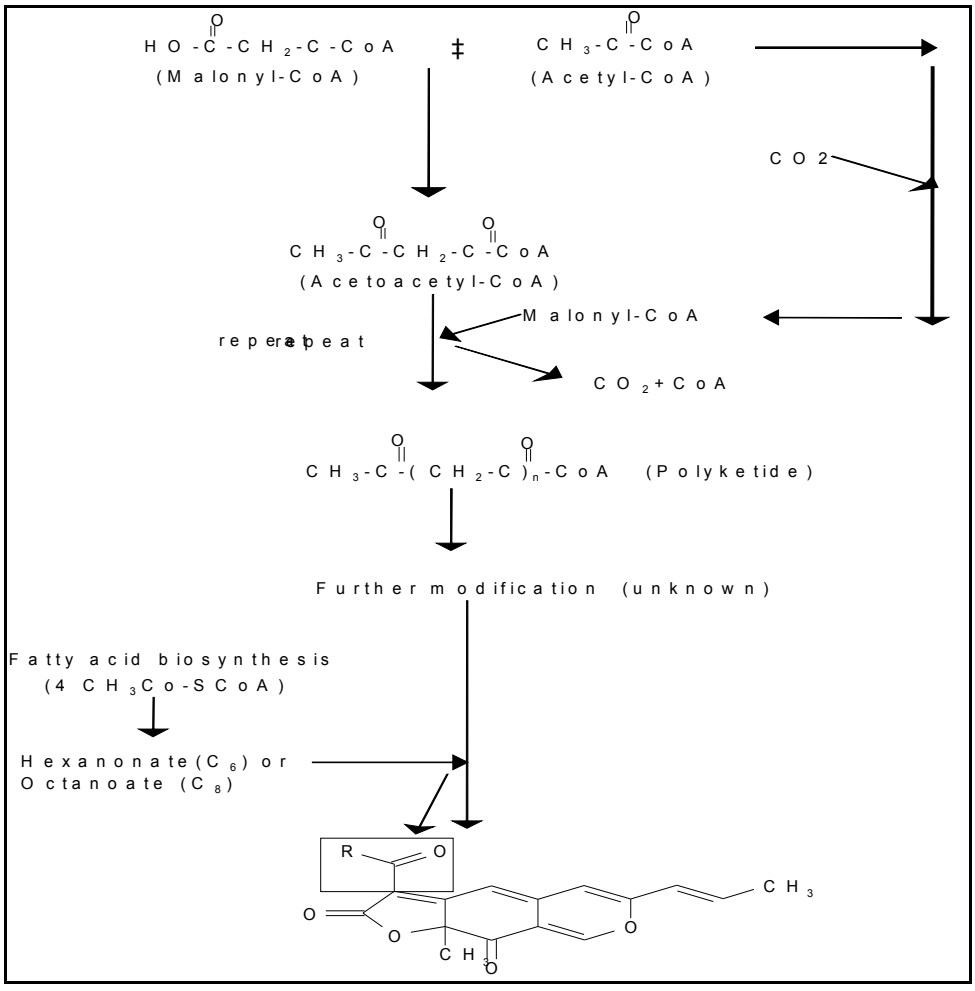


Figure 3. Biosynthesis of orange pigments in *Monascus* (Juzlova *et al.*, 1996a)

Two new *Monascus* metabolites with similar strong blue fluorescent spectra (λem=396 nm, *λ*em=460 nm) and UV absorption spectra (*λ*max=386 nm) detected were named as monasfluore A and monasfluore B. Theses metabolites contain a alkyl side chain, *γ*-lactone, and propenyl group, whereas, the more lipophilic compound, monasfluore B, is a higher homologue of monasfluore A, with the more lipophilic octanoyl instead of the hexanoyl side chain (Huang *et al.*, 2008).

The amidase enzymes secreted by this fungus hydrolyze the amino acid releasing ammonia and respective acid as product. The released ammonia reacts (**Figure 4**) with orange pigment to produce respective red pigments (Dhale *et al.*, 2009). Reductions of orange derivatives produce the yellow pigments monascin and ankafavin (Kurono *et al.*, 1963; Manchand *et al.*, 1973; Tseng *et al.*, 2000). Moreover, a yellowish colorant named Xanthomonasin A in the mutant of *M. anka* was identified (Martinkova *et al.*, 1999). The content of *Monascus* pigment varies according to culture condition, pH, humidity, oxygen supply and nutrients (Eisenbrand, 2006).

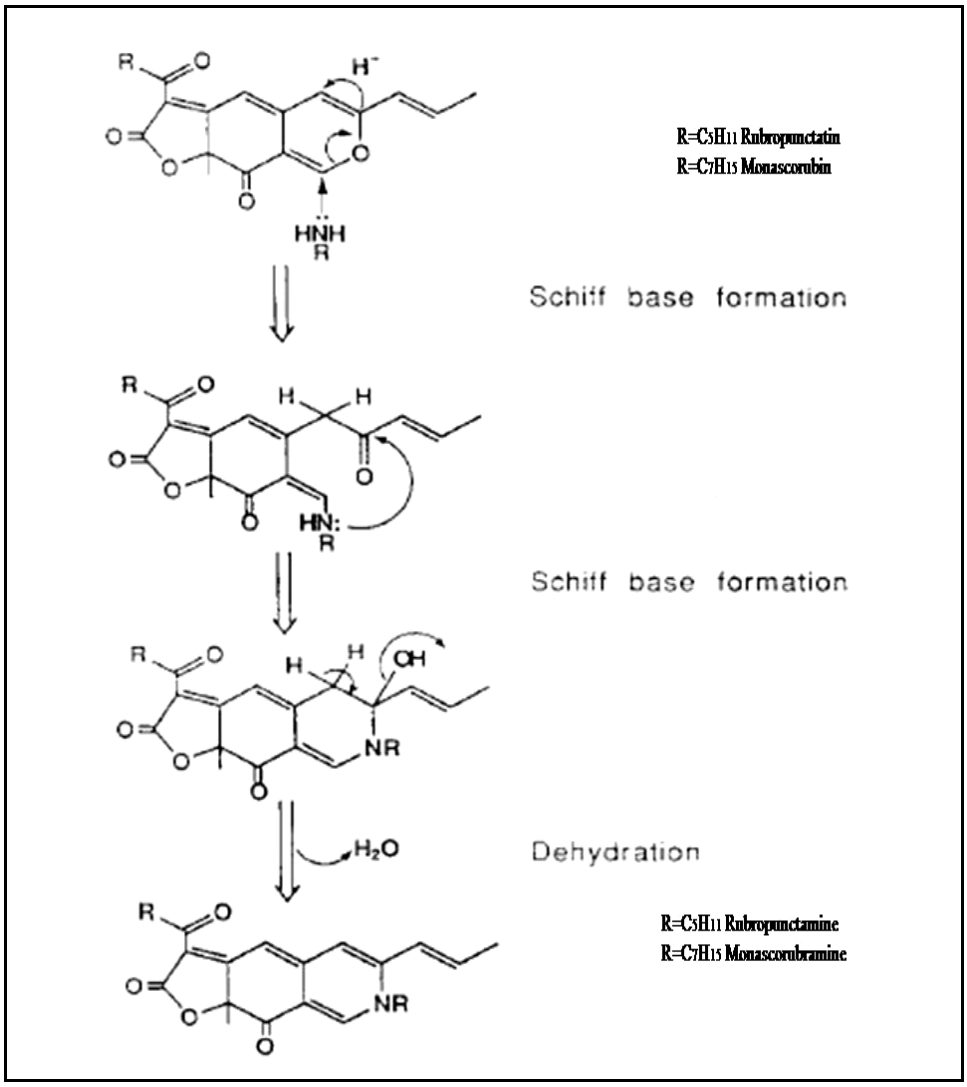


Figure 4. Formation of red pigments by Schiff's base reaction (Juzlova *et al.*, 1996a)

CULTIVATION OF *M. PURPUREUS*

SHAKE FLASK CULTURE

The production of *Monascus* secondary metabolites by shake flask culture has been extensively studied. Various culturing parameters including water supplement, temperature, nitrogen source, medium components, and pH value have been investigated (McHan & Johnson, 1970; Sato & Naito, 1935). Lin was the first to study shake flask

culture conditions on the pigment production by *Monascus* (Lin, 1973) and polished rice powder gave higher pigment productions (Su & Huang, 1976). Effect of different nitrogen sources on pigment production during shake flask fermentation were investigated by (Carels & Shepherd, 1977). During shake flask cultivation, pH of the medium play important role. At pH 6.5 using yeast extract or nitrate as the nitrogen source, red pigments are formed. While, using ammonium or ammonium nitrate, the pH is around 2.5 and the pigments are orange (Chen & Johns, 1993b). Oxygen concentration in the shake flask cultivation also influences the biosynthesis citrinin (Hajjaj *et al.*, 2000). The absence of potassium phosphate in the medium also reduces the red pigment production in the culture of *M. pilosus*. Spectrum of light also affects the composition of *Monascus* secondary metabolites (Miyake *et al.*, 2005).

The production of biomass and lovastatin by spore-initiated shake flask fermentations of *A. terreus* ATCC 20542 was shown to depend on the age of spores used for inoculation. Cultures started from older spores produced significantly higher titers of lovastatin and sporulation was reduced in dark (Rodriguez Porcel *et al.*, 2007). A two-stage feeding strategy improves the rate of lovastatin production by *A. terreus* compared to conventional batch fermentation (Rodriguez Porcel *et al.*, 2007) homogenity and stability of strain (*A. terreus*) to utilize carbon source and conditions like, controlled pH and high level of dissolved O_2 tension are crucial for elevated lovastatin production (Buckland *et al.*, 1989; Gbewonyo *et al.*, 1992). Among several organic and inorganic defined nitrogen sources, glutamate and histidine were metabolized by *A. terreus* produced highest lovastatin (Hajjaj *et al.*, 2001).

In *M. purpureus* MTCC 369 cultured in medium containing 29.59g/l dextrose, 3.86g/l NH_4Cl, 1.73 g/L KH_2PO_4, 0.86 g/l $MgSO_4 \cdot 7H_2O$, and 0.19 g/l $MnSO_4 \cdot H_2O$ using response surface methodology produced maximum lovastatin production of 351 mg/L (Sadik Ali *et al.*, 2006). A relatively low supplement of dissolved O_2 (DO) by the fungus

almost stopped performing product formation. With the DO controlled at 20%, lovastatin production enhanced by 38%, biomass production decreased by 25% and sugar utilization increased by 18%, as compared with the shaking-flask culture (Long-Shan *et al.*, 2005). Pelleted growth of *A. terreus* has yielded higher titers of lovastatin than obtained with filamentous growth. The rapid increase in viscosity accompanied by filamentous growth greatly impedes oxygen transfer and this is said to explain the low titers of lovastatin (Kumar *et al.*, 2000). Response surface methodology (RSM) was employed to study the effect of culture medium on the production of lovastatin in mixed solid-liquid state cultures by *M. ruber*. The maximal lovastatin yield (131 mg/L) observed at the region where the respective concentrations of rice powder, peptone, glycerin, and glucose were around 34.4 g/L, 10.8 g/L, 26.4 ml/L, and 129.2 g/L, respectively (Chang *et al.*, 2002).

(Suh & Shin, 2000) reported that the novel co-culture 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.

SOLID-STATE CULTURE

Solid-state fermentation (SSF) uses economical substrates (agricultural residues), requires fewer processing and down-streaming stages, utilizes lesser power and generates lesser effluent. Moreover, SSF has higher product yield and offers better product stability. Because of the reasons, SSF was used mainly for the production of industrial enzymes but nowadays, it is also being exploited for the production of secondary metabolites.

The contemporary method of RMR production is still by the traditional solid-state fermentation on cooked whole rice kernel. Successful productions of RMR are often determined by the following factors: the type of substrates (predominantly nonglutinous rice kernel), type of selected *Monascus* strains, temperature and moisture content of the fermentation mixture throughout the process, and control of contamination factors (Su, 2001). Until monacolins and GABA were reported in these products, good production quality of RMR was long being regarded as higher pigment accumulation in the fermentation mixture. (Juzlova *et al.*, 1996a) summarized several articles in a review that addressed solid-state cultivation of *Monascus* sp. in laboratorial scale for pigment production. Of the rice substrates used in producing RMR, nonglutinous soft rice was superior to glutinous rice in which the former has higher nutritious value (starch content) to the molds and is easier to absorb water (Su, 2001). Recently, a commercial koji maker with a rotary perforated bed was adopted for RMR mass production (Chiu *et al.*, 2006).

On the control of water content during RMR production, it is reported that the optimal substrate humidity should be adjusted to approximately 40-50% at initial and maintained by temporarily moistening the substrates in favor of fungal growth (Hesseltine, 1965; Su & Wang, 1977). Recent studies suggested that a lower initial moisture content (25-30%) helps to keep a low glucoamylase activity and, thus, is benefit to the pigment yields (Lotong & Suwanarit, 1990; Teng & Feldheim, 2000). However, (Chen & Hu, 2005) concluded that cultivation of a mutant strain *M. pilosus* M12-69

yielded the best monacolin K/citrinin ratio when the water contents is between 55 and 75%. Sufficient aeration is also a key parameter to pigment production than growth. Pigment formation was dramatically blocked when excess CO_2 accumulated in the incubator (Teng & Feldheim, 2000).

To increase the beneficial ingredients and decrease the toxic components, random mutation to *Monascus* has been performed and have acquired a genetically modified strain with higher monacolin K productivity and lower citrinin content (Chen & Hu, 2005; Wang *et al.*, 2004). In the culture of *M. purpureus* NTU 601, the addition of 0.5% ethanol as the carbon source elevated the monacolin K, GABA and reduced the citrinin content (Wang *et al.*, 2003). Moreover, substrates suitable for production of specific metabolites have also been studied. *Dioscorea batatas* is reported as an enhancer substrate for *Monascus* species to the production of monacolin K and monascin (Lee *et al.*, 2006).

Lovastatin produced by solid state fermentation (SSF) using *A. flavipes* BICC 5174 showed wheat bran was found to be the most suitable substrate yielding 13.49 mg g^{-1} dry solid. Wheat bran of particle size ranging from 0.3 to 0.5mm having moisture level of 60% and pH 5.0 gave the highest yield of lovastatin (Valera *et al.*, 2005). A simple temperature-shift method (28-23° C) showed that the maximum of lovastatin production was further enhanced by 25% (572 mg/l at day 10) in comparison with that when the fungus was cultured at 28° C. Red yeast rice prepared from several kinds of Thai glutinous rice (Oryza sativa L.) cv. Korkor 6 (RD6), Kam (Kam), and Sanpatong1 (SPT1) produced several monacolins. It was found that, highest amount of compactin and monacolin K were 21.98 and 33.79 mg/g respectively (Chairote *et al.*, 2008).

METABOLITES OF *MONASCUS PURPUREUS*

CITRININ

The mycotoxin citrinin **(Figure 5)** is produced by various sp of *Penicillium, Aspergillus* and *Monascus*. *Monascus* sp. namely *M. purpureus*, and *M. ruber* are reported to produce a mycotoxin citrinin as fermentation byproduct along with coloured pigments and monacolins. Citrinin is synthesized through the polyketide pathway, through which many secondary metabolites are synthesized, especially pigments. However, the synthesis of pigments and citrinin were not necessarily correlated (Wang *et al.*, 2005).

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.*, 1995a Hajjaj, 1999), a nephro and hepatotoxin. This discovery led to screening *Monascus* sp available in public collections for identifying toxinogenic strains and species. The applications of the high-producing pigments industrial strain *Monascus* sp have been greatly limited by the synchronous production of mycotoxin citrinin. Citrinin has been considered to have hepato-nephrotoxic properties (Lockard *et al.*, 1980; Kogika *et al.*, 1993), which causes functional and structural kidney damage and alterations in liver metabolism (Da Lozzo *et al.*, 1998). It inhibits several enzymes linked to the respiratory chain of the kidney cortex and liver mitochondria, as well as malate and glutamate dehydrogenases and the ATP-synthetase complex (Da Lozzo *et al.*, 1998).

The formation of citrinin is always a troublesome problem for the development of *Monascus* as functional food. The traditional mutagenesis and metabolic engineering methods to eliminate the production of citrinin have been established (Jia *et al.*, 2009). The formation of citrinin depends on the culture conditions. *Monascus* sp on rice is reported to produce citrinin 2.5 g/kg dry matter, while shake flask cultures up to 56 mg/kg dry matter. Citrinin was found to be nephrotoxic and teratogenic in chronic

toxicity studies in rats at a dosage of 50 mg/kg body weight/day after 60 weeks in the test animals (Eisenbrand, 2006).

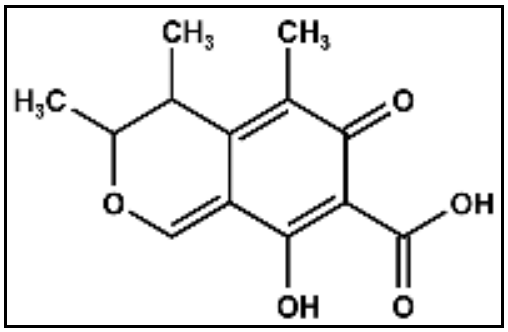


Figure 5. Structure of citrinin [$C_{13}H_{14}O_5$; IUPAC, (3*R*, 4S-*trans*)-4, 6-dihydro-8-hydroxy-3, 4, 5-trimethyl-6-oxo-3H-2-benzopyrane-7-carboxylic acid.

The safety of *Monascus* product containing high citrinin level was a concern even though many health functions including hypolipidemia, hypotensive, anti-fatigue and anti-cancer have been reported by many researchers and developed as the popular commercial product (Wang *et al.*, 2006a; Su *et al.*, 2005). Some of the *Monascus* strains produced pigments without citrinin (Pisareva *et al.*, 2005). The citrinin concentration in RMR produced by *M. purpureus* MTCC 410 is 1.427 mg/kg fermented rice (Mohan Kumari *et al.*, 2009), is much lower than the acute nephrotoxic level 18.4 g/kg (Krejci *et al.*, 1996).

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.*, 2000) 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.*, 2000). Ethanol has been added to repress citrinin formation under the solid fermentation of *Monascus* species (Wang *et al.*,

2003). The separation of growth phase and by temperature shifting from 30 to 23º C increased lovastatin production by nearly 20 times compared to temperature-constant cultivation of *M. pilosus*. In addition, citrinin was not produced even in the lovastatin production phase, although the pigment was increased (Tsukahara *et al.*, 2009).

STEROLS

Sterols are natural organic compounds widely distributed in all eukaryotic organisms and are significant part of membrane. Their functional role is evident through the participation in the control of membrane associated metabolic processes, such as, regulations of membrane permeability and fluidity, signal transduction and activity of membrane bound enzymes (Piironen *et al.*, 2000). Sterols are the precursors of steroid hormones and bile acids in humans, brassinosteroids-phytohormones in plants (Lindsey *et al.*, 2003). The recent identifications of sterol mutants have revealed their importance in growth and developmental processes in living organisms (Hartmann, 1998). Plants have more than 40 well-identified and studied sterols (Law, 2000) predominantly present in oilseed and are commonly termed as phytosterols. The most abundant phytosterols are: β-sitosterol, campesterol and stigmasterol **(Figure 6)**. Other phytosterols like avenasterol and cycloartenol are synthesized earlier in the biosynthetic pathway and as sterol precursors they usually occur in relatively smaller amounts (Maatat *et al.*, 1999).

Phytosterols are the naturally occurring equivalents of mammalian sterol (cholesterol) in plants. They have a structure similar to cholesterol, but with some modifications in the side chain, such as the addition of a double bond at C-22 and/or an alkyl (methyl or ethyl) group at C-24. In fungi and yeast, the natural sterol is ergosterol, which contains additional double bonds at C-7 and C-22, and a methyl group at C-24, as compared with cholesterol. The most common dietary sources of plant sterols are nuts, seeds, unrefined plant oils and legumes (Piironen *et al.*, 2000). β- sitosterol and campesterol account for up to 95% of all dietary phytosterol, the remaining 5% of the

plant sterols comprising mainly stigmasterol (Piironen *et al.*, 2000). Phytosterols have been shown to reduce blood cholesterol, as well as to decrease the risk of certain types of cancer and enhance immune function (Moreau *et al.*, 2002).

Plant sterols and stanols may displace cholesterol in the micelles, since there is a competition between phytosterols/phytostanols and intestinal cholesterol with regard to incorporation into micelles. This effect will result in a decreased incorporation of cholesterol into micelles, a consequent reduced availability of cholesterol for absorption and lower serum LDL cholesterol concentrations (Mensink *et al.*, 2002); (Miettinen & Gylling, 2004). Being an integral part of membrane, determine the characteristics of plasma membrane, endoplasmic reticulum and mitochondria membranes. Sterols certain function in the membrane adaptation to temperature variations (Piironen *et al.*, 2000). Participating in the control of metabolic processes, such as regulation of membrane permeability, fluidity, signal transduction events for cell division and even activity of membrane-bound enzymes, they fulfil their metabolic role (Hartmann, 1998; Lindsey *et al.*, 2003).

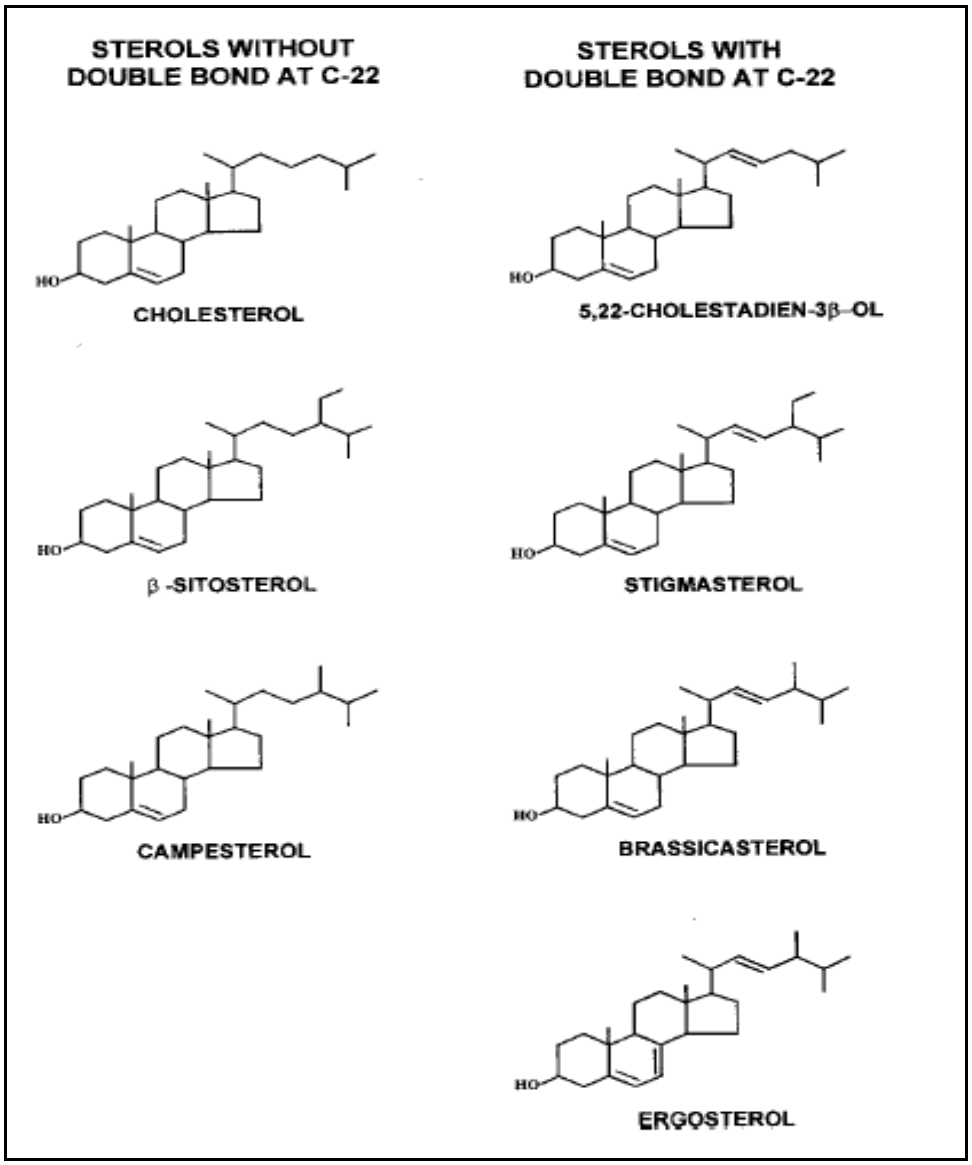


Figure 6. Chemical structures of the sterols and phytosterols.

Cholesterol performs the similar functions in humans and animals as phytosterols in plants. Cholesterol either synthesised *de novo* in liver, or taken up from the food. During the process of cholesterol absorption, it is being transported from the lumen of intestine, across the intestinal wall and into the blood. Low-density lipoprotein (LDL) transports cholesterol through the blood system. LDL cholesterol restrict the blood flow by narrowing blood vessels channels and people with high cholesterol levels eventually

become more susceptible to Cardiovascular Diseases (CVD) such as, coronary heart disease (CHD), hypertension (high blood pressure), cerebrovascular (stroke) and peripheral vascular diseases (Salo *et al.*, 2003).

According to "World Health Report 2003" of United Nations World Health Organisation, heart attacks and strokes kill 12 million people around the world every year. Among this 75% of CVD can be attributed to the major risks of high cholesterol, high blood pressure and low fruit and vegetable intake. Phytosterols decreases serum cholesterol, LDL cholesterol (Apo A1 and Apo B), plasma total cholesterol (TC) and increase high density lipoprotein (HDL) cholesterol concentration. (Miettinen & Gylling, 2004; Nozaki *et al.*, 1991; Trautwein *et al.*, 2003 ; Shirley *et al.*, 2009).

MONACOLINS (STATINS)

In the early 1970s, Endo et al (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, 1985). The enzyme became important since it controlled cholesterol synthetic pathway (Endo *et al.*, 1986b). 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).

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

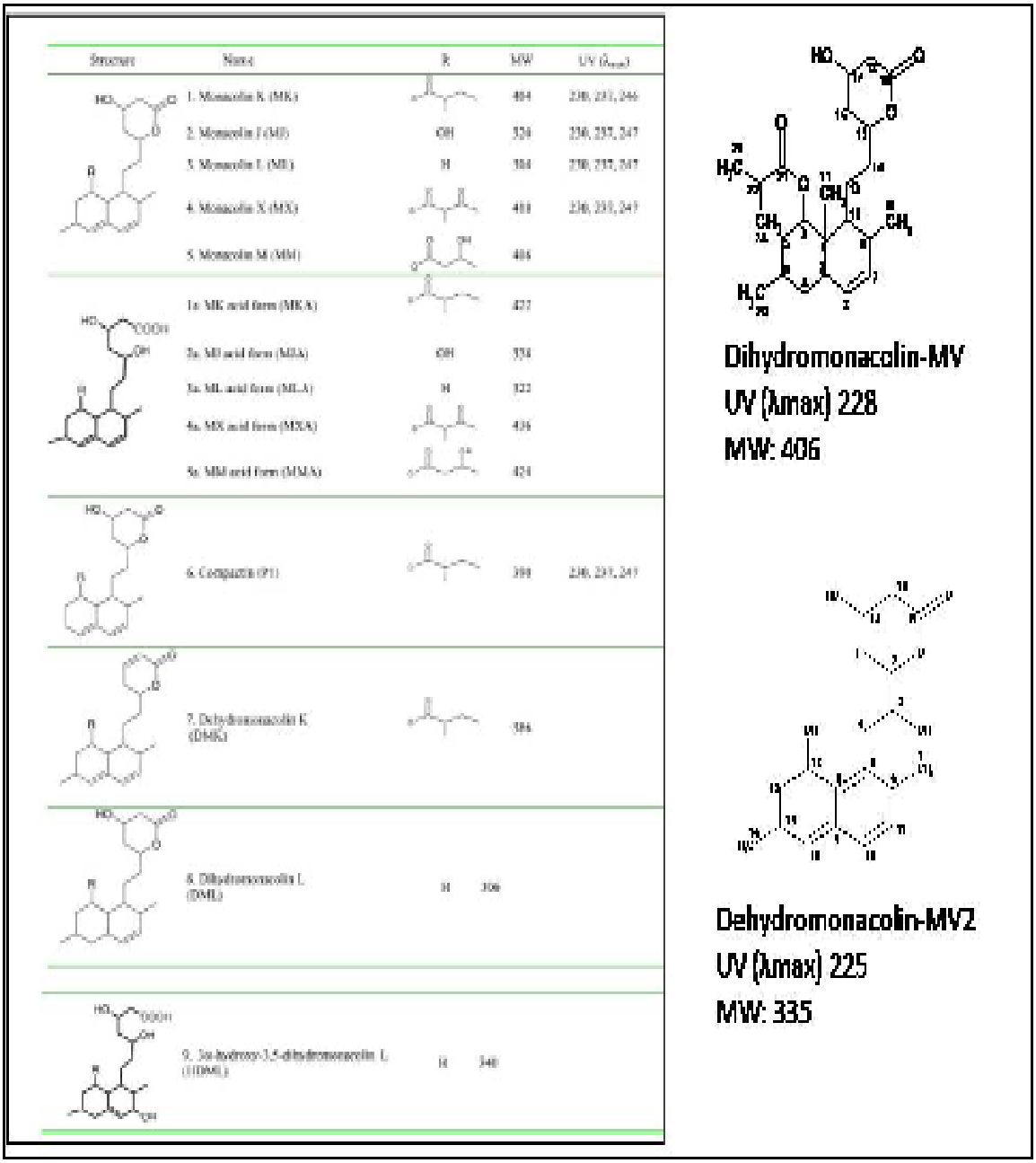
lovastatin production. Although glucose strongly repressed lovastatin production, maltose increased its productivity. Interestingly, glycerol combined with maltose enhanced lovastatin production, up to 444 mg/l. Moreover mutant, in which glucose repression was relieved, produced the highest level of 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).

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

Till date 18 monacolin metabolites (**Table 2**) 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 (Li *et al.*, 2004; Ma *et al.*, 2000).

Monacolins X and M have a different functional group at the C8 side chain. Monacolins J and L lack the lovastatin methylbutyric side chain. In monacolin J a hydroxyl group is present in the C8 position, while it is substituted by a hydrogen in monacolin L and dihydromonacolin L **(Table 2)** (Endo *et al.*, 1985b; Endo *et al.*, 1985b; Endo *et al.*, 1986; Juzlova *et al.*, 1996a; Kimura *et al.*, 1990; Komagata *et al.*, 1989).

Table 2. Monacolins identified from *Monascus* sp (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; Dhale *et al.*, 2007b). The beneficial

effects of statins in preventing cardiovascular disease have been described in literature (**Table 3**).

Table 3 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.

MECHANISM OF ACTION OF STATIN

Statins inhibit HMG-CoA reductase, the rate-limiting enzyme in cholesterol biosynthesis, thereby reducing serum cholesterol level. The mechanism involved in the hypocholesterolemic activity of statins is based on the competitive inhibition of HMG-CoA reductase (HMGR) is the rate-limiting enzyme in cholesterol biosynthesis. The statins have a moiety like HMG CoA and inhibit HMGR at nanomolar concentration in a competitive dose dependent and reversible fashion is due to the structural homology between the β-hydroxyacid form of statins and HMG-CoA **(Figure 7)**. The bulky hydrophobic group of statins that is covalently linked to the HMG-like moiety occupies the HMG-binding pocket and part of the binding surface for CoA, thus sterically preventing substrate (HMG-CoA) from binding to the enzyme. The substrate-binding pocket of the enzyme also undergoes a rearrangement that enables the rigid, hydrophobic ring structures of the statins to be accommodated. The tight binding is probably due to the large number of van der Waals interactions between the statin and HMG-CoA reductase (Istvan & Deisenhofer, 2001).

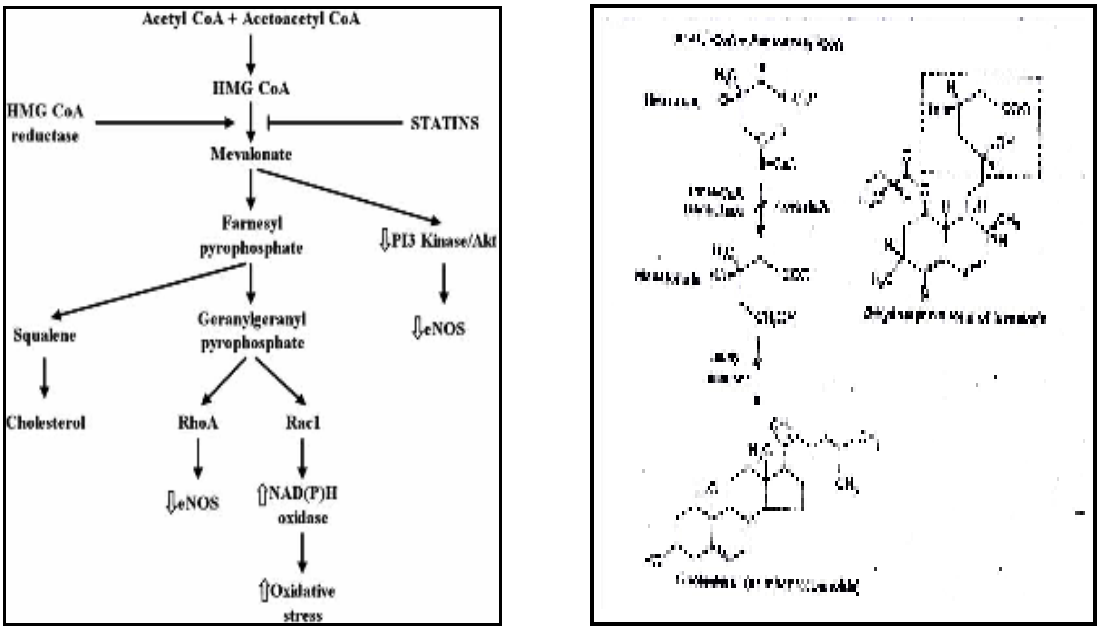


Figure 7. Mevalonate pathway for cholesterol biosynthesis and its inhibition by statin.

METABOLISM OF STATINS IN LIVER

All statins have the liver as target organ. The percentage of the dose retained by the liver is as follows: > 70% for fluvastatin and lovastatin, >80% for simvastatin and 46% for pravastatin (Blum, 1994). For their liver metabolism, lovastatin, simvastatin, atorvastatin and cerivastatin follow the cytochrome P450 (CYP 3A4) pathway. Fluvastatin follows the CYP 2C9 pathway and pravastatin is metabolized differently (Lennernas, 1997). The majority of the statins have a low circulation concentration: 12% for atorvastatin, 17% for pravastatin, 20-30 % for fluvastatin, 5% for simvastatin and lovastatin. Cerivastatin has a circulation distribution of more than 60%.

FORMS OF STATINS

Statins are currently among the most commonly prescribed agents for the prevention of cardiovascular disease. Mounting evidence suggests that in addition to their vascular effects such as stabilization of atherosclerotic plaques and decreased carotid intimal-medial thickness, statins have additional properties such as endothelial protection via actions on the nitric oxide synthase system as well as antioxidant, anti-inflammatory and anti-platelet effects (Krysiak *et al.*, 2003); (Liao & Ulrich, 2005); (Davignon, 2004). These effects of statins might have potential therapeutic implications in various neurological disorders such as stroke, Alzheimer's disease, Parkinson's disease, multiple sclerosis and primary brain tumors.

Currently, six statins with U.S. Food and Drug Association (FDA) approval are available for lowering cholesterol (rosuvastatin- Crestor, atorvastatin-Lipitor, simvastatin- Zocor/ Lipex, pravastatin- Pravachol/ Selektine/ Lipostat, fluvastatin- Lescol and lovastatin- Mevacor/ Altocor). Statins are administered orally, are well tolerated and have a good overall safety profile till date. The chemical structures of the different statins are shown in **(Figure 8).** Statins are classified into natural and synthetic statins. The natural statins (lovastatin and pravastatin) are obtained directly by fermentation, and the

semi-synthetic (simvastatin) and synthetic statins (atorvastatin and fluvastatin) obtained by chemical synthesis. Cerivastatin, a fully synthesized statin approved in the United States in 1997, has been employed until the recent withdrawal from the market (**FDA Talk Paper 2001**). Natural statins have a similar chemical structure with a common main polyketide portion, a hydroxy-hexahydro naphthalene ring system **(Figure 9a)**, to which different side chains are linked at C8 (R1) and C6 (R2) **(Figure 9b)**. Lovastatin, pravastatin, and simvastatin contain a substituted decalin ring structure. Fluvastatin, atorvastatin, and rosuvastatin are synthetic HMGR inhibitors with larger fluorophenyl groups linked to the HMG-like moiety.

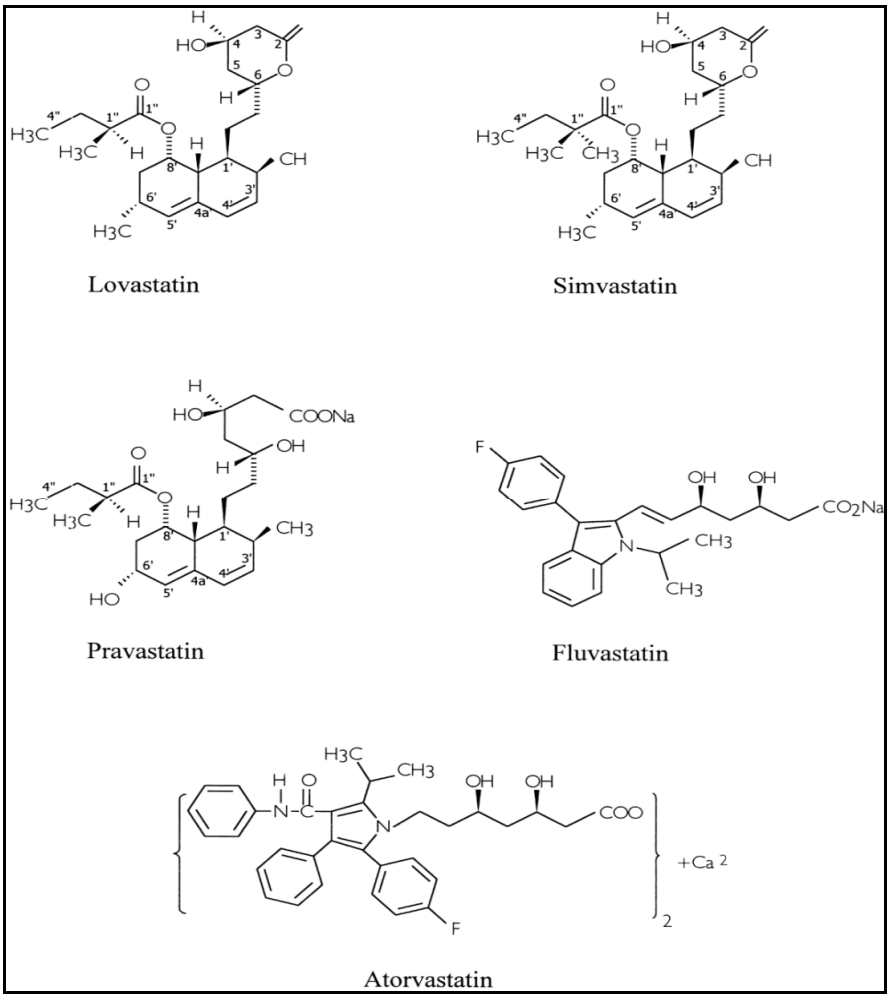
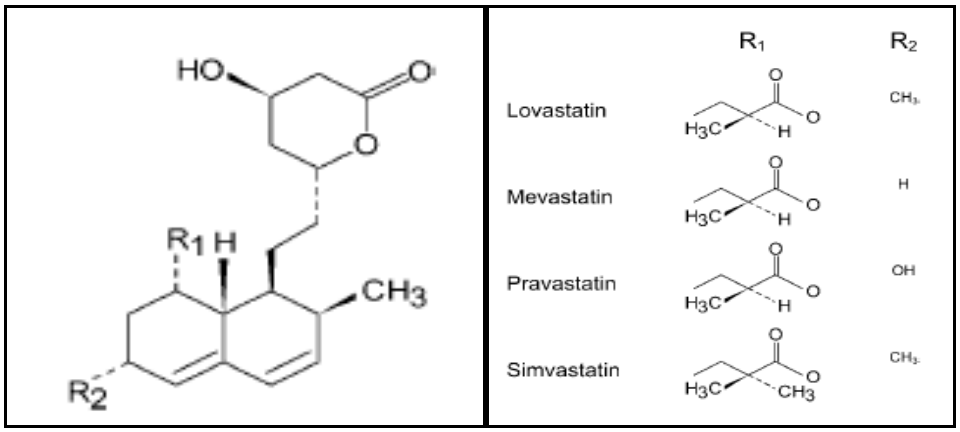


Figure 8. Chemical structures of different statins



A B

Figure 9. Base structure of statins (naphthalene ring and β-hydroxylactone) (A); Statin side chains linked at C8 (R1) and C6 (R2) of the base structure (B).

Lovastatin (or mevinolin, monacolin K, and Mevacor, Merck) contains a methylbutyric side chain (R1) and a 6-α methyl group (R2), which is lacking in mevastatin (or compactin, ML-236B, and CS-500). Pravastatin (or eptastatin and Pravachol, Bristol-Myers Squibb/Sankyo) has the β-hydroxylactone in the 6-hydroxy sodium salt form and is the C6-hydroxy analogue of mevastatin. Simvastatin (or Synvinolin and Zocor, Merck) contains an additional methyl group in the 2' position of the side chain. In addition to these most-important compounds, several lovastatin- or mevastatin-related metabolites have been isolated and characterized.

The natural statins like lovastatin and simvastatin are lactone form, while, synthetic statins are obtained as hydroxy acid form. Even though the structures of synthetic statins atorvastatin, fluvastatin and cerivastatin are dissimilar and are quite different from the natural statins, the HMG-CoA-like moiety responsible for HMG-CoA reductase inhibition is common in both natural and synthetic statins. Fluvastatin derived from mevalolactone was the first entirely synthetic statin available (Levy *et al.*, 1993),

while atorvastatin and cerivastatin, pyridine derivatives are a new generation of highly purified statins (Barker, 1903).

BIOSYNTHESIS OF STATINS

LOVASTATIN

Various fungi such as *Aspergillus* (*A.terreus*) species, *Monascus* (*M. ruber, M. purpureus, M. pilosus, M. vitreus, M. pubigerus and M. anka*) species, *paecilomyces viridis* and *pencillium (P. citrinum*) species have been found to produce lovastatin (Manzoni & Rollini, 2002).

Early biogenetic investigations of statins carried out on C^{14}-labelled monacolin J and L, employing a strain of *Monascus ruber,* suggested that these compounds are precursors of lovastatin and, consequently can be classified as isolated intermediate metabolites in the lovastatin biosynthetic pathway (Endo et al. 1985b). Studies on the pathway involved in monacolin synthesis (**Figure 10**) have demonstrated that monacolin L is the precursor of monacolin J. In fact in the hydroxylation reaction 18 O_2 was incorporated into monacolin J through the action of a monooxygenase system involving cytochrome P-450, present in the cell-free extract of *M. ruber* (Komagata et al. 1989). Subsequent experiments again employing the cell-free extract of *M. ruber* and living cells of *Paecilomyces viridis* have demonstrated the transformation of monacolin J to lovastatin (Kimura et al. 1990). Moreover, a combination of physical techniques indicates monacolin M derivation from monacolin J, via a pathway that is quite distinct from that for the synthesis of lovastatin, the α-methylbutyryl ester of monacolin J (Endo et al. 1986a).

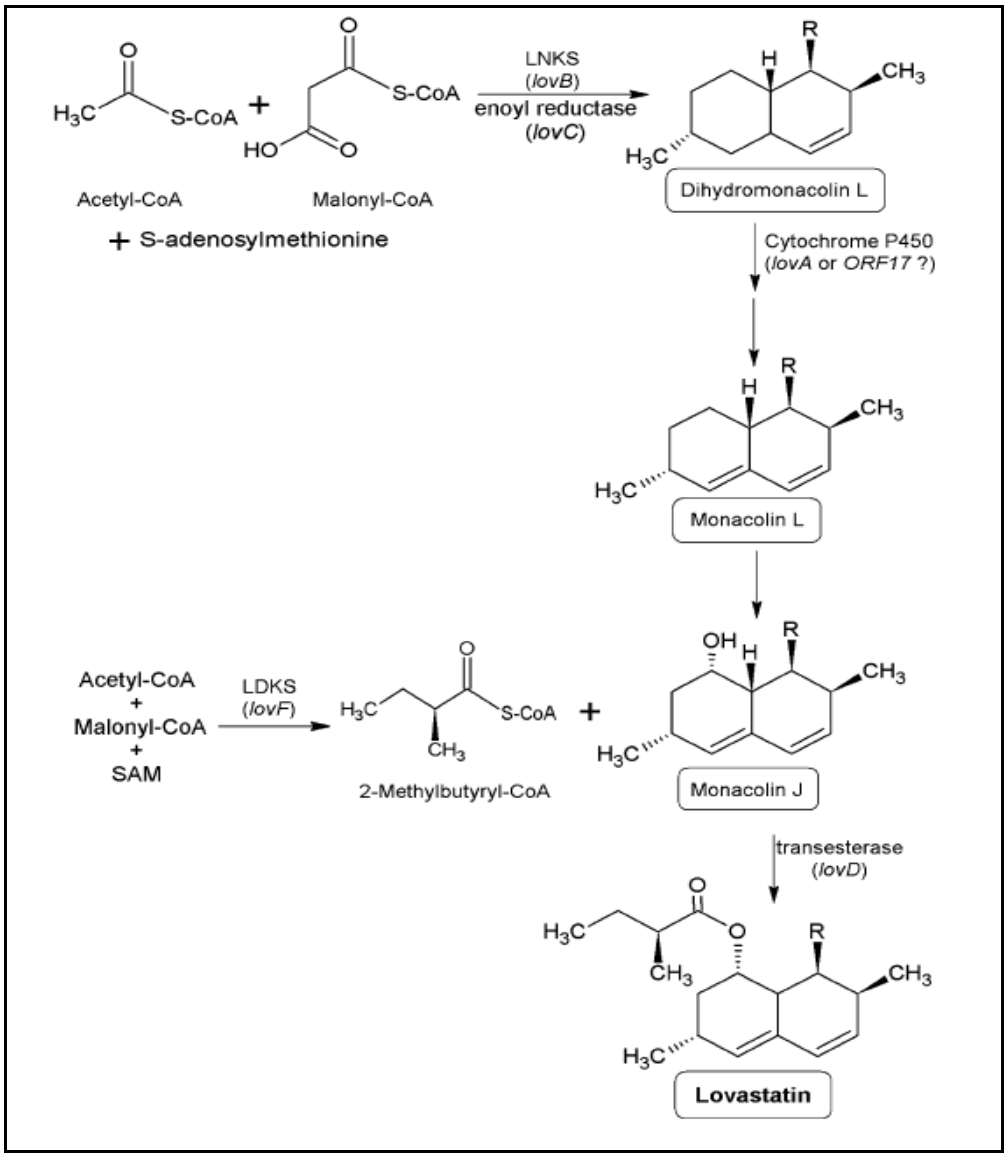


Figure 10. Lovastatin biosynthetic pathway, showing enzymes involved and their encoding genes.

PRAVASTATIN

Although compactin is not used as a medicine, it is an important source for producing pravastatin. At first stage, a strain of *Penicillium citrinum* produces compactin. Pravastatin is normally produced in a second stage by biotransformation (hydroxylation)

of compactin by *Streptomyces carbophilus* (Serizawa *et al.*, 1983; Serizawa & Watanabe, 1997). In order to develop more efficient processes for the industrial production of pravastatin several research groups have performed studies to achieve a higher conversion rate by adding high concentrations of compactin into the culture medium. But increased concentration of compactin in the culture medium inhibits cell growth and causes autolysis of the mycelia, resulting in a decrease in the conversion of compactin into pravastatin (Masahiko *et al.*, 1993). Park *et al.*, (2003) designed a process where compactin was fed continuously, keeping its concentration constant at 100 mg l^{-1}. Pravastatin production kinetics was about 1.5-fold higher than that on intermittent feeding of compactin (reaching 1.5 g of pravastatin per liter). On the other hand, (Chen *et al.*, 2006) designed a rational screening method to isolate compactin-resistant microbial strains with high hydroxylation activity at the 6β position of compactin. (Ykema *et al.*, 1999) developed a one-step biosynthesis process to produce pravastatin, by transforming the *P. citrinum* strain with a *S. carbophilus* hydroxylase gene that converts compactin to pravastatin.

OTHER BIOACTIVE METABOLITES OF *MONASCUS*

γ-AMINOBUTYRIC ACID (GABA)

Crude extract of RMR containing GABA could alleviate hypertension in rats (Kohama *et al.*, 1987). GABA has been widely researched on its role of an inhibitory neuronal signal transmitter. It has two receptors: $GABA_A$, which couples to chloride ion channels, and $GABA_B$, which are G protein-coupled receptors. Because GABA receptors exist extensively in the neuronal system and tissues, its pharmacological functions were intensively studied (Blein *et al.*, 2000; Kerr & Ong, 1995; Watanabe *et al.*, 2006). Gamma-aminobutyric acid (GABA) is a non-protein amino acid compound (Huang *et al.*, 2007; Komatsuzaki *et al.*, 2005; Park & Oh, 2007) produced in plants, microorganisms and mammals by decarboxylation of glutamic acid via the glutamate decarboxylase (GAD) enzyme (Kono & Himeno, 2000; Su *et al.*, 2003; Ueno, 2000); Komatsuzaki *et*

al., 2007). It has pharmacological functions such as, as an antihypertension agent, as a diuretic, as well as having a tranquilizing effect and as being an inhibitory neurotransmitter in sympathetic brain functions (Su *et al.*, 2003; Komatsuzaki *et al.*, 2005; Huang *et al.*, 2007; Komatsuzaki *et al.*, 2007). GABA can regulate blood pressure, heart rate, sensations of pain and anxiety (Kono & Himeno, 2000), lipid levels in serum (Miura *et al.*, 2006) and assist in insulin secretion to prevent diabetes (Huang *et al.*, 2007). Moreover, consumption of GABA-enriched foods can inhibit cancer cell proliferation (Park & Oh, 2007) and improve memory and the learning abilities in rats (Miura *et al.*, 2006).

DIMERUMIC ACID

The *Monascus* extract protected the liver injury (induced by carbon tetrachloride) in mice (Aniya *et al.*, 1999), and known for hepatoprotective activity. Dimerumic acid isolated from *Monascus* showed in vitro antioxidant activity in DPPH assay and was identified as the major constituent responsible for hepatoprotective antioxidant activity (Aniya *et al.*, 2000). Dimerumic acid was further found to inhibit the NADPH- and iron (II)-dependent lipid peroxidation of rat liver microsomes at 20 and 200 μM, respectively. The antioxidative property was contributed by the electron donation of the hydroxamic acid group to the oxidants (Taira *et al.*, 2002).

MONASCUSONES A and B

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).

ANKAFLAVIN

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).

MONASCIN

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.

DIHYDROMONACOLIN-MV

M. purpureus extract showed strong 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity. The activity-guided repeated fractionation of *M. purpureus* extract yielded a compound that exhibited strong antioxidant activity. The spectroscopic analysis by UV, IR, 1H NMR, 13C NMR, 2D-HSQCT NMR, and MS, the antioxidant isolated was elucidated as dihydromonacolin-MV. The dihydromonacolin-MV has significantly scavenged DPPH and superoxide radicals and strongly inhibited lipid peroxidation in a liposome model (Dhale et al 2007a).

DEHYDROMONACOLIN-MV2

Hyper pigment producing mutant of *M. purpureus* (CFR 410-11) was found to contain metabolites that inhibited the growth of *Bacillus*, *Pseudomonas* and *Streptococcus*. The extract inhibited lipid peroxidation and scavenged 2, 2-diphenyl-1-pycrylhydrazyl and hydroxyl radicals. The carbon, proton and 2D HSQCT assignments of purified compound identified dehydromonacolin-MV2 as the bioactive metabolite. Dehydromonacolin-MV2 apparently originated in the mutant by hydroxylation and oxidation of monacolin-J, an intermediate of monacolin biosynthetic pathway (Dhale et al 2007b).

FUNCTIONAL PROPERTIES OF *MONASCUS PURPUREUS*

M. purpureus rice (red mould rice/ red yeast rice) a traditional Chinese medicine has been shown in animal and pilot human studies to effectively lower serum lipid levels. *M. purpureus* rice is prepared by traditional fermentation of rice to produce red biomass known as red mould rice (RMR). It has been shown that RMR contains compounds with HMG-CoA reductase inhibitor activity, which is responsible for the inhibition of cholesterol synthesis in the liver. In addition to protein, fibre, sterols and fatty acids, RMR contains numerous active constituents, including Monacolin K, dihydromoncolin, and Monacolin I to VI.

RED RICE PROCESSING

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, 1998 #109). 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, 2004b).

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 4.**

Recently the extracts of *Monascus* sp fermented with garlic, turmeric and ginger have shown improved hypocholesterolemic, antiatherosclerotic, antioxidant and hypotensive activities. *Monascus* Garlic fermented extract is a unique material produced from garlic fermented using *M. pilosus* contains characteristic compounds such as dimerumic acid and monacolin K. The atherogenic index, triglyceride calculated was significantly reduced, and lipid peroxide levels were reduced. No abnormal changes in blood biochemical parameters or adverse effects were observed in any of the subjects (Sumioka *et al.*, 2006). Whereas, *M. pilosus* fermented in PDB medium containing turmeric extract showed higher antioxidant activity than uninoculated. The phenolic and curcuminoid contents were significantly increased, during fermentation process. As compared control (uninoculated), *M. pilosus* fermented with turmeric had a more significant effect on down-regulating the production of NO and TNF-alpha as well as the expression of inducible nitric oxide synthase, cyclooxygenase-2, glutathione peroxidase,

superoxidase dismutase, and catalase. Modified *M. pilosus* fermented product demonstrated a higher antiatherosclerotic value than the unmodified product (Kuo *et al.*, 2009b).

Table 4. Health benefits of red rice in human

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%
(Hutter & Niederberger, 1983)	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.*, 1999a)	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.*, 2007;.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).

RMR also contains unsaturated fatty acids such as oleic, linoleic, and linolenic acids (Ma *et al.*, 2000) that may also help reduce serum lipids (Wang *et al.*, 1997). RMR extract may help to reduce total cholesterol levels, lower levels of LDH (bad) cholesterol, increase levels of HDL (good) cholesterol, and lower the level unhealthy fats called triglycerides. It appears to accomplish this by restricting the liver's production of cholesterol itself. Interestingly, the compound responsible for the effect-mevinolin is chemically identical to the cholesterol-lowering compound lovastatin, sold as the prescription drug Mevacor. Mevinolin is also similar to the active ingredients in such cholesterol medications as Zocor (simvastatin) and Lipiton (atorvastatin). Unsaturated fatty acids in RMR extract are also believed to help, possibly in lowering triglycerides (Heber *et al.*, 1999; Wang *et al.*, 1997; Qin *et al.*, 1999b).

There's still another reason for regarding RMR as a food, and that is the fact that the product contains many other synergistic nutrients with lipid lowering properties in addition to monacolins. RMR has been reported to contain sterols such as beta-sitosterol and campesterol (Heber *et al.*, 1999), which are known to interfere with cholesterol absorption in the intestines (Moghadasian & Frohlich, 1999). Effects of dietary phytosterols on cholesterol metabolism and atherosclerosis: clinical and experimental evidence. The combination of such dietary sterols with statin drugs has in fact been

suggested as a more effective means of lowering cholesterol than statins alone (Plat & Mensink, 2001) so it makes sense to consume a single food which naturally combines both kinds of anti-cholesterol activity.

RMR also contains fiber, trace elements such as magnesium and B-complex vitamins such as niacin (Palo *et al.*, 1960) all of which have known benefits in decreasing serum lipids such as triglycerides and cholesterol. RMR, an Asian dietary staple made by fermenting yeast (*M. purpureus*) on rice, is rapidly gaining recognition as a cholesterol-lowering agent in United States. Indonesia, Japan, Taiwan, and Philippine people are been used as *Monascus*-nata complex (Sheu *et al.*, 2000).

RMR has been reported to function in lowering of plasma glucose, cholesterol, and triacylglyceride. In the model of chicken, the addition of RMR powder to their fodder lowers the level of cholesterol, triglyceride, and low density lipoprotein (LDL) in serum and reduces the cholesterol content in egg yolk. This approach suggests a healthier source of meat or egg products for people who need to control cholesterol intake in their diets (Wang *et al.*, 2003; Wang *et al.*, 2006b). In the model of Wistar rats, oral administration of MFR increases the release of acetylcholine from the nerve terminal, which in turn interacts with muscarinic M3 receptor in pancreatic cells, promotes insulin release, and thus reduces the plasma glucose (Chen & Liu, 2006).

Oral administration of RMR to streptozotocin-induced diabetic rats decreases their plasma glucose in a dosage-dependent manner from 50 to 350 mg/kg. In normal rats with intravenous glucose injection, oral administration of RMR (350 mg/kg) also attenuates the elevation of plasma glucose (Chen & Liu, 2006). In a clinical study, 79 patients with hyperlipidemia are randomly and double-blindedly grouped to receive RMR or placebo daily. After 8 weeks, the patients with RMR administration demonstrates

reduced levels of LDL cholesterol, total cholesterol, triglycerides, and apolipoprotein B (Lin *et al.*, 2005).

In the cell culture model, RMR extracts are known to significantly decrease the enzyme activity and gene expressions, which are related to the adipocyte differentiation. For example, the glycerol-3-phosphate dehydrogenase activity and lipid accumulation are lowered in 3T3-L1 cells. Moreover, the messenger ribonucleic acid levels of CCAAT/enhancer-binding protein and peroxisome proliferator-activated receptor are decreased. The results suggest the inhibitory function of RMR to the susceptibility of adipocyte differentiation (Jeon *et al.*, 2004).

The oral administration of RMR extract can inhibit the metastatic ability of murine Lewis lung carcinoma (LLC) cells in syngeneic C57BL/6 mice caused by the decline of serum vascular endothelial growth factor (VEGF) levels compared with untreated metastatic groups. Monacolin-k is a key antimetastatic and antiangiogenesis compound in RMR extract, as shown by down-regulation of VEGF-stimulated invasive activity in LLC cells. The application of RMR extract serve as chemopreventive or antineoplastic agent in the development of cancer adjuvant chemotherapy (Ho & Pan, 2009).

Monascus-fermented soybean extracts (MFSE) enriched with bioactive mevinolins (natural statins) and aglycone isoflavones (daidzein, glycitein, and genistein) perform an additive hypolipidemic effect in hyperlipidemic rats than unfermented soybean extracts. The oral administration of MFSE significantly lowered the serum total cholesterol, triglyceride and low-density lipoprotein cholesterol (LDL-C) levels and raised high-density lipoprotein cholesterol (HDL-C) levels in hyperlipidemic rats. The MFSE group had a significantly lower 3-hydroxyl-3-methylglutaryl-coenzyme A (HMG-CoA) reductase activity and higher atherogenic index (calculated as HDL-C/LDL-C) when compared with the UFSE group. Treatment of MFSE for 40 days significantly reduced the activities of serum aspartate aminotransferase and alanine aminotransferase

by averages of 35.6 and 43.2%, respectively, as compared to the high-fat diet group (Pyo & Seong, 2009).

Lovastatin is associated with various adverse effects such as myopathy and abnormal liver function test results, which can lead to serious problems if patients are not monitored and treated. The red yeast rice products can be beneficial in lowering serum cholesterol levels, but they are not without risk. Furthermore, product uniformity, purity, labeling, and safety cannot be guaranteed (Klimek *et al.*, 2009). Antihypertensive effects of red mold rice (RMR) and red mold dioscorea (RMD) by low-dose oral administration to spontaneously hypertensive rats were studied. A single oral dose RMD significantly decreased systolic blood pressure and diastolic blood pressure after 8 h of administration, but RMR showed no significant effect. RMR and RMD can improve the vascular elastin structure remodeling. In comparison to RMR, RMD contained a higher amount of gamma-aminobutyric acid and anti-inflammatory yellow pigments (monascin and ankaflavin). RMD exhibited higher angiotensin-I-converting enzyme inhibitory activity than RMR (Wu *et al.*, 2009).

Acute and sub-chronic toxicity studies of *M. purpureus* MTCC 410-fermented rice (red mould rice) on both sexes of albino rats were studied. Feeding acute doses of RMR at 0.5, 1.0, 2.5 and 5.0 g/kg body weight to rats did not cause any symptoms of toxicity or mortality. Similarly, dietary feeding of RMR at 2.0%, 4.0%, 8.0% and 12.0% level (w/w) for 14 weeks did not produce any significant changes in food intake or gain in body weight of the experimental rats compared to control rats. There were no significant differences in the relative weight of vital organs, hematological parameters, macroscopic and microscopic changes in vital organs and serum clinical enzyme levels between the experimental and control groups. The toxicity studies with RMR of *M. purpureus* did not cause any toxic effects in albino rats (Mohan Kumari *et al.*, 2009).

Nephrotic dyslipidemia is a risk factor for development of systemic atherosclerosis; also it may aggravate glomerulosclerosis and enhance progression of glomerular disease. *M. purpureus* rice is safe, effective cholesterol lowering agent for nephrotic dyslipidemia both in adults and children. The efficacy and safety of *M. purpureus* rice vs. fluvastatin therapy in the management of nephrotic dyslipidemia has been studied in seventy-two patients with idiopathic persistent nephrotic syndrome with secondary dyslipidemia. Both fluvastatin and *M. purpureus* rice were well-tolerated with no significant side effects significantly reduced cholesterol after 6 and 12 month (Gheith *et al.*, 2009).

The 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}). 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 (Kim *et al.*, 2006).

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).

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 belonging 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:single-aromatic-ring PKSs, multi-aromatic-ring PKSs, and reduced-complex- type PKSs (Hutchinson, 1999). 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).

Among the enzymes that biosynthesize lovastatin are two polyketide synthases (PKS) and numerous accessory enzymes (Kennedy, 1999). The two mega synthases are the lovastatin nonaketide synthase (LovB, 335 kDa) and lovastatin diketide synthase (LovF, 277 kDa), which catalyze the assembly of the decalin core and the 2-methylbutyrate side chain, respectively. Both LovB and LovF are multidomain enzymes with domain architectures and activities related to animal fatty acid synthases (FAS) and bacterial type I PKS. Central to PKS and FAS are the minimal catalytic domains (Hopwood 1990), consisting of the ketosynthase (KS) that performs decarboxylative claisen condensation for chain elongation (Heath & Rock, 2002); the malonyl-CoA:ACP acyltransferase (MAT) that selects and transfers the extender unit in the form of malonic esters; and the acyl carrier protein (ACP) that serves as the tether for the extender unit and the growing chain. In addition, tailoring enzymes such as ketoreductase (KR), dehydratase, methyltransferase and enoylreductase modify the carbon backbone and introduce structural diversity (Staunton & Weissman, 2001).

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 dextrines, disaccharides and monosaccharides. The number of amylases produced by the microorganisms in culture is many and has been described (Ravi-Kumar & 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 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).

AMIDASES

Free amino acids can be converted into flavour compounds through the action of both muscle and microbial enzymes. The microbial enzymes are particularly significant because, depending on the microorganisms inoculated, different flavours can be obtained. Among the various amino acid degradation pathways, deamidation of both glutamine and asparagine has an important role from the point of view of sausage production, especially the deamidation of glutamine, since hydrolysis of the glutamine amide group produces ammonia, an acidity neutraliser, and glutamate, a flavour enhancer.

Amidases catalyse the hydrolysis of amino acids. Identification of L-asparaginase and L-glutaminase activities in the extracellular fluids of *M. pupureus* suggested their

role in the production of red pigment (Dhale *et al.*, 2009). 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). The amidase enzymes produced by *Monascus* sp. may play major role in tumor inhibition.

PROBIOTICS

Probiotics have been used for centuries in the manufacture of cultured dairy products. Probiotics are now widely consumed in the form of fermented milk products such as yogurt or as freeze-dried culture. Probiotics are defined as selected viable microorganisms used as dietary supplements for their potential benefits on human health or disease prevention (Schrezenmeir & de Vrese, 2001). The primary probiotic bacteria associated with dairy products have been *Lactobacillus acidophilus, Lactobacillus casei,* and Bifidobacteria. The reduction of total cholesterol or low-density lipoproteins found in plasma is reported to lower the risk of coronary heart disease. There are a few studies that suggest that probiotics may also reduce LDL cholesterol, although the research to date is equivocal (Taylor & Williams, 1998). Studies on *Lactobacillus acidophilus* NCFM (Gilliland *et al.*, 1985; Gilliland & Walker, 1990) have shown that it is able to remove cholesterol from a laboratory growth medium. There are also reports claiming that probiotics may have beneficial effect in obesity, but the evidence to support such an effect is still lacking.

The Lactobacillus strain has been shown to cleave β-glycosidic isoflavones during fermentation of milk supplemented with soygerm powder. The interactions between the Lactobacillus strain and soygerm powder suggest that combining both can exhibit advantageous probiotic effect (De Doever *et al.*, 2001).

Probiotic bacteria are defined as “live microorganisms that when administered in adequate amounts confer a health benefit on the host” (FAO, 2001). Fermented foods that have potential probiotic properties are produced worldwide from a variety of food substrates (Farnworth, 2005). Probiotics have been used for the treatment of various types of diarrhoea (Sarker *et al.*, 2005; Szymanski *et al.*, 2006), urogenital infections (Reid & Hammond, 2005), and gastrointestinal diseases such as Crohn's disease (Bousvaros *et al.*, 2005) and pouchitis (Kuehbacher *et al.*, 2006), although there is still no consensus about their effectiveness (Lin, 2003; Reid *et al.*, 2003; Senok *et al.*, 2005). Lactic acid bacteria including *lactobacilli* and *bifidobacteria* are the most common bacterial species considered as potential probiotics.

THE PROBLEM

Polyketide pigments of *Monascus* sp are non-toxic azaphilones used as traditional food colourant by Chinese, Taiwanese, Indonesians, Japanese, Thais and Pilipinos since ancient times. Even though *M. purpureus* has been used in Chinese folk medicine for long, only recently, the pharmaceutical applications of its metabolites were described. These metabolites have demonstrated antioxidant, antibacterial, 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 records, the literature on comparative metabolites production and application of these metabolites is still scarce.

M. purpureus MTCC 410 produced pigments, statins, sterols, saturated and unsaturated fatty acids during growth. The hyper pigment producing mutant CFR 410-11 secreted more of red pigment in solid state cultures than shake flasks due to the production of amidase enzyme. Cultural parameters for enhanced production of the above metabolites have been optimized. The details of the study are described in the **First Chapter**

Acute and sub-chronic toxicity studies were conducted with both the sexes of albino rats did not show any significant differences between experimental rats when compared to controls. A compound isolated from this fungus was identified as Ankaflavin after NMR and other spectroscopic characterizations. *Monascus* extract when incorporated into the growth medium of beneficial organisms like LAB did not affect their viability. The **Second Chapter** describes the results of these experiments.

Since the above results suggested the safety of RMR, further it was characterized for hypolipidemic property. *M. purpureus* significantly lowered serum and hepatic cholesterol and triacylglycerol levels. Antioxidative defense mechanism in oxidatively stressed male albino rats fed on high fat diet is dueto induced production of antioxidant enzymes and molecules. The details are described in the **Third Chapter** of the thesis.

MATERIALS AND METHODS

MICROBIOLOGICAL METHODS

All media preparation and culturing of organism were carried out as routine microbiological methods. Culture media, glass and plastic wares are sterilized at 121° C for 20 min (15 lbs pressure). Inoculation of cultures was performed in laminar air hood and all other aseptic methods described for microbiological work were generally followed. All media preparations were carried out using double distilled water. Ultra pure water obtained from Milli Q filtration unit (Millipore) was used for buffer preparation for enzyme assay and antioxidant assay.

THE ORGANISM

Monascus purpureus MTTC 410 used in this study was obtained from Microbial Type Culture Collection (MTCC), Institute of Microbial Technology (IMTECH) Chandigarh, India. The culture was maintained on potato dextrose agar (PDA) medium at 4° C and sub cultured every 30 days. After cultivation for 7 days on PDA the spore suspension was obtained by washing the slants with 7 ml sterilized aqueous solution of 0.85% NaCl. About 1 ml of spore suspension (containing 3.5x 10^7 spores) was used as inoculum. The mutants, hyper pigment (CFR 410-11) and albino (CFR 410-22) were obtained from the culture collection of Food Microbiology Department, CFTRI, Mysore.

Strains of lactic acid bacteria (LAB) namely *Lactobacillus acidophilus* B4496 (La), *Lactobacillus bulgaricus* CFR 2028 (Lb), *Lactobacillus casei* B1922 (Lc), *Lactobacillus plantarum* B4495 (Lp) and *Lactobacillus helviticus* B4526 (Lh) were maintained on DeMan, Rogosa Sharpe (MRS) agar medium. After two successive transfers of the test organisms in MRS broth at 37° C for 12-16 h, the actively growing cultures were again inoculated into MRS broth incubated at 37° C for 16 h and maintained at 4° C.

CHEMICALS

Culture media for cultivation such as potato dextrose agar (PDA), potato dextrose broth (PDB), DeMan Rogosa Sharp (MRS) agar and broth, L-glutamine and L-asparagine were purchased from Hi-media Laboratories, Mumbai, India. Glucose, peptone, glycerin, $MgSO_4.7H_2O$, KNO_3, ethylenediamine tetraacetic acid (EDTA), sodium arsenite, dimethyl sulfoxide (DMSO), analytical grade phenol red dye, solvents and other reagents were purchased from Qualigens Fine Chemicals Mumbai, India. Choline chloride and mercuric iodide were obtained from Loba chemie Pvt. Ltd (Mumbai, India). Solvents were of HPLC purity from Merck Limited, Mumbai, India. All other reagents used were of analytical grade. Rice for solid-state cultures was obtained from local supermarket.

Lovastatin, pravastatin, β-sitosterol, fatty acid standards, cholesterol, bile acid mixtures, isoflavones (genistin, daidzin, genistein and daidzein), 2, 2, diphenyl-1-pycrylhydrazyl (DPPH) and butylated hydroxy anisole (BHA), 3-hydroxy-3-methylglutaryl CoA, 5, 5'-dithiobis (2-nitrobenzoic acid) (DTNB), triethanolamine-HC1, tris (hydroxymethy1) amino methane, NADPH and dithiothreitol and chemicals for antioxidant enzymes were purchased from Sigma chemical (St. Louis, MO, USA).

L-ascorbic acid, potassium ferricyanide, trichloroacetic acid, mineral mixture, vitamins, cellulose were obtained from Sisco Research Laboratories, Mumbai, India. Casein was purchased from Nimesh Corporation, Mumbai. Creatinine, urea, total cholesterol (TC), triacylglycerol (TG), glucose and assay kits for biochemical studies like Serum Glutamyl Oxaloacetic Transaminase (SGOT), Serum Glutamyl Pyruvic Transaminase (SGPT), Lactate Dehydrogenase (LDH), Alkaline Phosphatase (ALP) were purchased from Agappe Diagnostics Ltd. (Kerala, India). Rats were fed with commercial semi synthetic diet obtained from M/S Gold Mohar, Lipton India Ltd, Bangalore.

EXPERIMETAL ANIMALS

Animals (rats) were obtained from the stock colony of the Animal House Facility (Central Food Technological Research Institute, Mysore, India). Animal experiments were carried out based on the ethical guidelines laid down by the committee, control and supervision of experiments on animals by the Ministry of Social Justice and Empowerment, Government of India. The rats were kept in separate individual stainless steel cage with screen bottom. They were housed in a room maintained at 25± 2º C with a relative humidity of 60-70% and exposed to a light and dark cycle of 12h duration.

ISOLATION OF THE MUTANTS

Spore suspension of *M. purpureus* (MTCC 410) prepared from one week old slants were 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 (Dhale, 2007c). Two different mutants hyper mutant (CFR 410-11) and albino (CFR 410-22) were obtained. Both parent and its mutants were maintained on slants of PDA at 4° C and sub-cultured every 30 days.

PREPARATION OF SPORE SUSPENSION

Spore suspension was prepared by adding 8 ml of 0.85% sterile NaCl to the actively growing (7 days at 28 ± 2° C) *M. purpureus* slants. The surface was gently scraped and the spore suspension pooled together was diluted to reach $3.5x10^7$ spores/ml.

POLYKETIDES OF *M. PURPUREUS* MTCC 410 AND IN MUTANTS

SHAKE FLASK CULTURES

The basic synthetic medium for shake flask cultures of *M. purpureus* was prepared according to (Lin & Demain, 1991a).

Composition of growth medium (gL^{-1})

Maltose	50.0
MSG	12.6
K_2HPO_4	2.40
KH_2PO_4	2.40
$MgSO_4$	1.00
KCl	0.50
$ZnSO_4.7H_2O$	0.011
$FeSO_4.7H_2O$	0.010
$MnSO_4.H_2O$	0.030
NH_4NO_3	0.286
pH	5.5

GROWTH AND CULTURE CONDITIONS

2.0 ml of spore suspension was inoculated into 500 ml Erlenmeyer flask containing 100 ml of sterile synthetic medium. This was incubated on a rotary shaker (200 rpm) at 28 ± 2° C for 7 days. At the end of 7 days, pH of the culture broth was noted, the biomass was harvested and total pigments were extracted. The pigments were spectrometrically measured and were expressed as OD units/g dry cell mass.

SOLID-STATE CULTURES

Red Mould Rice (RMR) 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° C. 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° C (Adolf Khuner Therm-Lab Switzerland) for 11 days. The flasks were shaken periodically to ensure uniform mixing.

EXTRACTION AND QUANTIFICATION OF PIGMENTS

SHAKE FLASK CULTURES

At the end of 11 days of fermentation, the fermented broth was filtered using Whatman No. 1 and the mycelia was washed two times with distilled water. This was made up to 100 ml and used extracellular pigments. The resultant mycelia were repeatedly (2-3 times) used for extraction of intercellular pigments in 25 ml of 95% alcohol on a rotary shaker (180 rpm) at 28 ± 2^{o} C for 30 minutes. This was made up to known volume after filtration as above and used as intracellular pigments. The biomass was determined after extraction of pigment by drying the mycelia at 105^{o} C for 3h.

SOLID-STATE CULTURES

Pigments from rice fermented with *M. purpureus* were extracted sequentially using hexane, benzene, chloroform, acetone, ethanol and water. The extraction was carried out at 30° C by keeping the flasks for 60 min on rotary shaker (150 rpm). The insoluble debris was removed by filtration and used quantification of the pigments.

The pigments from shake flask and solid state cultures after suitable dilution were determined spectrophotometrically by measuring the optical density (OD). Optical

density was measured at 375, 475 and 500 nm for yellow, orange and red pigments respectively. Pigment yield was calculated as OD Units using following formula.

$$\text{OD Units} = \frac{\text{OD X Total vol of extraction X Dilution}}{\text{Red mould rice (g)}}$$

STATINS OF *M. PURPUREUS* MTCC 410 AND ITS MUTANTS

The statin production was estimated both in shake flask and solid state cultures of *M. purpureus* and its mutants.

SHAKE FLASK CULTURES

Composition of monacolin-k production medium gL^{-1} (Chang *et al.*, 2002)

Rice powder	30.0
Peptone	9.0
Glycerin	30.0
Glucose	110.0
$MgSO_4.7H_2O$	1.0
KNO_3	2.0
pH	5.0

Inoculum preparation

Spore suspension was inoculated into 100 ml of potato dextrose broth (PDB), and incubated at 28° C for 4 days with shaking at 150 rpm. 5% (v/v) of the above broth was inoculated into 500 ml flasks containing statin production medium. The culture was

incubated at 28° C on a rotary shaker at 150 rpm for 10 days (Chang *et al.*, 2002). The biomass was determined after drying the mycelia at 105° C for 3h.

SOLID-STATE CULTURES

Components of basal medium (gL^{-1}):

Glucose	100.0
Peptone	10.0
KNO_3	2.0
$NH_4H_2PO_4$	2.0
$MgSO_4.7H_2O$	0.5.0
$CaCl_2$	0.1.0
pH	6.0

For solid state fermentation the seed culture inoculum was prepared according to (Su *et al.*, 2003) by inoculating the loop full of spores in 50 ml basal medium. This was incubated at 28±2º C on a rotary shaker at 110 rpm for 48 hrs. After incubation 5% of seed culture was used as inoculum in solid-state fermentation to prepare RMR.

LOSS OF ORGANIC MATTER

Material was dried before and after fermentation up to constant weight. LOM was calculated as the weight difference and expressed as a percentage of the initial dry weight of the samples.

$$LOM = \frac{W_i - W_f}{W_i} \times 100\ \%$$

Where, W_i is the initial dry weight of solid material before fermentation and W_f is the final dry weight of solid material after fermentation.

Since there is no direct method for biomass estimation in solid state cultures, LOM was used to express biomass in an indirect way (Kumar *et al.*, 2003).

RED MOULD RICE (RMR) PREPARATION

The traditional RMR preparation was carried out according to the method described by (Su et al., 2003). The sterilized rice was inoculated with a 5% (v/w) seed culture inoculum of *M. purpureus*. The inoculated substrate was incubated at 30º C (Adolf Khuner Therm-Lab, Birfelden (Basel), Switzerland) in a slanting position for 14 days with intermittent mixing of rice by shaking. After incubation, RMR was dried at 45-50° C for 24 h. This was crushed to coarse powder and used to carry out further experiments.

EXTRACTION OF STATINS (LOVASTATIN AND PRAVASTATIN)

Lovastatin from commercial tablet (Dr. Reddy's) was extracted in 5 ml of acetonitrile: water (4:1, v/v) mixture by mechanical disintegration and sonication (Dr. Hielscher Gmb_H, UP 50H ultraschallprozessor sonicator) for 5 mins. This was centrifuged to collect supernatant and concentrated under nitrogen. The final residue was dissolved in acetonitrile (HPLC grade) and this was compared with the standard lovastatin according to method of (Walter *et al.*, 1999).

Twenty microlitre of lovastatin standard preparation was accurately transferred into another 25 ml actinic volumetric flask and 5ml of 0.5N NaOH was added and the solution was allowed to stand for 30 min, and used as standard for hydroxyl acid form of

lovastatin. Standards from sigma dissolved in acetonitrile can be directly used for HPLC. Methods for determination of the statin concentration under different conditions were mentioned below.

SHAKE FLASK AND SOLID-STATE CULTURES

After 10 days of shake flask cultivation, the fermented broth (extracellular) was filtered. The remaining mycelia was extracted with 50 ml (95%) ethanol (intracellular) for 24 h in a rotary shaker at 180 rpm (Adolf Khuner Therm-Lab, Birfelden (Basel), Switzerland) (Chang *et al.*, 2002). The mixture was centrifuged at 1500x g for 15 min and filtered through 0.45 µm millex-LH (Millipore Corp., Bedford, MA 01730) membranes to quantify statin by HPLC.

Statin from RMR obtained by solid state cultures (1g powder) was extracted with 10 ml of 75% ethanol at 30° C for 1h on rotary shaker at 180 rpm. After centrifuging for 10 min at 10,000 rpm, samples were filtered through a 0.45 µm filter before injection. The identification and quantification of statins were carried out by HPLC (Su et al., 2003) (Samiee et al. 2003).

PURIFICATION OF STATINS BY THIN LAYER CHROMATOGRAPHY

Statins from mycelial extract of fermented broth and fermented rice was purified by loading on a heat activated silica gel TLC plates (alumina silica gel 60 plates 20x20 cm Merck, Germany). The plates were allowed to run in ascending direction to 15 cm with the dichloromethane and ethyl acetate (70:30, v/v) as mobile phase. The plates were air dried and exposed to iodine to locate the spots and scraped.

QUANTITATIVE ANALYSIS BY HPLC

The identification and quantification of statins in both shake flask and solid state cultures were monitored by high performance liquid chromatography (HPLC) equipped with linear isocratic system, using Shimadzu Liquid Chromatograph LC-10A (Shimadzu, Japan). The samples were eluted with a mobile phase comprising 72% acetonitrile at a flow rate of 1.0 ml/min fitted with water's ODS (5 µm, 25 cmX4.6 mm i.d.) column. The column temperature was set at 30° C and the injection volume was 20 µl. The chromatogram was monitored at 237 nm. Standard lactone and hydroxyl acid form of lovastatin and lactone form of pravastatin were used to construct a calibration curve to determine the statin concentration in shake flask and solid state cultures.

PROXIMATE ANALYSIS OF *M. PURPUREUS* RED MOULD RICE (RMR)

The moisture content of the RMR was determined after drying at 105^{o} C until a constant weight was attained. The micro-Kjeldahl method was employed to determine the total nitrogen and the crude protein (N x/5.95) (AOAC, 2000). The total lipids were extracted using hexane by Soxhlet apparatus and ash content (gravimetric) were determined based on methods outlined in (AOAC, 2000). The total carbohydrate content (%) was calculated as 100 - (crude protein+ crude lipid +crude fibre+ash).

DETECTION OF CITRININ IN RMR

RMR (1 g dry weight) extracted with 10 ml methanol at 50° C for 1.5 h was defatted twice with isooctane. After adding an equal volume of water and acidifying to pH 4.5 with H_2SO_4 (50:50, v/v), the extract was partitioned with $CHCl_3$. The lower phase was evaporated to dryness and dissolved in methanol. This was filtered through 0.45 µm pore size membrane filter before injecting into HPLC (Blanc *et al.*, 1995a). Citrinin was determined by HPLC using C18 column (LiChroCART 250-4, Merck, Darmstadat, Germany) with the composition of water: acetonitrile: triflouacetate (450:550:0.5 v/v/v)

as a mobile phase. The flow rate was set at 1.0 ml min^{-1} and the fluorescence detector was used. The excitation and emission wavelength was set at 330 and 500 nm respectively (Kycko *et al.*, 1998).

STEROL AND FATTY ACID OF *M. PURPUREUS* AND IN ITS MUTANTS

SHAKE FLASK CULTURES

Shake flask culture for sterol and fatty acid production was carried out by using Chapec Dox (CD) medium with yeast extract (Rasheva *et al.*, 1997). 3 ml of spore suspension was inoculated into flask with 100 ml CD medium and incubated in a rotary shaker at 220 rpm at 30º C for 5 days.

Components of CD medium (gL^{-1}):

Glucose	200.0
NaCl	5.0
$MgSO_4.7H_2O$	5.0
KH_2PO_4	10.0
$FeSO_4.7H_2O$	0.10
$ZnSO_4.7H_2O$	0.010
NH_4Cl	25.0
pH	6.0

SOLID-STATE CULTURE

One gram dry RMR was extracted with 5 ml of chloroform: methanol mixture (2:1) for 2 hrs and the supernatant was filtered. The extraction was repeated till the

extract become colorless. The residue was dried completely and the amount of lipid extracted was measured by weight.

ESTIMATION OF STEROL BY LIBERMAN-BURCHARD METHOD

About 10-20 mg of extracted lipid (described above) was subjected to saponification by methanolic KOH (6%, w/v) and refluxed for 2 hours at 80º C in boiling water bath, cooled and diluted with 1 vol. of water. Total sterol was repeatedly extracted 3 times with hexane. The total extract was pooled together and evaporated to dryness. The final residue was dissolved in 1 ml chloroform and estimated by Liberman-Burchard method (Syed Mubbasher *et al.*, 2003). Briefly to 200 µl sample, 800 µl chloroform, 2 ml of the Liberman-Burchard reagent (5 ml of H_2SO_4 dissolved in 10 ml acetic anhydride) was added in a graduated test tube. The final volume was made up to 7 ml with chloroform and incubated in dark for 15 mins. The absorbance of resultant solution was determined spectrophotometrically at 640 nm (Shimadzu 160 UV A). Sitosterol was used as standard for comparison.

LIPID EXTRACTION AND FATTY ACID ESTIMATION

Total lipid extraction and analysis of fatty acids from dry biomass were carried out according to the method of (Somashekar *et al.*, 2003). The fatty acids were identified with the retention time of authentic fatty acid standards obtained from Sigma chemicals.

FATTY ACID DETERMINATION BY GC

Fatty acid profile was estimated by converting the fatty acids to methyl esters (FAMEs). FAMEs were prepared according the method of (Kate, 1964) with slight modification. Fatty Acid methyl esters were separated by Shimadzu GC-15A equipped with an instrument with flame ionization detector. GC conditions were as follows: DEGS

packed column with isothermal temperature. The injector temperature was set at 220º C, detector temperature was maintained at 230º C and column temperature at 180º C. N_2 was used as carrier gas at a flow rate of 40 ml min^{-1}.

OPTIMIZATION OF CULTURAL CONDITIONS FOR *M. PURPUREUS* STATINS

SHAKE FLASK CULTURES

Fungi are morphologically complex organisms and growth conditions influences their morphogenetic behaviour. In shake flask cultures, a large number of factors affect fungal development and differentiation. These include the type and concentration of carbon, nitrogen, phosphate, trace minerals, dissolved oxygen, carbon dioxide, pH and temperature.

EFFECT OF CARBON AND NITROGEN ON STATIN PRODUCTION

Effects of different carbon sources on lovastatin production were studied by using glucose, maltose, fructose, lactose and sucrose. Production medium was supplemented individually with the above carbon sources. The carbon sources did not contain any nitrogen content. The carbon content in production medium was kept constant (4.4%) for all carbon source. Effect of different nitrogen sources like, peptone, yeast extract, ammonium chloride, ammonium sulphate and ammonium nitrate on lovastatin yield from *M. purpureus* was determined by incorporating nitrogen sources in the growth medium. Total nitrogen content in the media was maintained at 0.14%. Fermentation was carried out at 28° C with shaking at 150 rpm for 10 days. After fermentation, the mycelia and culture filtrate were separated using cheesecloth. Statin was extracted and determined as described above.

EFFECT OF TEMPERATURE AND pH ON STATIN PRODUCTION

Primary inoculum (seed culture) grown at 28° C was inoculated to production medium and cultivated at different temperatures (20, 25, 28, 30 and 35° C) to check the effect of temperature on statin production by *M. purpureus*. Similarly, the seed culture was inoculated to production medium, where, initial pH of the medium was set to 3.0, 5.0, 7.0, 9.0, 11.0 and 13.0 using 1 N HCl or 1 N NaOH. Fermentation was carried out at 28° C with shaking on a rotary shaker at 150 rpm for 10 days and biomass (mycelia) was separated from the culture filtrate. Statin was extracted and determined as described in Materials and Methods section.

MEASUREMENT OF DRY CELL WEIGHT (BIOMASS)

Known volume of fermented broth was filtered through a filter membrane (Whatman No.1). The mycelia obtained were washed with distilled water and dried at 102° C for 24 h and were then equilibrated at room temperature for the measurement of dry weight. Biomass was measured till the weight was constant.

SOLID-STATE CULTURE

EFFECT OF CARBON AND NITROGEN SOURCE ON STATIN PRODUCTION

Effect of different carbon and nitrogen sources on statin production during solid state fermentation was studied as above. The sterilized seed media containing different carbon and nitrogen sources independently was inoculated into rice. The concentrations of carbon and nitrogen sources were 4.4 and 0.14% respectively. Fermentation was carried out at 28° C with intermittent mixing with shaking for 14 days. At the end of fermentation the RMR was dried at 45-50° C for 24 h and powdered. Estimation of statin was carried out as above.

EFFECT OF pH AND TEMPERATURE ON STATIN PRODUCTION

To study the effect of the pH of the growth medium on statin production, experiments were performed with media of different initial pH. Initial pH of the medium (3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0 and 10.0) was adjusted using 1 N HCl or 1 N NaOH. Temperature is another major factor affecting the fungal metabolism. Primary inoculum (seed culture) grown at 30° C was inoculated to sterilized rice and incubated at different temperatures (20, 25, 30, 35 and 40° C). At the end of the incubation statin was extracted and determined as described above.

STATIN PRODUCTION DURING GROWTH

The production of statin was determined during growth of *M. purpureus* for 20 days. The lovastatin was extracted on alternate day from second day for estimation as mentioned above.

GROWTH TEMPERATURE AND STEROL CONCENTRATION

Solid-state fermentation was performed as mentioned in Materials and Methods. Both *M. purpureus* and its mutants cultures were inoculated to rice. The cultures were incubated at different temperatures (20°, 30° and 40° C) for 14 days. RMR prepared above was used for sterol extraction. 1 g RMR was extracted with 5 ml of chloroform: methanol mixture (2:1) for 2 hrs and the supernatant was filtered. The extraction was repeated till the extract become colorless. The residue was dried completely and the lipid extracted was weighed. The total sterol was estimated by Liberman-Burchard method (Syed Mubbasher Sabir et al. 2003) as mentioned earlier in Materials and Methods.

AMIDASE ACTIVITY

CULTURE CONDITIONS

The culture medium was prepared by sterilizing 4% rice flour for 20 min at 115° C in 500 mL conical flasks. The medium was inoculated with 1.0 mL of spore suspension in saline (0.85% NaCl). Inoculated rice flour was incubated at 30° C for 12 days at 180 rpm. The culture medium containing (1%) L-asparagine and L-glutamine as sole carbon and nitrogen source was inoculated as above and $NaNO_3$ was used as control.

ENZYME ASSAY

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 up to 1 L. The supernatant was used as the reagent.

The culture filtrates of fermented rice flour were used as enzyme source. The substrate for enzyme reaction was prepared in phosphate buffer (0.1 M, pH 8). L-glutaminase and L-asparaginase activities were determined in the fermented rice extracts by direct nesslerization of NH_3 (Imada *et al.*, 1973). The reaction mixture was containing,

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 adding of 0.5 mL TCA (1.5 M). To 0.25 mL reaction mixture, 6.75 mL distilled water and 0.5 mL Nessler's reagent were added. After 20 min of incubation at 18° C the absorbance was measured at 480 nm. The enzyme activity was expressed as international units (IU) mL^{-1}. One IU of activity is defined as that amount of enzyme that catalyzes the formation of 1 µmol of NH_3 min^{-1}. The products (L-aspartic acid and L-glutamic acid) were detected by paper chromatography using ninhydrine reagent.

QUANTIFICATION OF PIGMENTS

Pigments from *M. purpureus* MTCC 410 and its mutants were extracted using ethanol as mentioned in Materials and Methods. The extraction was carried out at 30° C on rotary incubator shaker (110 rpm) for 60 min. The mycelial extract was directly used to estimate the pigment.

ISOLATION OF BIOACTIVE COMPOUND

Dried fermented RMR (48-50° C) was powdered to 60-80 mesh and extracted with HPLC grade methanol. The extraction procedure continued till the extract becomes colourless. 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 OF COMPOUND

About 5 g of lyophilized methanol extract was passed through a column prepared using silica gel (60-120 mesh) for purification. Sufficient *n*-hexane was passed through the column to remove the oily hydrophobic materials. Stepwise elution was performed

with a linear gradient of hexane: chloroform: ethyl acetate: methanol (100:0; 100; 25:75; 50:50; 75:25 v/v). About 13 fractions, each measuring about 100-150 ml was collected depending upon colour intensity of the eluting fraction. The fractions were concentrated by flash evaporation and pooled together after TLC (silica gel 60 F_{254} plates 20X20 cm Merck, Germany) analysis for purity and the fractions having same Rf values were pooled, concentrated and again subjected to column chromatography as above. About 13 fractions were collected, concentrated by flash evaporation and assayed for DPPH radical scavenging activity. The chloroform and chloroform: ethyl acetate fractions (100 and 75:25 v/v) showed maximum activity. The sample was again eluted with linear gradient of chloroform to ethyl acetate with different concentration.

The fractions (chloroform and chloroform: ethyl acetate fractions) with DPPH activity were further separated by rechromatography on a silica gel (60-120 mesh) column. Stepwise elution was performed with a linear gradient of hexane, chloroform and ethyl acetate to give 2 fine fractions measuring 50-80 mL based on colour intensity. The fractions were concentrated by flash evaporation and pooled together after analysis by TLC. Finally, the fraction with the desired compound was concentrated to dryness and then resuspended in chloroform. These resultant fractions were further analyzed by HPLC and then fractions with a similar single peak profile were combined.

THIN LAYER CHROMATOGRAPHY

The collected fractions were spotted on silica gel TLC (alumina silica gel 60 F_{254} plates 20X20 cm Merck, Germany) plates. The plates were developed in ascending direction of 12 to 15 cm height with mobile phase made of dichloromethane and ethyl acetate (7:3 v/v), dichloromethane, ethyl acetate and methanol solvent (9:0.5:0.5 v/v) and ethyl acetate and *n*-hexane (3:7 v/v). The developed spots were visualized directly or under UV light (254 and 360 nm) irradiation. Combination of 3:7 v/v of ethyl acetate and *n*-hexane mobile phase showed best separation. The plates were air-dried and spots were located.

HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC)

Purified bioactive compounds were dissolved in HPLC grade acetonitrile to analyze purity by subjecting it to HPLC (Shimadzu Liquid Chromatograph, LC-10AVP, Shimadzu, Japan) fitted with Waters C_{18}, 5 μm, 4.6 X 250 mm analytical column (Waters Corporation, 34 Maples Street, Milford, MA 01757-3696 USA). 20 μl of purified 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 acetonitrile was scanned for the absorbance between 200-700 nm.

FOURIER TRANSFORMER INFRA RED (FTIR) SPECTROSCOPY

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

MASS SPECTROSCOPY

Mass spectrum of the bioactive compound was 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 0.2-1.0 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 OPTICAL ROTATION (POLARIMETRY)

Optical rotations of the isolated glycosides were recorded on a Perkin-Elmner 243 Polarimeter. Sodium lamp at 599 nm was used as the light source. Sample concentration of 0.5 to 1% in H_2O was used for the rotation measurements and specific rotations were calculated using the equation.

$$[\alpha]_D^{25^{\circ}C} = \frac{[\alpha]_{obs} \times 100}{C \times l}$$

Where, $[\alpha]_D$ is the specific rotation in degrees at 25 ^{o}C, $[\alpha]_{obs}$ is the observed rotation, C is the concentration of the sample in percentage and l is the path length in dm.

DETERMINATION OF MELTING POINT (MELTING POINT)

Melting point of the ankaflavin was determined by capillary method. A thin walled capillary tube 10-15 cm long of about 1 mm inside diameter sealed at one end containing the sample was employed. The sample capillary was pressed gently into the sample several times and then pushed to the bottom of the tube by repeatedly dropping

onto a table through a glass tube of 1m in length. The sample capillary was tightly packed to a depth of 2-3 mm. The capillary, containing the sample was pressed on to a thermometer and then suspended into paraffin is heated slowly and evenly with the help of burner flame. A narrow temperature range over which the sample was observed to melt was taken as the melting point.

DETERMINATION OF ANTIOXIDANT ACTIVITY

Antioxidant activity of purified compound was determined by estimating DPPH radical scavenging activity, inhibition of ascorbate autoxidation and reducing activity. All the experiments were carried out in triplicates by maintaining appropriate blanks and controls.

DETERMINATION OF TOTAL PHENOLICS

Total phenolics were determined using the method described by (Singleton & Rossi, 1965). The values were expressed as gallic acid equivalents. Dried RMR extract (1 mg) prepared from fermented rice was dissolved in 1 ml methanol and water (6:4 v/v) mixture. From this 0.2 ml sample was added to 0.8 ml water, 1 ml of Folin-Ciocalteu reagent (10 fold dilutions) and 2 ml sodium carbonate (10 %) solution. The reaction mixture was incubated for 30 min at room temperature and absorbance was measured at 765 nm with Shimadzu UV-Visible spectrophotometer. The experiments were carried out in triplicates. Results were expressed as milligram gallic acid equivalent per 100 g dry weight.

DPPH FREE RADICAL-SCAVENGING ASSAY

The DPPH radical scavenging activity (Blois, 1958) of purified compound was measured accordingly (Moon & Terao, 1998). The reaction mixture was containing 1ml DPPH (500μM in ethanol), 0.2 ml of purified compound (dissolved in ethanol) and final

volume made to 2.0 ml with Tris-HCl buffer (100mM, pH 7.4). The reaction mixture was shaken vigorously and incubated at room temperature for 30 min. The absorbance of the resulting solution was measured at 517 nm using Shimadzu 160 UV A spectrophotometer. Blank and control were maintained without DPPH and sample respectively. Antioxidant activity was calculated with the following formula.

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

MEASUREMENT OF INHIBITION OF ASCORBATE AUTOXIDATION

The method described by (Mishra & Kovachich, 1984) was used to determine the inhibition of ascorbate autoxidation. The reaction mixture containing 0.1 ml of sample, 0.1 ml ascorbate solution (5.0 mM, Sigma) was added to 9.8 ml phosphate buffer (0.2 M, pH 7.0). The reaction mixture was incubated at 37° C for 10 min and the absorbance of this mixture was read at 265 nm using water as control. The ascorbate autoxidation inhibition rate of the sample was then calculated.

$$\text{Inhibition effect (\%)} = \left(\frac{A_{\text{sample}}}{A_{\text{control}}} - 1\right) x100$$

MEASUREMENT OF REDUCING ACTIVITY

The reducing power was determined according to the method of (Oyaizu, 1986). The sample (0.5 ml) was mixed with 0.5 ml of 200 mM sodium phosphate buffer (pH 7.0) and 0.5 ml of 1% potassium ferricyanide. This mixture was incubated at 50° C for 20 min, and then 0.5 ml of 10% trichloroacetic acid (w/v) was added. The mixture was centrifuged at 1000g for 10 min. The upper layer (1.5 ml) was mixed with 0.2 ml of 0.1% ferric chloride and the absorbance was measured at 700 nm against a blank. Butylated hydroxyl anisole was used standard as for comparison.

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 (20-120 µg ml^{-1}) dissolved in ethanol were 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 kept in a boiling water bath for 15 min. After cooling, it was centrifuged at 10,000 rpm for 5 min and the absorbance of the supernatant was measured at 532 nm. Blank and control were maintained without liposome and compound respectively. Percentage of lipid peroxidation inhibition activity was determined using the formula.

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

SAFETY EVALUATION OF *M. PURPUREUS* RMR IN ALBINO RATS

ANIMALS AND MAINTENANCE

Adult male and female rats (CFT-Wistar strain- 8 week old, 180-200 g) were employed for the acute toxicity study. The weanling young male and female rats (CFT-Wistar strain, 55 g) were employed for the sub-acute toxicity studies. The animals were obtained from the stock colony of the Institute Animal House Facility and were kept in separate individual stainless steel cage with screen bottom. They were housed in a room maintained at 25± 2º C with a relative humidity of 60-70% and exposed to a light and

dark cycle of 12h duration. Animal experiments were carried out based on the ethical guidelines laid down by the committee for the purpose of control and supervision of experiments on animals by the Government of India, Ministry of Social Justice and Empowerment.

DIETS AND THEIR PREPARATION

Rats were fed with commercial diet (M/S Gold Mohar, Lipton India Ltd.). Freeze dried *M. purpureus* RMR was incorporated into the diet at levels based on body weight for acute toxicity studies and at different dietary levels for sub-chronic toxicity studies.

ACUTE TOXICITY STUDY

A graded dose of RMR was fed through diet at levels of 0, 0.5, 1.0, 2.5 and 5.0 g/kg body weight on day one only. Since the nature of fungal biomass (RMR) did not permit administration through oral intubation, feeding of RMR through dietary means was opted. Prior to dosing, rats were fasted overnight. Rats were observed thoroughly for onset of any immediate toxic signs and also during the observation period of one week for any delayed acute effects. All the animals were sacrificed humanely after twenty one days and selected vital organs were excised, blotted, weighed and processed for routine histological examination.

SUB-CHRONIC TOXICITY STUDY

Weanling rats of both sexes were randomly assigned to control and treatment groups. The rats were fed with diets containing 0, 2.0, 4.0, 8.0 and 12.0% freeze dried fungal biomass (RMR) to assess the cumulative effect of low doses of lovastatin containing fungal biomass (RMR). Diet and water were given *ad libitum* for a continuous period of 14 weeks. Daily food intake and weekly body weight gain were monitored and

all the animals were observed thoroughly for the onset of any sign of toxicity. At the end of experimental period all the animals were killed humanely under light ether anesthesia.

ORGAN WEIGHT AND HISTOPATHOLOGICAL STUDIES

The following vital organs (liver, lungs, kidney, spleen, brain, adrenals, heart, testis and ovaries) from each rat were excised, blotted, weighed and the organ/body weight ratio was calculated. Portion of tissues were cut and fixed in Bouin's fixative. The tissues processed to obtain 5µm thick sections were stained using hematoxylin and eosin and observed for any histological changes.

HEMATOLOGICAL STUDY

Blood samples collected from a minimum of four rats of each group of the experimental and control groups were examined for haematological profiles like Hemoglobin Concentration (Hb), Red Blood Cells (RBC), White Blood Cells (WBC), Packed Cell Volume (PCV), Mean Corpuscular Hemoglobin (MCH), Mean Corpuscular Hemoglobin Concentration (MCHC), Differential Count (DC), Lymphocytes (L), Polymorph Neutrophils (P), Monocyte (M), Eosinophils (E), Basophils (B). MCH was calculated from hemoglobin and erythrocytes counts, whereas MCHC was determined from hemoglobin concentration (Bharucha *et al.*, 1976).

SERUM ENZYMES AND OTHER BIOCHEMICAL PARAMETERS

Serum separated from blood was analyzed for SGOT (Serum Glutamate Oxaloacetate Transaminase, SGPT (Serum Glutamate Pyruvate Transaminase, (Moss & Henderson, 1999), lactate dehydrogenase (Henry, 1979), alkaline phosphatase (Moss & Henderson, 1999), creatinine (Tanganelli *et al.*, 1982) and urea (Thomas, 1998). Serum cholesterol and triacylglycerol were estimated according to (Schaefer & McNamara,

1997; Rifai *et al.*, 1999), respectively. Liver cholesterol and triglycerides were estimated according to (Searcy & Bergquist, 1960; Fletcher, 1968) respectively.

HYPOLIPIDEMIC ACTION OF RMR IN HYPERLIPIDEMIC ALBINO RATS

EXPERIMENTAL ANIMALS AND DIETS

Sixty four male albino rats (CFT-Wistar strain) weighing 150-180 g bred in animal house facility at the Central Food Technological Research Institute, Mysore, India were used as experimental animals as mentioned above. Four animals in control group were sacrificed immediately and analyzed for cholesterol and triacylglycerol levels in serum and liver to establish a base line lipid profile data.

DOSAGE AND GROUPING

Experimental diets were provided in accordance with American Institute of Nutrition (AIN)-76 diet formulation (American Institute of Nutrition 1977), with slight modification. Animals were divided into six groups of 10 animals each. The control group of rats (I group) were fed a normal diet of AIN-76 formulation. Group II were fed with high fat diet (HFD, cholesterol). Group III to V rats were fed with high fat diet (HFD) supplemented with 8, 12 and 16% of RMR powder, while Group VI rats were fed with HFD supplemented with lovastatin at 0.08 g/kg. Cholesterol, bile salts, RMR and standard lovastatin were supplemented in the required proportion to the control diet at the expense of corn starch. Cholesterol and bile acids were added to the diet at 1.0 and 0.15% respectively to induce hyperlipidemia in rats. Diets were prepared every week and stored at 4º C. The animals had free access to food and water *ad libitum* throughout the study. The rats were fed for a period of 14 weeks. Daily food intake and gain in weekly body weight were monitored and all the animals were observed thoroughly for the onset of any signs of toxicity. At the end of the experimental period (7^{th} and 14^{th} week), overnight fasted rats were sacrificed under mild ether anesthesia. Blood samples were collected by

cardiac puncture and serum separated by centrifugation at 6000*g*. Organs like liver, heart, brain, kidney, adipose, lungs, spleen, ovaries and testis were excised, blotted, weighed and stored at -20º C till they were processed. A portion of the liver was stored in 10% neutral formalin and processed for histological examination.

SERUM ENZYMES AND BIOCHEMICAL PARAMETERS

Serum separated from blood was used for biochemical analysis for estimation of creatinine, urea and were also analyzed for serum enzymes like SGOT, SGPT, LDH, ALP using commercial enzymatic kits

DETERMINATION OF SERUM LIPID PARAMETERS

Serum cholesterol and triacylglycerol were measured using commercial enzymatic kits. HDL cholesterol was estimated after precipitation of LDL with heparin-$MnCl_2$ reagent (Warnick & Albers, 1978).

DETERMINATION OF HEPATIC CHOLESTEROL AND TRIACYLGLYCEROL

Triacylglycerol in liver was estimated by the method of (Fletcher, 1968). Total lipids from liver after extraction were estimated by the method of (Folch *et al.*, 1957). Total cholesterol content of liver was estimated by the method of (Searcy & Bergquist, 1960).

3-HYDROXY-3-METHYLGLUTARYL-COA REDUCTASE ACTIVITY

PREPARATION OF MICROSOMES

Immediately after sacrificing the rats, liver was removed and placed in cold buffer (0.1 M triethanolamine HC1, 0.02 M ethylenediamine tetraacetic acid (EDTA) and 2.0 mM dithiothreitol, 0-4° C, pH 7.4). The liver homogenate was centrifuged at 4º C for 10 min at 12,000g. The supernatant was carefully removed to prevent contamination with mitochondria and recentrifuged at 4º C for 10 min at 12,000g. To ensure complete removal of mitochondria, supernatant was once again centrifuged at 4º C for 60 min at 60,000g (Sigma Laboratory Centrifuge 3K30, Germany). The microsomal pellet was rinsed with buffer and frozen in a freezer (-20° C). Frozen microsomes resuspended in 0.1 M triethanolamine buffer, pH 7.4, containing 0.02 M EDTA and 10 mM dithiothreitol were allowed to stand in ice pack for 60 min and centrifuged at 4° C for 45 min at 60,000g. Microsomes, either freshly prepared or frozen, treated with 10 mM DTT were resuspended in 2 ml of cold buffer (0.1 M triethanolamine HC1, 0.02 M EDTA, 2 mM dithiothreitol, 4° C, pH 7.4). Protein concentrations were determined with Folin Ciocalteu's phenol reagent using bovine serum albumin as a standard. The microsomes to be used for the assay were diluted with buffer to give a protein concentration of 5-10 mg/ml.

For the assay of HMG-CoA reductase, the reaction mixture contained 0.5-1.0 mg of microsomal protein, 150 nmoles of HMG-CoA, 2 μmoles of NADPH and 0.8 ml of 0.1 M triethanolamine in 0.02 M EDTA buffer (pH 7.4) without dithiothreitol. The final incubation volume was made up to1 ml. After incubation at 37° C for 30 min, 20 μl of 0.02 M sodium arsenite solution was added to facilitate removal of soluble proteins. Exactly after a min the reaction was terminated by adding 0.1 ml of 2.0 M citrate buffer (pH 3.5) containing 3% sodium tungstate to give a final pH of 4.0 and to precipitate the microsomal proteins. This reaction mixture was kept in water bath (37° C, 10 min) and centrifuged at 25,000 g for 15 min to remove proteins. 1 ml of the supernatant was transferred to a stoppered tube and the pH was adjusted to 8.0 just before the assay by addition of 0.2 ml 2.0 M Tris buffer (pH 10.6) and 0.1 ml of 2.0 M Tris buffer (pH 8.0).

The formation of the dithiol-arsenite complex was completed in 3-4 mins after the addition of 50 µl of 0.4 M sodium arsenite. The concentration of monothiol was determined by reacting DTNB with the reaction mixture. 20 µl of 3 mM DTNB in 0.1 M triethanolamine-0.2 M EDTA buffer (pH 7.4) was added to 1.0 ml of the reaction mixture in a 1.0-ml cuvette placed in a spectrophotometer. The contents were mixed and the absorbance was measured for 4 min at 412 nm (Hulcher & Wayne, 1973).

ANTIOXIDATIVE DEFENSE MECHANISM OF RMR IN OXIDATIVELY STRESSED MALE ALBINO RATS FED ON HIGH FAT DIET (HFD)

Sixty male albino rats (CFT-Wistar strain) weighing 150-180g bred in animal house facility at Central Food Technological Research Institute (CFTRI), Mysore, India were used as experimental animals. Animals were grouped by randomized design on a body weight basis and kept individually in stainless steel cages with 12h light/dark cycles. The relative humidity (60%) and temperature (25±2º C) was maintained for 14 weeks period.

TREATMENT GROUP AND DOSAGE

Experimental diets were provided in accordance with American Institute of Nutrition (AIN)-76 diet formulation (American Institute of Nutrition 1977) with slight modification as mentioned in Materials and Methods. The rats were given free access to food and water *ad libitum* throughout the study. Daily food intake and gain in weekly body weight were monitored. All the animals were observed thoroughly for onset of any signs of toxicity.

SAMPLING

Animals were anesthetized and sacrificed under mild ether anesthesia. Blood samples were collected by cardiac puncture and serum separated by centrifugation at 6000Xg for 15 mins and stored at -80° C until analysis. The liver tissue was quickly removed and rinsed frequently in 0.8% NaCl solution for eliminating any blood. This was homogenized in ice-cold phosphate buffer saline (PBS) and centrifuged (8000Xg, 15 min). The supernatant was collected and stored at -80° C for the assay of antioxidant molecules (total thiols, glutathione and ascorbic acid), superoxide dismutase (SOD), Glutathione reductase (GR), Glutathione peroxidase (GPx) and Catalase (CAT) activity.

DPPH RADICAL SCAVENGING ACTIVITY

The DPPH radical scavenging activity (Blois, 1958) of methanolic extract of RMR was measured accordingly (Moon & Terao, 1998). The IC_{50} was determined using different concentrations (20-120 μg ml^{-1}) of methanol extract (1 mg ml^{-1}). The mixture was shaken vigorously and incubated at room temperature for 30 mins. The absorbance of the solution (change in colour from deep violet to light yellow) was measured at 517 nm. Blank and control were maintained without DPPH and sample respectively. Antioxidant activity was calculated with the following formula.

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

ESTIMATION OF TOTAL THIOLS, GLUTATHIONE AND ASCORBIC ACID

Total thiols in serum and liver were measured spectrophotometrically by using Ellman's reagent according to (Sedlock & Lindsay, 1968). Glutathione was estimated by using Ellman's reagent according to (Beutler *et al.*, 1963). Ascorbic acid was estimated spectrophotometrically by measuring the 2, 4-dinitrophenylhydrazone derivative of dehydroascorbic acid according to (Omaye *et al.*, 1973).

DETERMINATION OF LIPID PEROXIDATION IN LIVER AND SERUM

Serum lipid peroxides were estimated by the fluorimetric measurement of thiobarbituric acid (TBA) complex. The fluorimetric measurement was carried out at an excitation wavelength of 515 nm and an emission wavelength of 553 nm (Yagi, 1984). Lipid peroxidation in liver was estimated by thiobarbituric acid reactive substances (TBARS) method (Ohkawa *et al.*, 1979). TBARS complex were measured by their reactivity with TBA in an acidic condition to generate pink coloured chromophore which was read at 532 nm and quantitated using extinction co-efficient of $1.56X10^{-5}$ cm^{-1}. Both serum and liver peroxides were compared with the standards prepared by reacting 0.5 nanomoles 1, 1, 3, 3-tetraethoxy-propane with TBA reagent.

DETERMINATION OF ANTIOXIDANT ACTIVITIES IN LIVER AND SERUM

Glutathione Reductase (GR) activity in serum and liver was estimated by the formation of 2-nitro-5-thiobenzoic acid (yellow color compound) when dithionitrobenzoic acid was added to compounds containing sulfhydryl groups according to the method of (Ellman, 1959). The yellow color developed was read at 412 nm. Glutathione peroxidase (GPx) activity in serum and liver was determined by following NADPH oxidation in a coupled reduction system consisting of hydrogen peroxide and oxidized glutathione as described by (Flohe & Gunzler, 1984 a) (Flohe & Otting, 1984 b). Catalase (CAT) activity in serum and liver homogenate was determined based on the reduction of hydrogen peroxide according to the method of (Aebi, 1984). Fifty microliters of diluted sample and 1 mL of 30 mmol/L of hydrogen peroxide (H_2O_2) were added to 1.95 mL of 50 mmol/L phosphate buffer, pH 7.0. Change of absorbance at 240 nm was recorded for 30 seconds at 30° C by spectrophotometer. CAT activity was expressed as nanomoles of H_2O_2 consumed per minute per milligram of protein. Superoxide dismutase (SOD) activity in serum and liver homogenate was measured by the inhibition of cytochrome c reductase mediated by superoxide anions generated by

xanthine-xanthine oxidase and monitored at 550 nm (Flohe & Otting, 1984 b). Protein concentrations in liver homogenate were determined using (Lowry *et al.*, 1951).

STATISTICAL ANALYSIS

Data obtained in the experiment were subjected to analysis of variance (ANOVA). Means were separated using Duncan's test for multiple comparisons. Statistical significance was determined based on $P<0.05$. These analyses were performed by using statistical analysis software SPSS, release 9.0 (SPSS, Inc.).

EFFECT OF *M. PURPUREUS* PIGMENT ON LACTOBACILLUS BACTERIA CULTURAL CONDITIONS

Five strains of lactic acid bacteria namely *Lactobacillus acidophilus* B4496 (La), *Lactobacillus bulgaricus* CFR 2028 (Lb), *Lactobacillus casei* B1922 (Lc), *Lactobacillus plantarum* B4495 (Lp) and *Lactobacillus helviticus* B4526 (Lh) were maintained in MRS Agar stabs. After two successive transfers of the test organisms in MRS broth at 37° C for 12-15 h, the activated cultures were again inoculated into MRS broth and incubated at 37° C for 16 h. It was diluted to obtain a population of 6-7 log10 CFU/ml and served as the inoculum. 16 h old LAB suspension with the OD_{600} of 1.0 was used as inoculum and incubated at 37° C for a period of 24 h.

EFFECT OF *M. PURPUREUS* PIGMENT ON VIABLE COUNT OF LAB

Viable cell counts of five strains of LAB and its combination with *M. purpureus* pigment were determined in duplicate using the pour plate method on MRS agar medium. Two set of experiment was conducted; one set containing only LAB and another set in combination with *M. purpureus* pigment. One ml of fermented rice extract in DMSO in combination with 1 ml of LAB was added to 8 ml of sterile 0.85% saline (w/v) and

vortexed for 30 sec. The resulting suspension was serially diluted with sterile 9 ml saline and 1 ml of the appropriate dilution was used for selective enumeration by pour plate technique. The cell growth of each organism was assessed by enumerating bacterial population on MRS agar after 24h of fermentation. Plates containing 25 to 250 colonies were counted and recorded as colony forming units (CFU). Same experiment was carried out with LAB cultures.

ANALYSIS OF ISOFLAVONES

Isoflavones were analysed in *M. purpureus* RMR. The analysis of isoflavones was carried out according to (Chiou & Cheng, 2001). In brief, fermented rice (1 g) mixed with 4 ml of methanol was vortexed in a screw cap tube and incubated at 70° C for 30 min. During incubation, the tubes were shaken manually at 5-min intervals. The tubes were centrifuged at 18,000 rpm for 30 min at 20° C. One ml of sample withdrawn from the middle layer was membrane-filtered (0.45 µm) and 20 µl of sample was injected into the HPLC system (Shimadzu, Japan, LC 10A UV-Vis detector 265 nm) for analysis. A reversed-phase water C18 Column (Spherisorb ODS 2, 4.6 X 250 mm) was used. A gradient solvent system started with 20% solvent A (methanol) and 80% solvent B (water) and progressed to 80% of A and 20% of B within 16 min followed by holding for an additional 2 min was followed with a flow rate of 1ml/min. Peaks were identified with the help of standards injected under similar conditions.

ANTIOXIDANT ACTIVITY OF LAB GROWN WITH *M. PURPUREUS* PIGMENT

The antioxidant activity of *M. purpureus* pigment and also LAB fermented with *M. purpureus* pigment was assessed using different methods like 2, 2-diphenyl-1-picrylhydrazyl free radical scavenging assay, inhibition of ascorbate autoxidation, and measurement of reducing activity as described above (Materials and Methods).

CHAPTER 1

METABOLITES OF *MONASCUS PURPUREUS* AND ITS MUTANTS

The species of the genus *Monascus, M. pilosus, M. purpureus, M. ruber* and *M. floridanus,* belong to the class A*scomycetes* and family *Monascaceae. Monascus* is a source for several metabolites of polyketide origin. Polyketides are synthesized by successive condensation of acetate, propionate, molanate and butyrate, due to polyketide synthase (PKS). The resulting linear chains, formed after several cycles of reactions, carry unreduced keto groups attached to β-carbons. The occurrence of these keto groups at many of the alternate carbon atoms gives rise to the name 'polyketides' (Hoopwood, 1997).

China, for thousands of years, is known for a traditional food additive called Red mould rice (RMR). RMR has also been used in food and folk medicines in many Asian countries. The traditional method of making RMR is to ferment a bed of cooked, nonglutinous whole rice kernels with *Monascus* sp. Secondary metabolites produced by the filamentous fungus *M. purpureus,* during fermentation include red, orange and yellow pigments (Su & Huang, 1976), dihydromonacolins, monacolin K, a HMG-CoA reductase inhibitor (Endo, 1979), γ-aminobutyric acid (GABA) an antihypertensive metabolite (Kono & Himeno, 2000). Dimerumic acid, an antioxidant (Aniya *et al.*, 2000), and metabolites with antibacterial (Wong & Koehler, 1981), antitumor (Yasukawa *et al.*, 1994) and immunosuppressive properties (Martinkova *et al.*, 1999). Addition of turmeric into the medium increased inherent levels of antioxidant and anti-inflammatory capacities of *M. pilosus* fermented products. The modified *M. pilosus* fermented product also demonstrated a higher antiatherosclerotic value (Kuo *et al.*, 2009b).

Dihydromonacolins are structural analogs to monacolins. Dihydromonacolin-MV derived from the methanolic extract of *M. purpureus* contained strong 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity and inhibition of lipid peroxidation in

a liposome model (Dhale, 2007c). Dihydromonacolin-L was also isolated from the culture of *M. ruber* and was identified as a potent inhibitor of cholesterol biosynthesis (Endo, 1985). A hyper pigment producing mutant of the *M. purpureus* isolated after UV irradiation was found to produce a monacolin with antibacterial properties (Dhale *et al.*, 2007). Monacolin K, one of the secondary metabolites of *M. purpureus* in RMR has been considered to be an anti-hyperlipidemic agent, since due to competitively inhibiting HMG-CoA reductase, enzyme involved in *de novo* cholesterol biosynthesis in liver. In male and female rats fed for 14 weeks, RMR of *M. purpureus* MTCC 410 decreased cholesterol and triglyceride levels in serum and liver (Mohan Kumari *et al.*, 2009). RMR also contain unsaturated fatty acids that help to reduce serum lipids, total cholesterol, triglyceride (Wang *et al.*, 1997; Qin *et al.*, 1999a) and phytosterols such as β-sitosterol and campesterol (Heber *et al.*, 1999). The effect is probably due to interference with cholesterol absorption in the intestine (Moghadasian & Frohlich, 1999). The combination of such dietary sterols with statin drugs has also been suggested as a more effective means of lowering cholesterol than statin alone (Plat & Mensink, 2001). Since RMR possesses properties for lowering cholesterol level and blood pressure, it has been developed as a health food in human dietary supplements (Manzoni *et al.*, 1999). The oral administration of *Monascus* extract dramatically inhibited the metastatic ability of murine Lewis lung carcinoma cells in syngeneic C57BL/6 mice due to decline of serum vascular endothelial growth factor (VEGF) levels compared to untreated metastatic groups. Monacolin K is a key antimetastatic and antiangiogenesis compound in *Monascus* extract, as shown by down-regulation of VEGF-stimulated invasive activity in LLC cells. Application of RMR may serve as a nontoxic natural chemopreventive or antineoplastic agent in the development of cancer adjuvant chemotherapy (Ho & Pan, 2009).

M. purpureus pigments of polyketides origin are used as food colorants. Lovastatin, a HMG CoA reductase inhibitor, produced by *M. purpureus* is widely used as a hypercholesterolemia drug. Phytosterols of this fungus interfere with cholesterol absorption in the intestine and there by reduces the cholesterol uptake. Unsaturated fatty acids reduce serum lipids, total cholesterol and triglyceride levels. These findings have

promoted the use of *Monascus* in food products as dietary supplements and as medicine. Since metabolites of *Monascus* have several applications, *M. purpureus* and its mutants were screened for characterising useful compounds produced by them.

1.1. POLYKETIDES OF *M. PURPUREUS* MTCC 410 AND ITS MUTANTS

M. purpureus polyketides (pigments, azophilones) are similar in molecular structure and chemical properties. In the fungal cytosol, they are synthesized due to condensation reaction of acetyl Co-A with malonyl Co-A. Chemical modifications in the lactone rings of the primary product give rise to typically coloured polyketides like orange; monascorubrine and rubropunctatin, yellow; ankaflavin and monascin and red; monascorubramine and rubropuntamine.

M. purpureus produces the pigments when grown in shake flask and solid-state culturing conditions. In order to ascertain their production by *M. purpureus* MTCC 410 and its mutants, the fungi were grown in shake flask containing the synthetic media made of maltose as carbon source and MSG as nitrogen source. The procedure for assaying pigment produced was as follows. The pigment from mycelia were extracted with 95% ethanol **(Figure 11)** and measured at 500, 475 and 375 nm for red, orange and yellow pigment respectively.

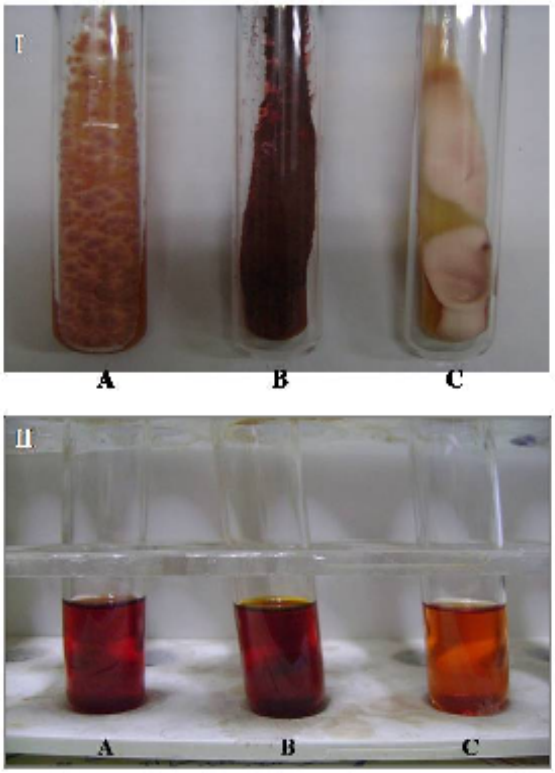


Figure 11. I. *M. purpureus* MTCC 410 (A); CFR 410-11(B); CFR 410-22 (C) and II. Mycelial ethanol extract of pigment.

M. purpureus MTCC 410 produced more red pigment (202.8 OD units) compared to orange (183.4 OD units) and yellow pigments (120.3 OD units) when grown in shake flasks. Similarly the mutant CFR 410-11 also produced significantly increased red pigment **(Table 5)** in shake flasks cultures. There was a 207% increase in pigment production by mutant CFR 410-11. However, CFR 410-22 showed decrease pigment production (27%) compared to the wild type (**Figure 12**).

Table 5 Polyketide pigment produced by *M. purpureus* MTCC 410 and mutants in shake flask

	OD Units /g dry cell mass			
Strain	Red (500 nm)	Orange (475 nm)	Yellow (375 nm)	Total pigments
MTCC 410	202.8±12.56	183.4±9.86	120.3±2.23	506.5
Mutant 1 (CFR 410-11)	382.0±11.95	343.2±8.99	324.8±2.59	1050.0
Mutant 2 (CFR 410-22)	146.5±12.06	115.0±9.56	109.8±2.76	371.3

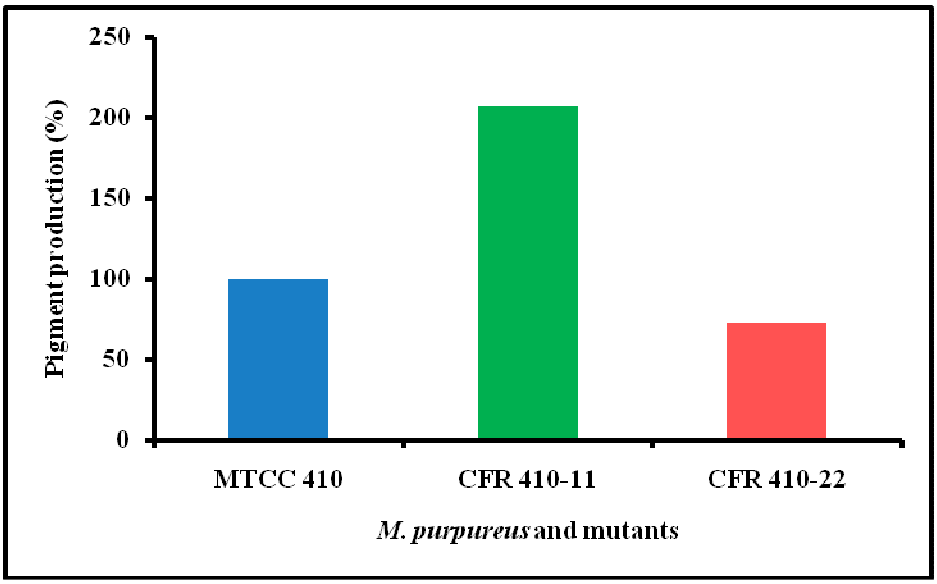


Figure 12. Quantities of pigment produced by mutants of *M. purpureus* MTCC during shake flask cultivation.

Solid-state fermentation was also carried out on rice (Materials and Methods). 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° C (Adolf Khuner

Therm-Lab Switzerland) for 11 days. The flasks were shaken periodically to ensure uniform mixing. The total polyketide pigments from RMR were extracted using ethanol as detailed in Materials and Methods.

Rice upon fermentation by *M. purpureus* appears to be red in colour **(Figure 13)**. The mutant CFR 410-11 over produced polyketide pigments (1148 OD Units) compared to *M. purpureus* MTCC 410 (604 OD Units). The mutant CFR 410-22 showed low quantity of polyketide pigments (473 OD Units) compared to MTCC 410 **(Figure 14)**.

Figure 13. Rice fermented with *M. purpureus* MTCC 410

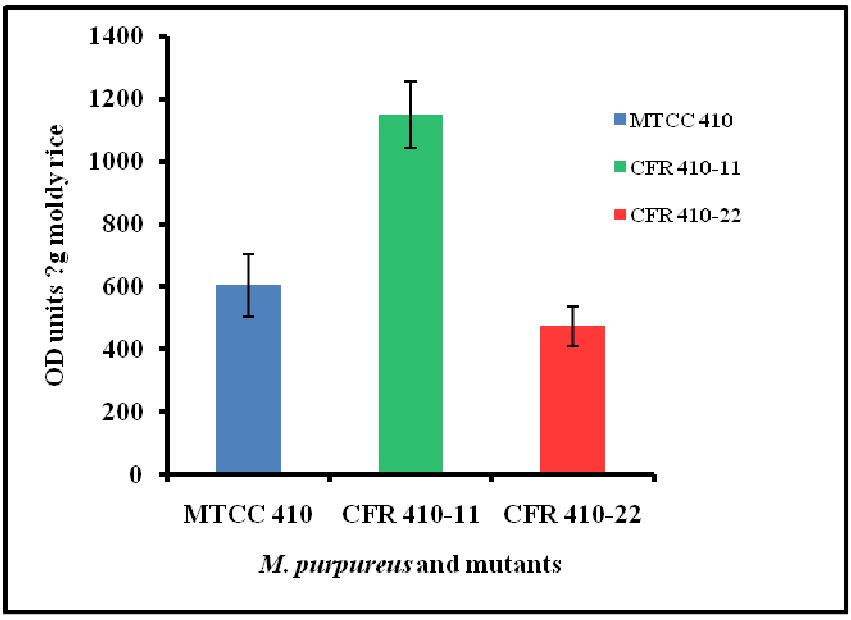


Figure 14. Total polyketide pigments produced by *M. purpureus* and its mutants

Polyketide pigment yield was higher in solid state as compared to shake flask cultures. The higher yield of pigments is due to release of synthesized pigment from cytoplasm to substrate (usually rice grains). However, during shake flask cultivation the pigments are mainly retained intracellularly, inhibiting production.

1.2. EXTRACTION OF THE PIGMENTS BASED ON POLARITY

The previous experiment showed higher pigment production by *M. purpureus* mutant (CFR 410-11) when grown in solid-state cultures made of rice. In order to quantify the total productivity of the pigment step wise extraction based on polarity was followed (Dhale, 2007c). The polyketide pigments from RMR were extracted step wise using different solvents as detailed in Materials and Methods.

Polyketide pigments were extracted from fermented rice based on their polarity using hexane, benzene, chloroform, acetone, ethanol and water. Since RMR extracted with hexane showed maximum absorption at 375 nm (115.20 OD units) it indicated the presence of yellow pigment. After hexane extraction, the RMR was extracted with benzene and chloroform. These fractions have also shown maximum absorption at 375 nm with OD units of 33.16, and 19.27 respectively. Thus all the yellow pigments were extracted only by one solvent **(Table 6a).** After chloroform extraction, RMR treated with acetone resulted in a fraction with highest absorption at 500 nm (126.13 OD units)**.** The absorbance of ethanol extract was like that of acetone with maximum absorption at 500 nm **(Table 6a).**

Similar results were observed when the polyketide pigments were extracted from mutants. The increase in concentration of yellow pigment **(Table 6b and c)** was observed in CFR 410-11 (674.79 OD units) and CFR 410 -22 (243.65 OD units). Among the two mutants, red, orange and yellow pigments produced were more in CFR 410-11 compared to wild type and CFR 410 -22 **(Table 6)**.

These results indicate that, *M. purpureus* and its mutants produced more pigment when grown on solid substrate compared to that grown in shake flasks.

Table 6. Polyketide pigments produced by *M. purpureus* MTCC 410 and its mutants during SSF.

Solvent	Red (500 nm)	Orange (475 nm)	Yellow (375 nm)
Hexane	1.114	2.556	115.205
Benzene	5.734	7.662	33.168
Chloroform	4.625	5.262	19.279
Acetone	126.13	100.1	83.4
Ethanol	28.51	22.32	8.35
Water	6.340	6.561	7.803
Total	172.453	164.461	267.205

A

Solvent	Red (500 nm)	Orange (475 nm)	Yellow (375 nm)
Hexane	5.264	3.652	356.01
Benzene	8.562	9.125	126.125
Chloroform	6.245	10.256	67.241
Acetone	205.23	128.91	95.78
Ethanol	55.262	32.15	18.35
Water	9.125	10.65	11.289
Total	289.688	194.653	674.795

B

Solvent	Red (500 nm)	Orange (475 nm)	Yellow (375 nm)
Hexane	1.235	0.965	125.3
Benzene	4.359	5.281	52.37
Chloroform	1.026	3.268	25.26
Acetone	95.32	25.368	26.358
Ethanol	59.67	21.59	9.135
Water	5.262	6.35	5.233
Total	166.872	62.822	243.656

C

1.3. STATINS OF *M. PURPUREUS*

Statins, due to their ability to influence the *de novo* synthesis of endogenous cholesterol have pharmaceutical applications. Many fungi produce them as secondary metabolites. Lovastatin, a well known inhibitor of HMG-CoA reductase, is reportedly produced by *A. terreus, M. purpureus* and other fungi. Since statins possess a common main polyketide portion, a hydroxyhexahydro naphthalene ring to which different side chains are linked at C6 and C8. *M. purpureus* can be cultivated for production of the drug.

Preliminary experiments showed that statin was identified in the mycelia of *M. purpureus* MTCC 410 when grown in rice powder as carbon source and peptone as nitrogen source. Hence the seed culture of the fungus and its mutants prepared by growing them in PDB for four days at 30° C was used to inoculate 100 ml rice powder medium. After 10 days growth at 30° C in a rotary shaker **(Figure 15)**, the mycelium obtained after filtration was used as a source for statins. Statins were extracted using ethanol. The details are described in Materials and Methods.

Figure 15. Ten day old shake flask culture of *M. purpureus* MTCC

Statins (lovastatin and pravastatin) from the mycelia of *M. purpureus* MTCC 410 and mutants, extracted with ethanol, were identified after TLC and HPLC analysis. For TLC, pravastatin and lovastatin standards obtained from sigma were used. After performing TLC, with the solvent system made of dichloromethane and ethyl acetate (70:30), R_f values were determined in relation to the standards. Standards showed an R_f values of 0.52 and 0.39 for pravastatin and lovastatin respectively. Spots appearing at this R_f, with the ethanol extract of *M. purpureus* identified (**Figure 16)** the presence of two statins. Hence for conformation, HPLC was carried out.

Standard pravastatin and lovastatin under standard HPLC conditions were eluted at 3.1 and 11 minutes, respectively (**Figure 17a and b)**. Ethanol extracts of *M. purpureus* MTCC 410 and its mutants when separated using C18 HPLC column also showed elution of compounds corresponding to pravastatin and lovastatin with 3.3 and 11 minutes, respectively **(Figure 17c and d)**. These results revealed the presence of pravastatin and lovastatin in the ethanol extracts of *M. purpureus* MTCC 410 and its mutants.

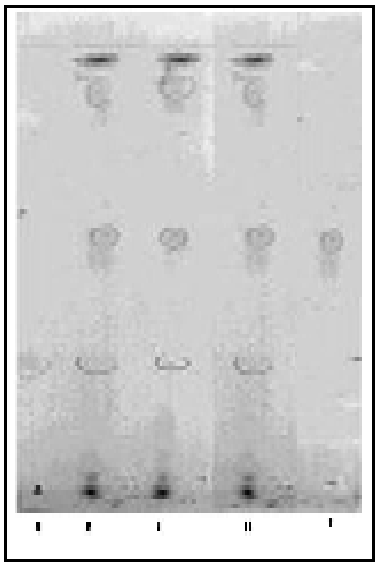


Figure 16. Ethanol extracts of *M. purpureus* for the identifications of statins; A) Standard lovastatin, B) *M. purpureus* MTCC 410, C) CFR 410-11, D) CFR 410-22, E) Standard pravastatin.

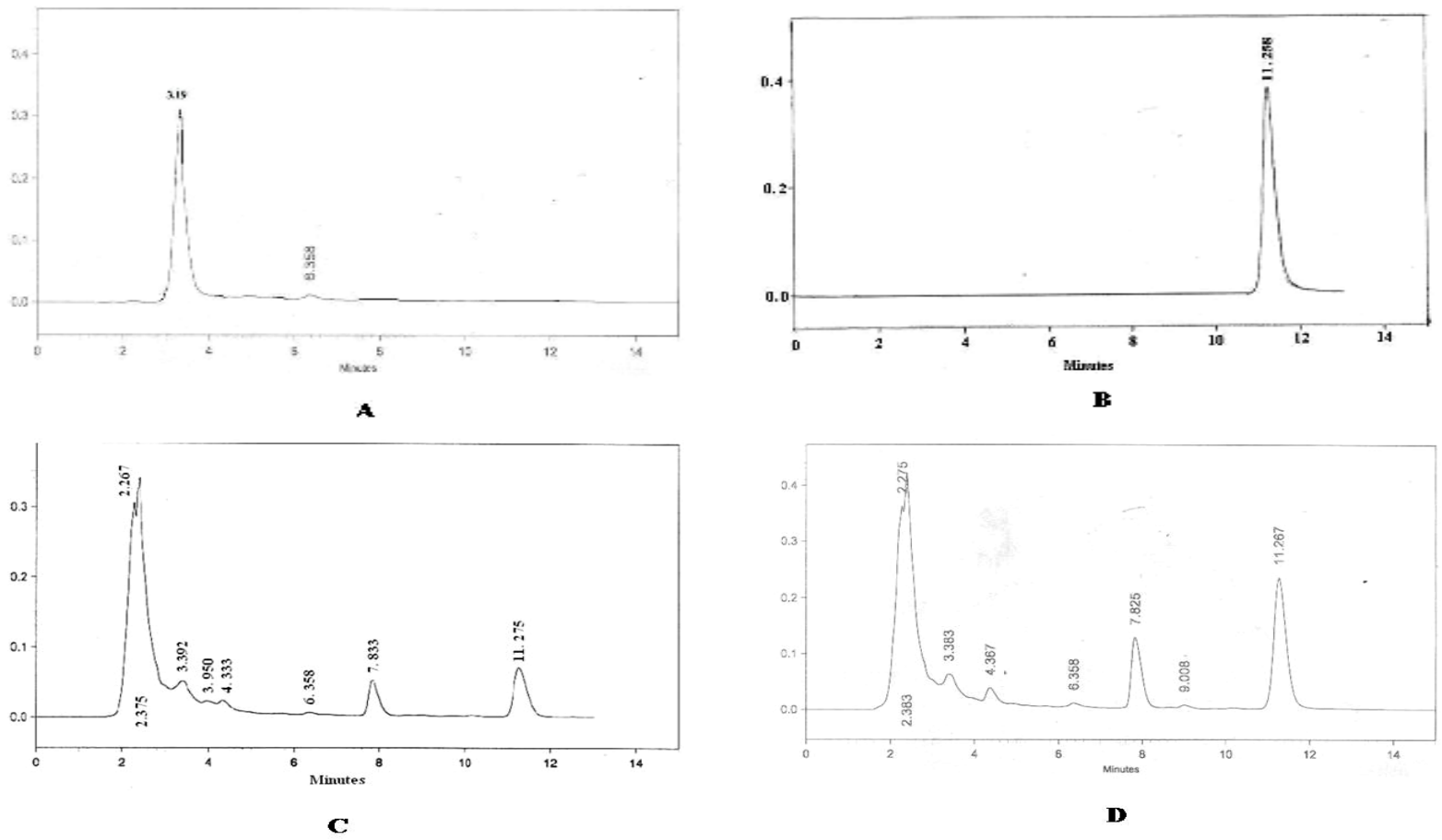


Figure 17. Identification of pravastatin and lovastatin after HPLC. A).Elution profile of standard pravastatin; B) Elution profile of standard lovastatin; C) Elution profile of extracellular statin (culture filtrate); D) Elution profile of intracellular statin.

The quantities of lovastatin and pravastatin in the biomass of *M. purpureus* MTCC 410 and mutants were also estimated by HPLC. The results showed (**Figure 18**) that the wild type had a concentration of 3.82 and 0.266 mg/g of lactone form of lovastatin and pravastatin respectively.

Of the total statins (4.57 mg/g), the wild type biomass had a high concentration of lovastatin (4.31 mg/g). Only negligible concentrations of lactone form of pravastatin (0.27 mg/g) and hydroxyl acid form of lovastatin (0.49 mg/g) were detected under the experimental conditions. Both mutants were affected in the statin biosynthesis. However, the ratio of pravastatin to hydroxyl acid form of lovastatin was higher in the mutants compared to the wild type (**Figure 18**).

The results are discussed in the latter part of the thesis.

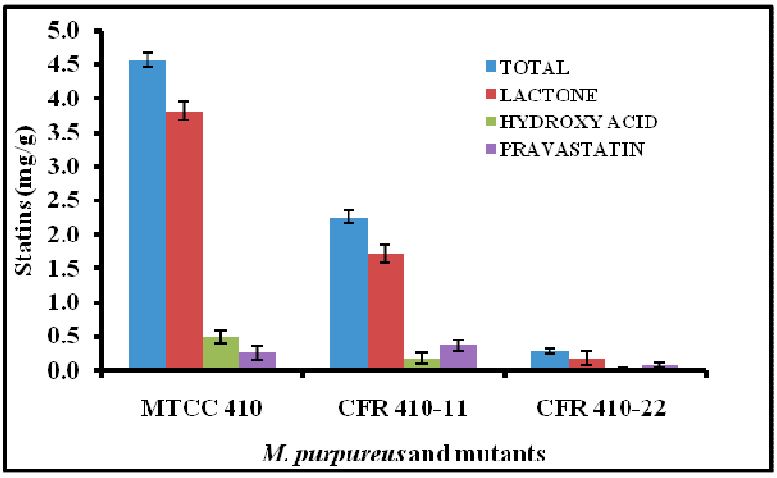


Figure 18. Statins produced by *M. purpureus* and its mutants by shake flask cultures

1.4. STATINS OF *M. PURPUREUS* AFTER SOLID-STATE FERMENTATION

In the fungal mycelia grown in solid-state conditions, more statin concentrations were estimated. HPLC quantitation showed that lovastatin and pravastatin in the *M. purpureus* wild type was 11.51 mg/g and 0.781mg/g of respectively **(Figure 19)**.

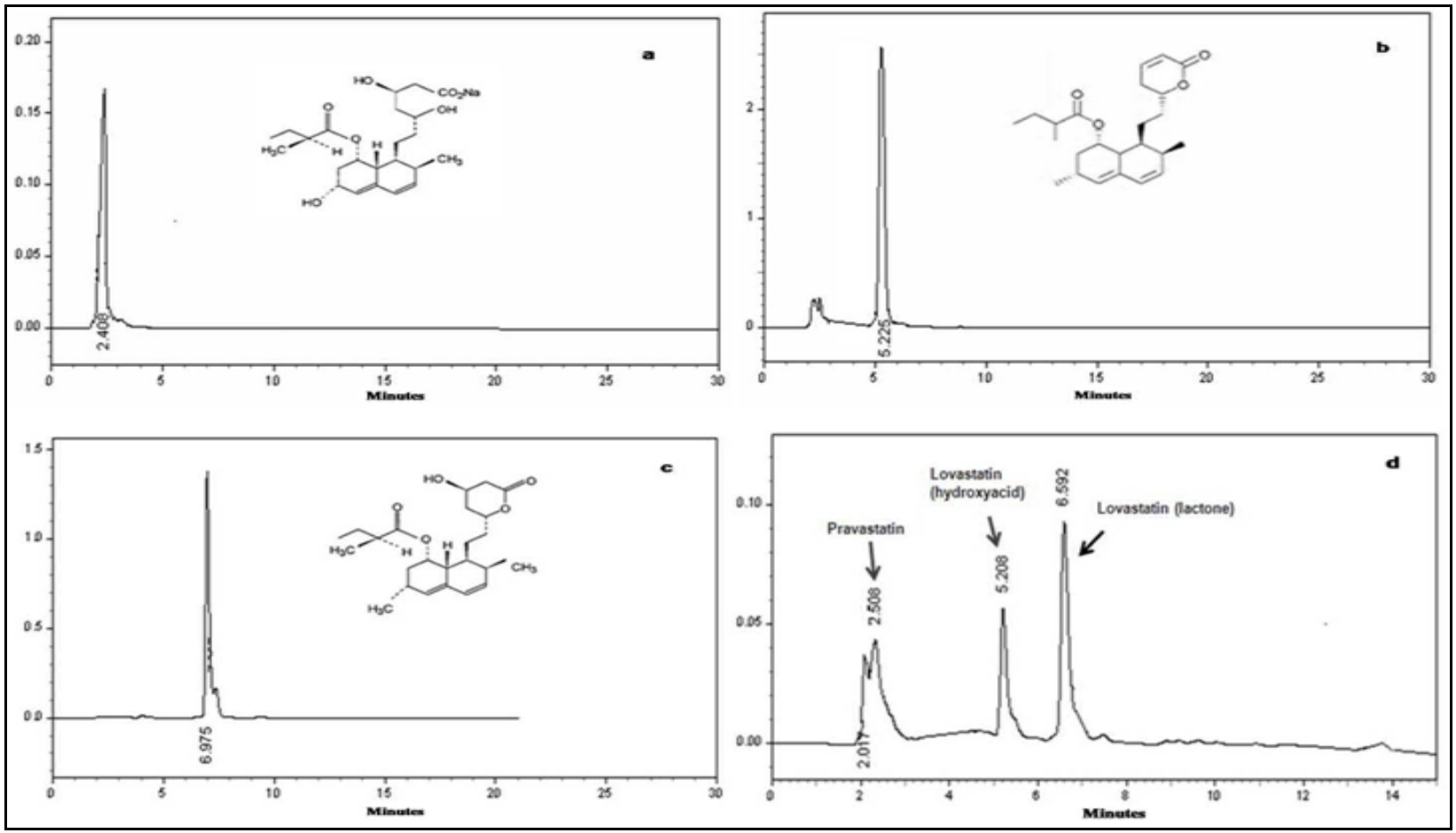


Figure 19. Identification of pravastatin and lovastatin after HPLC. A).Elution profile of standard pravastatin; B) Elution profile of standard hydroxyl acid form of lovastatin; C) Elution profile of standard lactone form of lovastatin; D) Elution profile of statins from RMR extract (d).

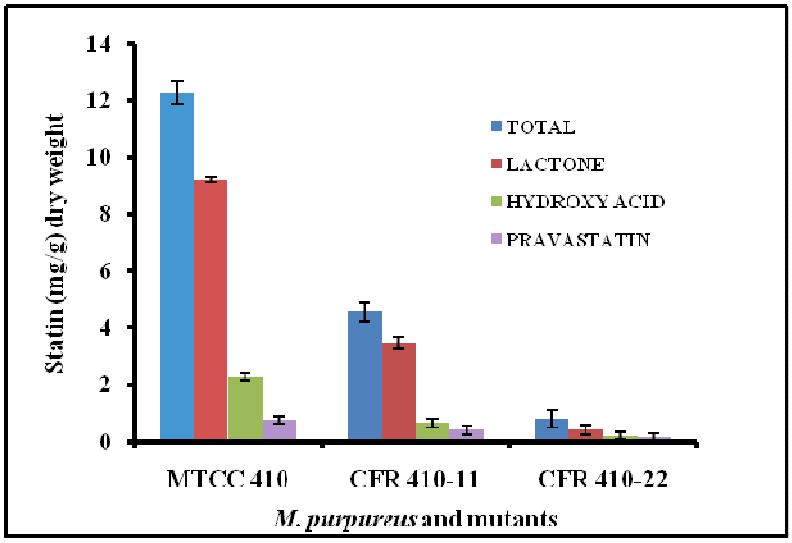


Figure 20. Statins produced during solid-state culture by *M. purpureus* and its mutants

Of the total lovastatin (12.28 mg/g), the lactone form was the major form constituting 9.23 mg/g. Very little hydroxyl acid form of lovastatin (2.27 mg/g) could only be assayed in the sample by HPLC.

Like in shake flask cultures, the concentrations of both lovastatin and pravastatin were low in the mycelia of both the mutants. Unlike in shake flasks, the ratio of hydroxyl acid form of lovastatin to pravastatin was higher **(Figure 20)**. The albino mutant (CFR 410-22) behaved as in shake flask cultures with regard to statin production **(Figure 20).**

In solid-state cultures, the fungus and the mutants appeared to synthesise substantial quantities of statins. The results are discussed (*vide* Discussions).

1.5. STEROL OF *M. PURPUREUS* MTCC 410 AND ITS MUTANTS

Sterols are essential for cell viability, fermentative ability and adaptation to temperature shifts (Piironen *et al.*, 2000). Sterol production in fungi during shake flask growth is dependent on oxygen availability and age of the mycelium (Jean &

Eric, 2001). However there are no reports on the effect of solid-state growth condition on fungal sterols.

Sterols, like the statins, are synthesized from acetyl CoA precursor. Since the previous experiment showed diversion of some of the precursors for the synthesis of statins, the effect of growth condition on *M. purpureus* total sterol was studied.

M. purpureus and its mutants were grown in shake flask and solid-state cultures as described in Materials and Methods. For shake flask cultivation the inoculated flasks were incubated on a rotary shaker (220 rpm) maintained at 30º C for 5 days. The broth was filtered and the biomass harvested was dried by lyophilizer and used for extraction.

Sterol from the dried biomass (dried at 45-50° C) obtained after solid-state cultivation of the fungi on rice was used for sterol extraction. They were ground in pestle and mortor and sterols were extracted with a mixture of chloroform and methanol (2:1 v/v). The extract dried in flash evaporator was reconstituted in 1.0 ml chloroform. The total sterol was estimated by Liebermann Burchard procedure as described in Materials and Methods.

Sterols produced during shake flask cultures by *M. purpureus* MTCC 410 and its mutant are presented in **Figure 21**. MTCC 410 has produced more sterols of 0.908±0.62 mg/g compared to CFR 410-11 (0.652±0.50 mg/g) and CFR 410-22 (0.24±0.83 mg/g). The results showed a reduced sterol in mutants compared to wild type. Reduction in the concentration of total sterol content in the mutants suggested a mutation that directed the precursor towards the synthesis of pigments and lovastatin.

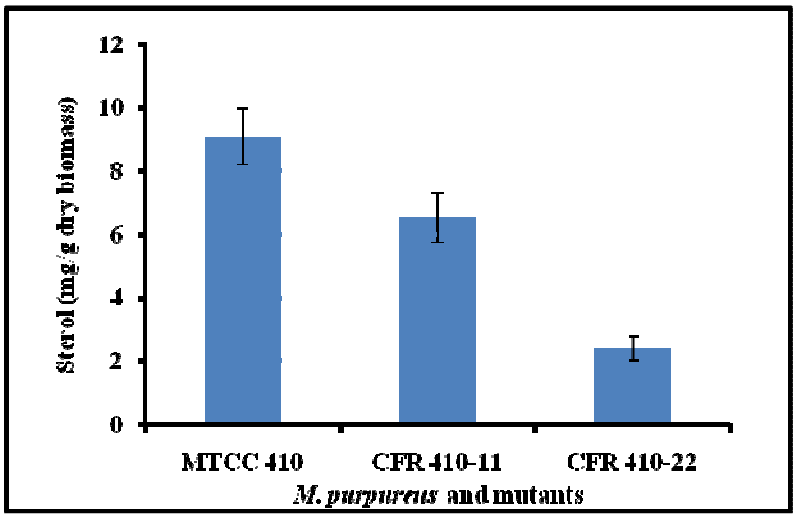


Figure 21. Sterols in the *M. purpureus* and mutants grown in shake flask.

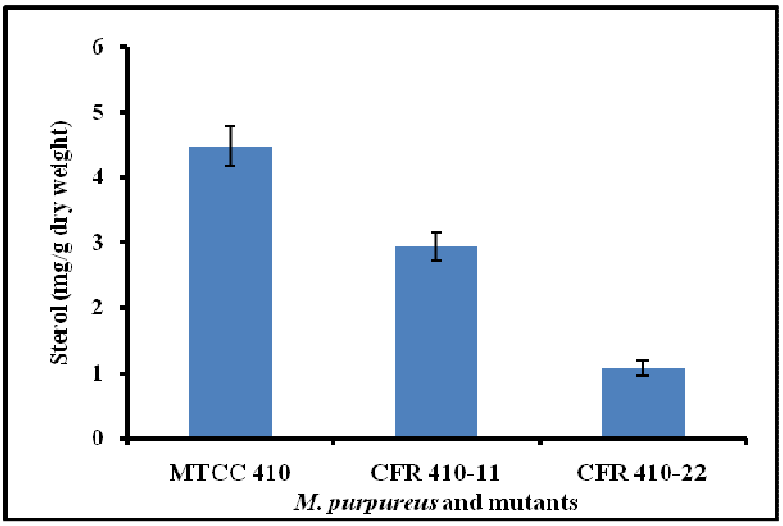


Figure 22. Sterols in the *M. purpureus* and mutants grown in solid-state.

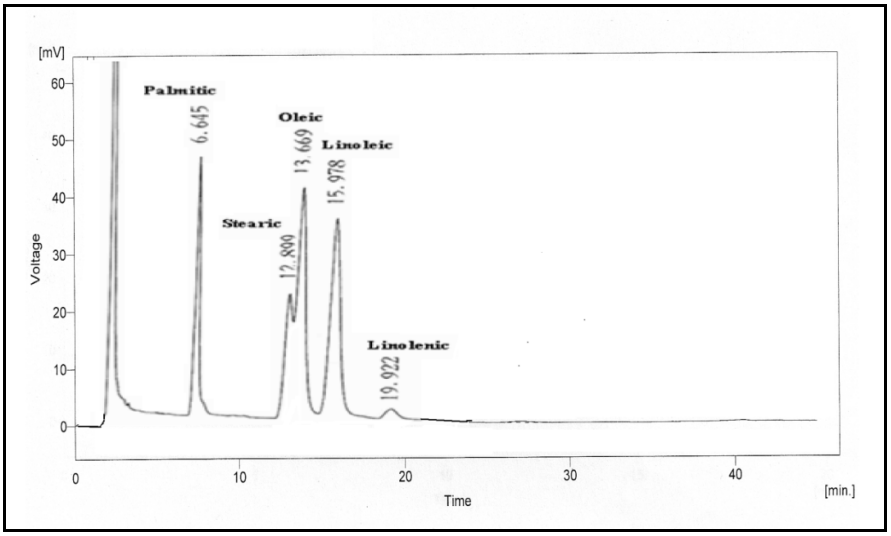
The sterol concentration in *M. purpureus* and mutants was higher in shake flask cultures compared to that estimated in the fungi grown in solid-state (**Figure 21 and Figure 22)**. Generally mutants had lower sterol concentration compared to the wild type.

The result suggests the effect of mutations on sterol biosynthesis.

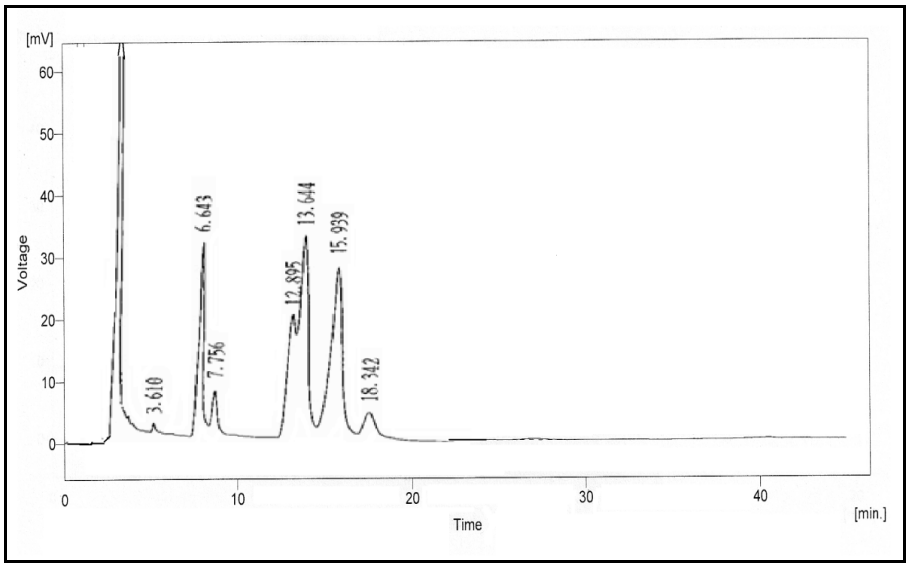
1.6. FATTY ACID PROFILE OF *M. PURPUREUS* AND ITS MUTANTS

Esential fatty acids are used to produce hormone-like substances that regulate a wide range of functions including blood pressure, blood clotting, blood lipid levels, immune response and inflamation response to injury infection. These are important in several human body systems like immune systems and in blood presure regulation, as they are invoved in synthesis of compounds like prostraglandin (Barnard, 2002). Unsaturated fatty acids such as oleic, linoleic, and linolenic acids also help to reduce serum lipids (Ma *et al.*, 2000). In order to quantitate useful fatty acids in *M. purpureus*, the profile were estimated by GC.

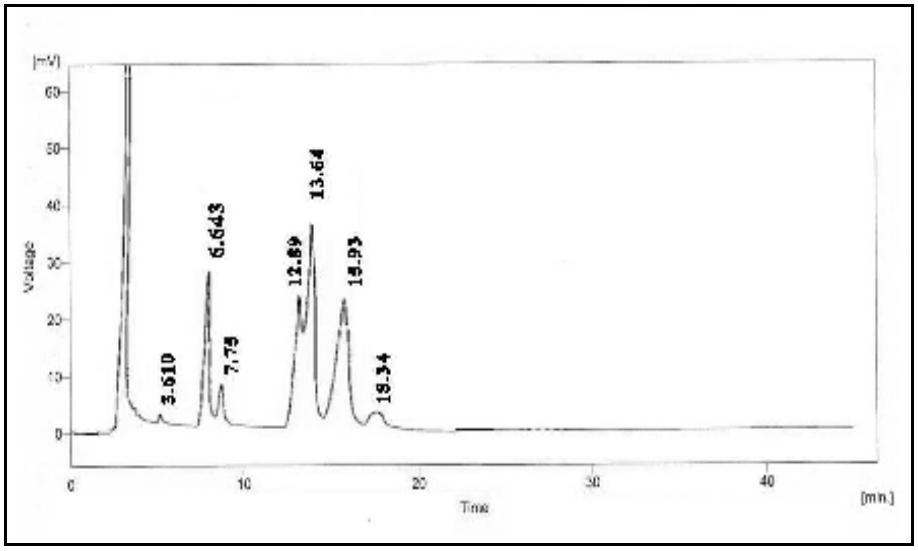
For fatty acids extraction from *M. purpureus* and mutants, the procedure followed was similar to that used for sterol extraction (Experiment 1.5). After reconstituting in chloroform, esterification was carried out with KOH (Materials and Methods). Gas Chromatography (GC) analysis was performed using Shimadzu GC-15A (DEGS packed column, with injector temperature of 220º C, detector temperature 230º C and column temperature 180º C, Materials and Methods). Each of the fatty acids was identified using the retention time with reference to that of the standard (Materials and Methods).



(a)



(b)



(c)

Figure 23. GC profiles of standard fatty acids (a), and the faty acids of *M. purpureus* MTCC 410 grown in (b), shake flasks and (c) solid-state.

Palmitic, stearic, oleic, linoleic and linolenic acids were identified as the major fatty acids in the mycelia of *M. purpureus* MTCC 410 grown in solid-state and the shake flasks (**Figure 23a, b and c)**. The mutants also showed a similar profile.

1.6.1. FATTY ACIDS OF *M. PURPUREUS* GROWN IN SHAKE FLASKS

The quantity of unsaturated fatty acids, oleic and linoleic acids, were higher. Linoleic acid concentration was more in mutant CFR 410-11 (36.0±0.20%) compared to the wild type (33.7±0.44%) and mutant CFR 410-22 (20.7±0.72%). Higher concentrations of oleic acid was estimated in CFR 410-22 (34.5±0.40%) than in CFR 410-11 (27.3±0.46%) and wild type (21.8±0.26%).

Linolenic acid was found to be higher in concentration in CFR 410-22 (15.1±0.72%) compared to CFR 410-11 (1.8±0.56%) and MTCC 410 (0.4±0.66%). The wild type had higher stearic acid concentration **(Figure 24).**

The results indicate that in the mutants, the concentration of unsaturated fatty acids were higher in than in the wild type.

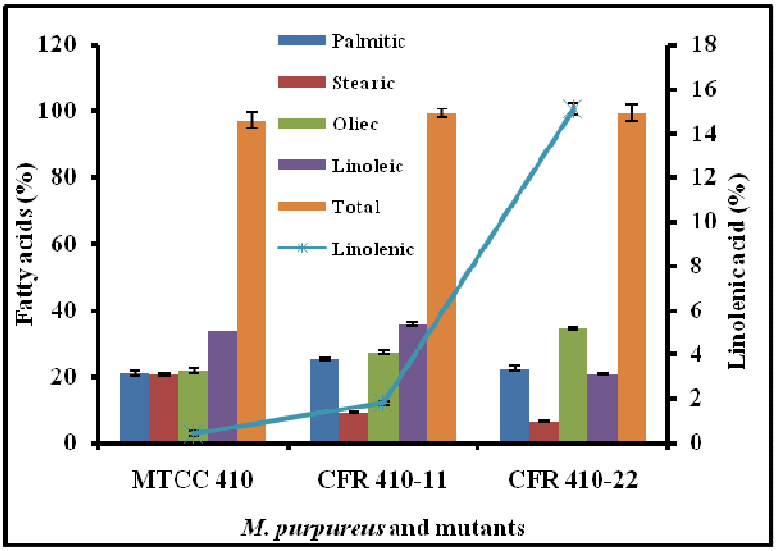


Figure 24. Fatty acid profile of *M. purpureus* and mutants (shake flask culture).

1.6.2. FATTY ACIDS OF *M. PURPUREUS* GROWN BY SOLID-STATE

Like in shake flask growth, the fungi growing on solid substrate also had higher concentrations of unsaturated fatty acids (linoleic and oleic acids) than

saturated fatty acids (palmitic and stearic acid). More of oleic acid was determined in CFR 410-22 (32.20±0.46%) compared to CFR 410-11 (31.60±0.26%) and wild type (27.90±0.90%). Linoleic acid concentration was in the following order; CFR 410-22 (29.90±0.72%) < CFR 410-11 (28.40±0.56%) < wild type (24.60±0.66%).

Linolenic acid concentration was more in CFR 410-22 (6±0.72%) compared to CFR 410-11 (5.6±0.56%) and wild type (0.2±0.66%). Only low quantities of linolenic acid were determined in the wild type. However stearic acid percentage was more in *M. purpureus* compared to its mutants CFR 410-11 and CFR 410-22 (**Figure 25**). Mutants CFR 410-22 (68.10%) and CFR 410-11 (65.50%) had higher concentration of unsaturated fatty acids than the wild type (52.70%).

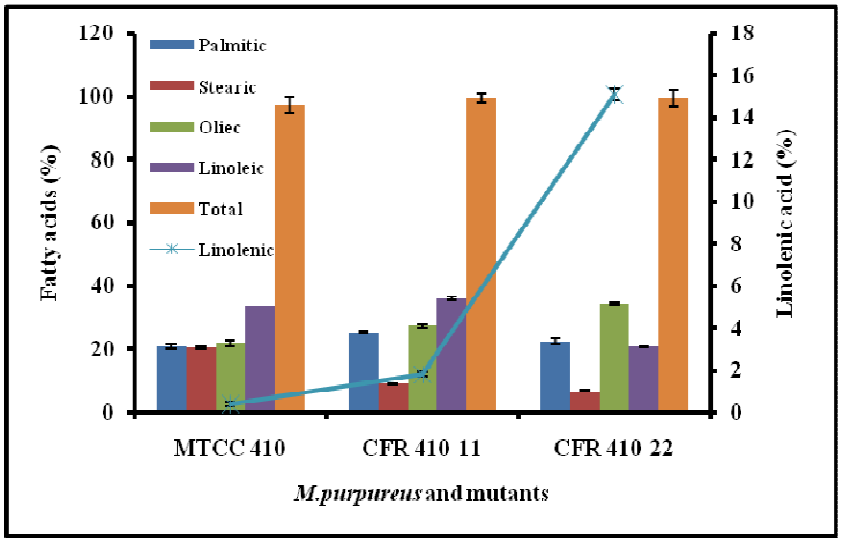


Figure 25. Fatty acid profile of *M. purpureus* and its mutants (solid-state culture).

Culture conditions did not affect fatty acid profiles of *M. purpureus* and its mutants. Presence of increased concentrations of unsaturated fatty acids in mutants revealed their possible use as a nutrient supplement.

1.7. OPTIMIZATION OF CULTURAL CONDITIONS FOR *M. PURPUREUS* STATINS

The composition of a fermentation medium influences the supply of nutrients and metabolism of cells. Accordingly the yields of a product in a fermentation process depend on the culture medium used. Of the major culture nutrients, carbon and nitrogen sources generally play a governing role in fermentation productivity because these nutrients are directly linked with the formation of biomass and metabolites. Also, the nature and concentration of the carbon source can regulate secondary metabolism through phenomena such as catabolic repression.

Lovastatin synthesis in *A. terreus* is associated with nitrogen limited growth when excess carbon is channeled for the secondary metabolite production. Hence lovastatin yield was improved in nitrogen limiting condition when growth was ceased as carbon was in excess. The variety of rice used as culture media is known to influence the statin production in *Monascus* (Chairote *et al.*, 2008). Nitrogenous compounds such as ammonia, glutamine and glutamate are preferentially used by filamentous fungi, and asparagine is a preferred nitrogen source in yeast. However, when these primary nitrogen sources are not available or are present at a concentration insufficient to support growth, other nitrogen sources like nitrate, nitrite, purines, amides, amino acids and proteins are used by fungi (George, 1997). There are various reports on the effect of nitrogen source on the production of different metabolites in fungi. Even the addition of turmeric and ginger to the fermentation media can influence the production of statin (Kuo *et al.*, 2009a)b).

The pH of the growth medium is very important because it can profoundly affect the cellular activities. Conidial fungi can grow over a wide range of pH. Most of them tolerate a pH range from 4 to 9 but grow and sporulate maximum near neutral pH (Braun & Vecht-Lifshitz, 1991). The composition of the medium can affect direction of pH drifts during growth of the fungus. Effect of pH of the medium strongly affects many enzymatic process and transport of various components across the cell membrane (Moon & Parulekar, 1991). Buckland *et al.*, (1989) reported that

because of enzyme activation or degeneration, the broth pH could play a crucial role in the behaviour of secondary metabolite production as well as cell growth of fungus.

Since *M. purpureus* MTCC 410 produced more statins compared to its mutants (previous experiments) it was used for the experiment. Growth parameters were optimized for statin production during shake flask and solid state cultures.

1.7.1 SHAKE FLASKS

Various cultural parameters including carbon and nitrogen source, temperature and pH were investigated. Production of lovastatin and microbial biomass by *M. purpureus* were influenced by the carbon and nitrogen sources. The culture conditions and extraction procedures are as described in Materials and Methods.

EFFECT OF CARBON SOURCE ON STATIN PRODUCTION

Among the different carbon sources, maltose had high contribution and effective in regulation of statin biosynthesis. Replacing maltose with glucose, fructose, lactose or sucrose as carbon sources, did not yield higher productivity of statins **(Figure 26)**. The lovastatin (lactone form and hydroxyl acid) concentration was recorded highest in culture grown in maltose (5.09 mg/g) as a carbon source, followed by glucose (2.98 mg/g), fructose (2.15 mg/g), lactose (0.91 mg/g) and sucrose (0.55 mg/g).

Glucose produced more biomass of 15.57 g/L compared to maltose (12.52 g/L). However, statin content in the mycelia was less. Very low biomass yield was obtained when grown in lactose (8.00 g/L) and sucrose (5.76 g/L).

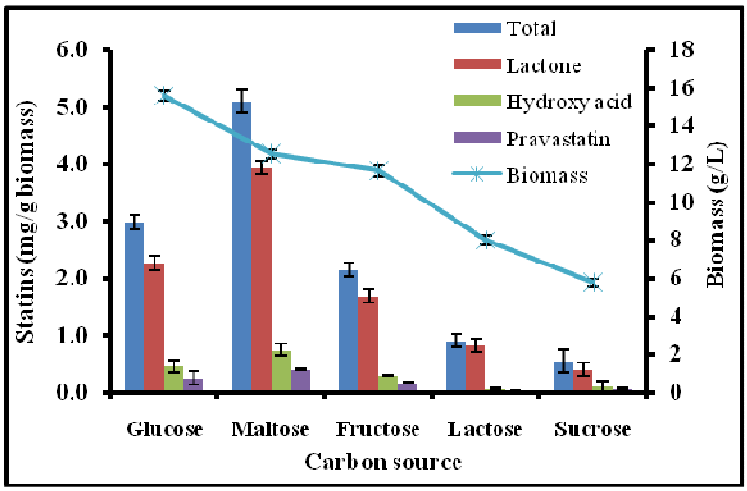


Figure 26. Effect of carbon source on statin production

EFFECT OF NITROGEN SOURCE ON STATIN PRODUCTION

Different nitrogen sources like peptone, yeast extract, ammonium sulphate, ammonium chloride and ammonium nitrate were added to the growth medium (pH 6.0) containing maltose as carbon source. Biomass was harvested after 10 day fermentation and statin extracted from mycelia was quantified as mentioned in Materials and Methods.

Among different nitrogen sources used, peptone was found to be better nitrogen source for statin yield with 5.53 mg/g compared to ammonium chloride (3.86 mg/g). Ammonium nitrate, yeast extract and ammonium sulphate when used as nitrogen sources results in very low yield of statins (3.14 mg/g; 2.78 mg/g; 2.04 mg/g**).** Ammonium sulfate did not improve statin yield since the pH of the culture broth reduced to less than 3.0 during fermentation process **(Figure 27)**. Under these conditions, only yellow and orange pigments were produced which resulted in lowest statin productivity. Use of peptone and ammonium sulphate as nitrogen source increased biomass production.

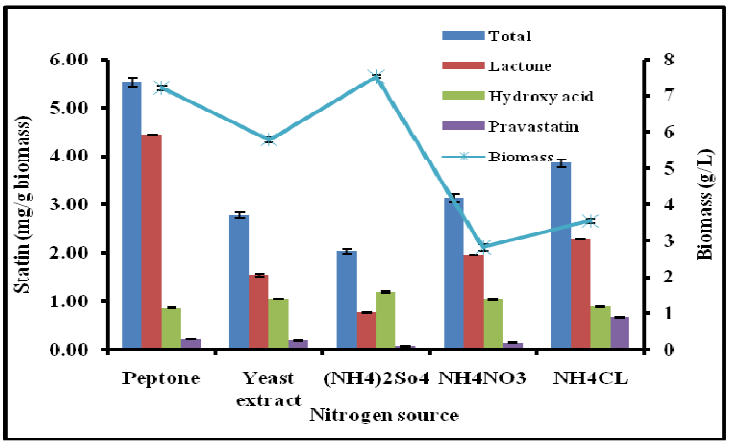


Figure 27. Effect of nitrogen source on statin production

INFLUENCE OF INITIAL PH OF THE MEDIUM ON STATIN PRODUCTION

For this experiment, maltose as carbon and peptone as nitrogen source was used. The initial pH of the medium was decreased or increased with the concentration of 0.1 N HCl or 0.01 N NaoH. The fungi grown for 10 days was harvested and statin concentration determined by HPLC (Materilas and Methods).

Reducing or increasing the pH from 5.0 decreased statin yield **(Figure 28).** Maximum statin production was recorded at pH 5 with 5.79 mg/g and was found to be optimum pH. Though pH 7.0 and 11.0 results in high biomass of 11.2 g/L and 10.55 g/L respectively, they did not contribute much for the production of statin. At acidic condition, the primary metabolite itaconic acid is produced and it is known to decrease biomass and statin. This acidic condition affects the formation of key enzymes necessary for statin production (Riscaltadi *et al.*, 2002).

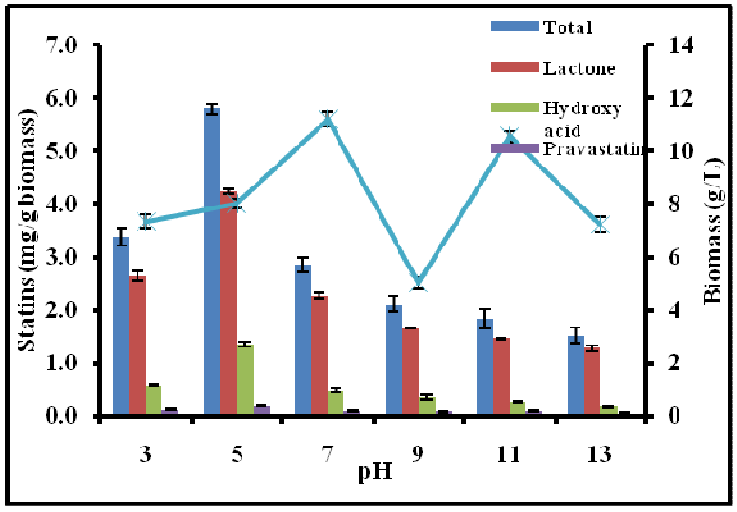


Figure 28. Effect of pH on statin production

EFFECT OF TEMPERATURE ON STATIN PRODUCTION

The primary inoculum grown at 28° C was transferred to production medium and cultivated at different temperatures (20, 25, 28, 30 and 35° C). Most favorable temperature for maximum statin production was found to be 28° C with total yield of 3.79 mg/g. Temperature below or above 28° C has altered stain production (**Figure 29).**

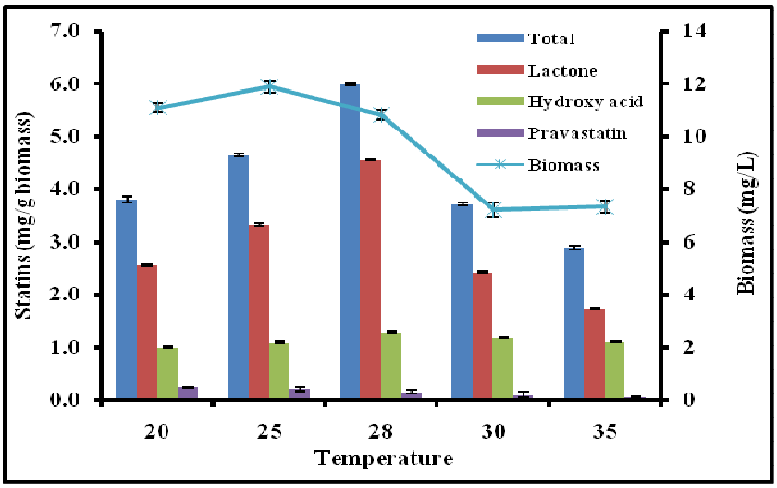


Figure 29. Effect of temperature on statin production

Thus based on the above results the growth condition favourable for statin production in shake flaks using *M. purpureus* was as follows:

Maltose	4.4%
Peptone	0.14%
pH	5.0
Temperature	28º C

1.7.2. SOLID-STATE FERMENTATION

In recent years, researchers have shown an increasing interest in solid state fermentation (SSF) as a potential alternative of shake flasks. Because it uses inexpensive substrates (agricultural residues), requires fewer processing and down-streaming stages, utilizes lesser power and generates lesser effluent (Kumar *et al.*, 2003). Moreover, SSF has higher product yield and offers better product stability.

Fermentation process parameters such as temperature, fermentation time periods, carbon, nitrogen and pH of the solid medium are selected for statin production. To identify the optimum levels of different process parameters influencing statin production, solid-state fermentation was carried out in conical flasks containing optimized nutrients. These four parameters were chosen for study as they mostly influence the growth of different fungal strains and secondary metabolite production during solid-state fermentation. The yield of statin was expressed as mg/g dry substrates.

EFFECT OF CARBON AND NITROGEN SOURCES ON STATIN PRODUCTION

The effect of the carbon and nitrogen supplementation on statin production was analyzed. After the rice was sterilized and cooled, various carbon and nitrogen

sources added to seed medium was inoculated to determine their effect on statin production.

Among the different carbon sources glucose was the best carbon source for statin biosynthesis. Seed media made up of glucose was recorded highest statin concentration with 12.30 mg/g. maltose (6.61 mg/g) was the second best carbon source. Lovastatin lactone form was produced in significant quantity in all carbon sources. Sucrose reduced the yield of statin to 3.72 mg/g (**Figure 30)**.

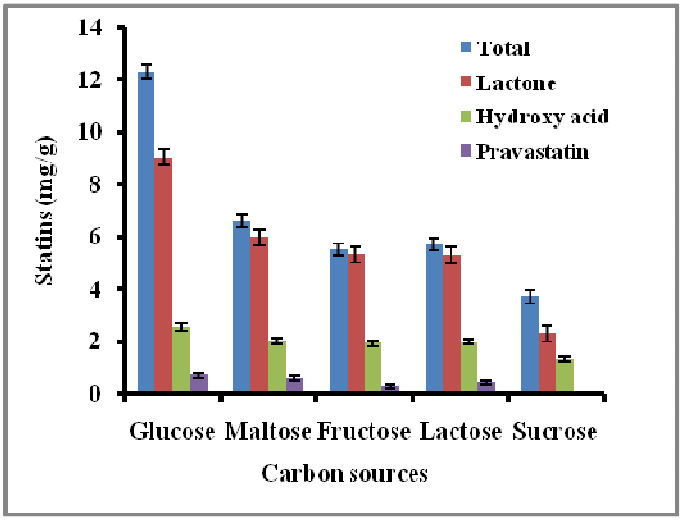


Figure 30. Effect of carbon source on statin production

Effect of nitrogen sources on statin production is represented in **Figure 31**. The nitrogen sources; peptone, ammonium nitrate, ammonium nitrate, MSG and yeast extract were added to growth medium containing glucose as a carbon source to evaluate their contribution towards statin production. Peptone in culture medium yielded total statin of 12.30 mg/g with 9.34, 2.42 and 0.54 mg of lactone, hydroxyl acid and pravastatin respectively per gram of dry mycelia. MSG has reduced the yield of total statin to 5.26 mg/g.

The media with peptone and glucose as a nitrogen and carbon source did not contribute much towards statin production.

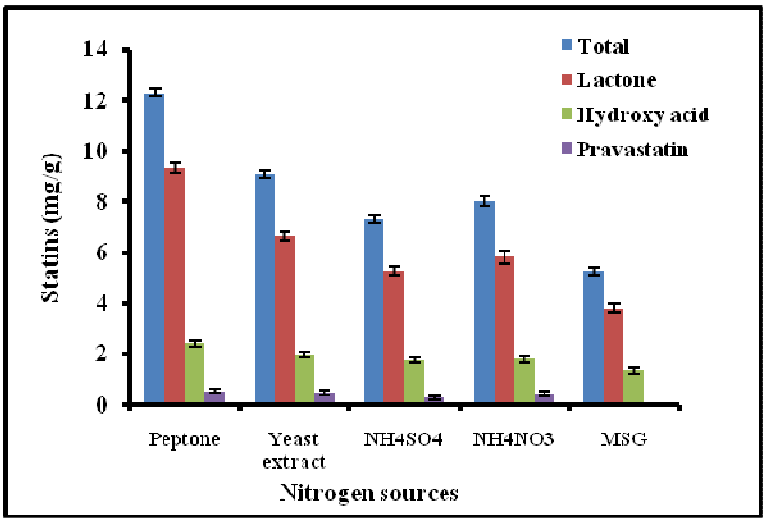


Figure 31. Effect of nitrogen source on statin production

EFFECT OF pH OF SUBSTRATE ON STATIN PRODUCTION

Statin production by *M. purpureus* is sensitive to pH. The water pH adjusted to 3-10 using HCl and NaOH was used to prepare the solid substrate for fermentation. Depending on the pH of water used to maintain the moisture content, production of statin showed a maximum yield of 12.81 mg/g at pH 6.0. The lactone form of statin was prominently produced at all pH compared to hydroxy acid form and pravastatin.

Statin production was altered very much by reducing or increasing the pH from 6.0 (**Figure 32**). These results suggested the extreme pH does not favour the statin production.

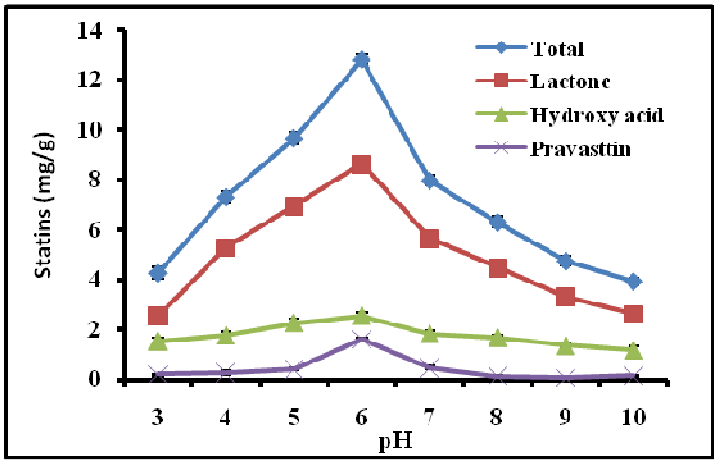


Figure 32. Effect of pH of substrate on lovastatin production

EFFECT OF TEMPERATURE ON STATIN PRODUCTION

Primary inoculum (seed culture) grown at 30° C was transferred to sterilized rice and cultivated at different temperatures (20, 25, 30, 35 and 40° C) to check the effect of temperature on statin production.

Production of statin at 20° C was poorly achieved (6.47 mg/g). However, at 30° C the yield of statin was raised to 13.57 mg/g. In contrast, at 40° C the yield of statin decreased (4.04 mg/g), perhaps because statin decomposed quickly above 30° C (**Figure 33)**. As mentioned earlier temperature shift plays an important role in statin production.

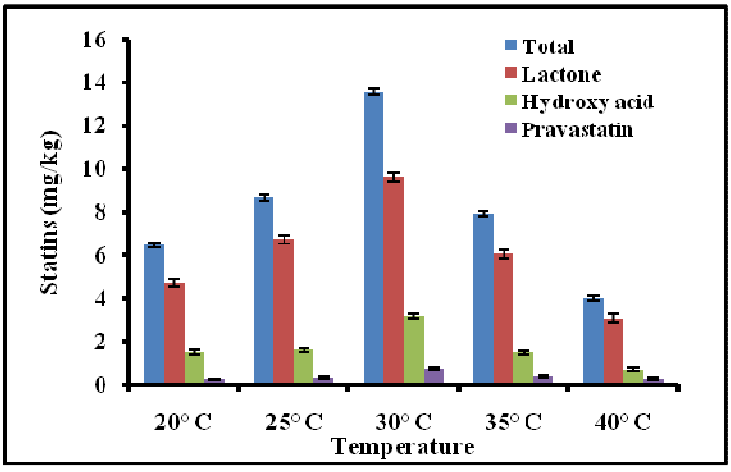


Figure 33. Effect of temperature on statin production

EFFECT OF INCUBATION PERIOD ON STATIN PRODUCTION

During the growth of the fungi the changes in the production of metabolites are observed. The primary inoculum grown at 30° C was transferred to sterilized rice and cultivated for 20 days. At alternative days the fermented sample was dried and used for statin quantification. Statins were extracted and estimated as described in Materials and Methods.

During initial condition of growth (2nd day) total statin yield was very low (2.27 mg/g). A gradual increase in the production of statin was recorded as growth progress. Increase in statin yield was observed till 14 days of incubation with maximum yield of 14.38 mg of statin per gram of dry biomass. After 14 days there is a gradual decrease in statin production **(Figure 34).**

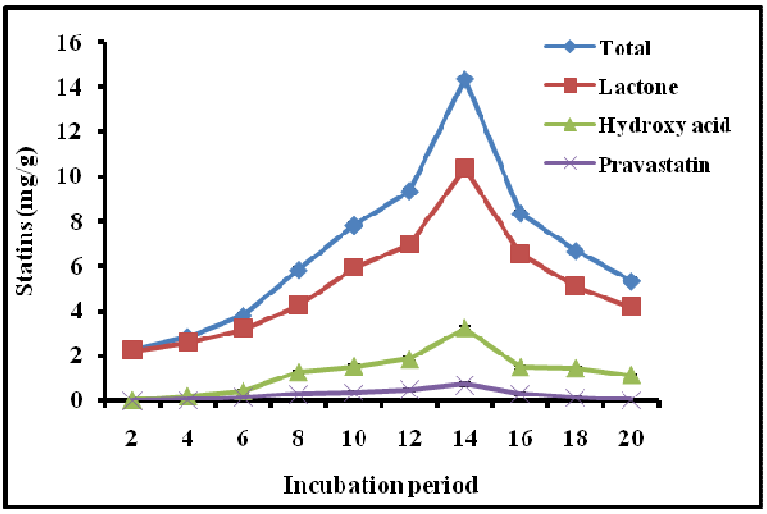


Figure 34. Effect of incubation period on statin production.

1.8. GROWTH TEMPERATURE AND STEROL CONCENTRATION

In eukaryotes, sterols function as a component of biological membranes by modulating their properties, especially their fluidity (Tamara *et al.*, 1997). Because of this function sterol is very important for the organism.

M. purpureus and mutants were grown at different temperatures and total sterol were quantitated (Materials and Methods). *M. purpureus* MTCC 410 produced more sterols compared to mutants CFR 410-11 and CFR 410-22 at various temperatures. The details are described in Materials and Methods.

M. purpureus MTCC 410 produced 4.4, 4.5, and 4.5 mg/g of sterols at 20, 30 and 40° C respectively. Reduction in the concentration of total sterol content in the mutants was observed. The mutants CFR 410-11 produced 2.9, 2.9 and 3.0 mg/g and CFR 410-22 produced 1.0, 1.1 and 1.1 mg/g of sterol at 20, 30, 40° C respectively. There is no variations in sterol content among the strains at different temperature indicating that temperature does not affect the sterol biosynthesis.

Even though mutation results in reduced sterol content since the organism need sterol for membarne fluidity and structure there is no variation at different temperatures among the mutants (**Figure 35**).

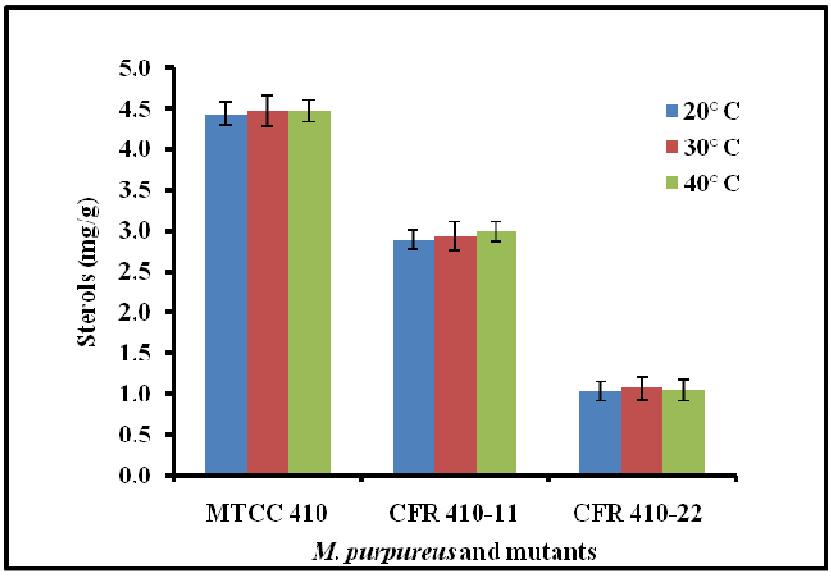


Figure 35. Influence of temperature on sterol content of *M. purpureus* isolates

1.9. AMIDASE ACTIVITIES

Amidase activities were determined in wild type, hyper pigment (CFR 410-11) and albino (CFR 410-22) mutants (**Figure 36**). Rapid plate assay showed more L-asparaginase production by CFR 410-11 compared to the MTCC 410. Very little L-asparaginase activity was visualized in CFR 410-22 during its growth (**Figure 36-C1**) (Fig 1-C1). The colony growth and pink zone width were measured as described by (Dhale, 2007c). This was further validated by amidase enzyme assays and estimation of pigment production by *M. purpureus* and its mutants in culture media.

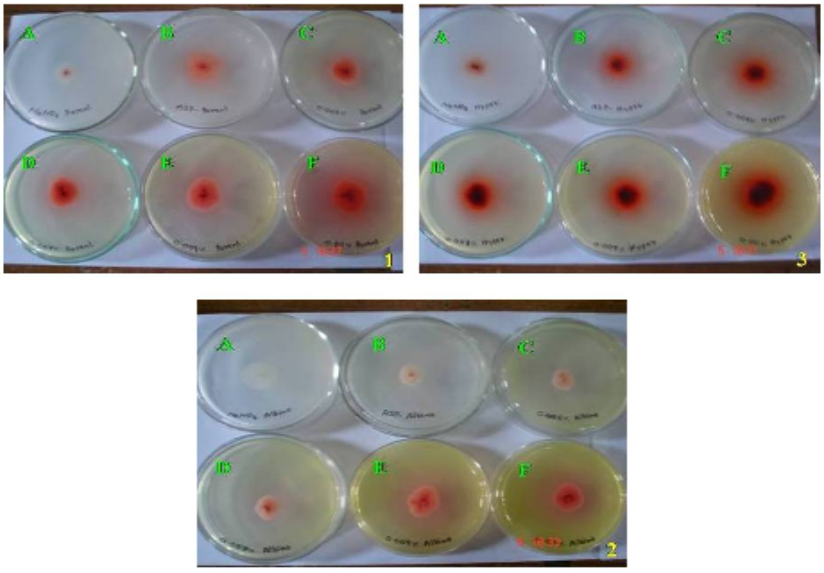


Figure 36. Rapid plate assay determined L-asparaginase activity of *M. purpureus* (1) and mutants (2-CFR 410-11 and 3- CFR 410-22). A (NaNO3, Control); B (L-asparagine-phenol red); C-E (L-asparagine+phenol red 0.005-0.011% concentration).

L-asparaginase and L-glutaminase activity of *M. purpureus* and its mutants were measured in the culture filtrate made up of 4% rice flour. The mutant CFR 410-11 showed higher L-asparaginase and L-glutaminase activity compared to MTCC 410 (0.103 and 0.139 IU v/s 0.072 and 0.105 IU respectively). L-asparaginase activity was not observed in mutant CFR 410-22. However, L-glutaminase activity was detectable (0.064 IU) **(Table 7)**. All the experiments were carried out in duplicate and data expressed as average values.

Table 7. L-asparaginase and L-glutaminase activities of MTCC 410 and its mutant (IU) cultured on rice flour.

Strain	L-asparaginase	L-glutaminase
MTCC 410	**0.072**	**0.105**
CFR 410-11	**0.103**	**0.139**
CFR 410-22	**-**	**0.064**

Pigments were quantified in the culture filtrate of *M. purpureus* and its mutants grown in 4% rice flour. The results suggested that more pigment synthesis occurred in CFR 410-11 due to increased amidase production by this fungus. Secretion of more quantity pigment was observed in CFR 410-11 compared to MTCC 410 and CFR 410-22 **(Table 8).** Pigment production was expressed as OD Units. All the experiments were carried out in duplicate and data expressed are average values.

Table 8. Pigment production by *M. purpureus* and its mutants on rice flour as substrate.

Strain	Yellow (375 nm)	Orange (475 nm)	Red (500 nm)
MTCC 410	**1.403**	**1.025**	**1.038**
CFR 410-11	**2.062**	**2.232**	**2.248**
CFR 410-22	**0.667**	**0.373**	**0.369**

In order to confirm the above results fungi were grown in medium containing L-asparagine and L-glutamine as sole carbon and nitrogen source.

The mutant CFR 410-11 cultured in L-glutamine secreted more red pigment (0.841 OD units). It secreted approximately 50% less pigment (0.465 OD units) in L-asparagine medium. The significant difference in the red pigment production was not observed in MTCC 410 cultured in L-asparagine (0.450 OD unit) and L-glutamine (0.320 OD unit). Mutant CFR 410-22 did not produce pigment either in L-asparagine nor L-glutamine (**Table 9**).

The paper chromatography of culture extract of *M. purpureus* MTCC 410 and CFR 410-11 confirmed the formation of L-aspartic acid and L-glutamic acid as the hydrolyzed product of L-asparagine and L-glutamine respectively. This evidently indicated the hydrolysis of L-glutamine and L-asparagine by amidase enzymes and released NH_3 in the media. Pigment production was expressed as OD Units. All the experiments were carried out in duplicate and data were expressed as average value.

Table 9. Pigment production by MTCC 410 and its mutants cultured on L-asparagine and L-glutamine as a sole carbon and nitrogen source.

Strain	**L-asparagine**			**L-glutamine**		
	Yellow (375 nm)	**Orange (475 nm)**	**Red (500nm)**	**Yellow (375 nm)**	**Orange (475 nm)**	**Red (500nm)**
MTCC 410	**0.281**	**0.447**	**0.450**	**0.382**	**0.373**	**0.371**
CFR 410-11	**0.245**	**0.462**	**0.465**	**0.822**	**0.819**	**0.841**
CFR 410-22	**nd**	**nd**	**nd**	**Nd**	**nd**	**Nd**

CHAPTER 2

SAFETY EVALUATION OF *MONASCUS PURPUREUS*

INTRODUCTION

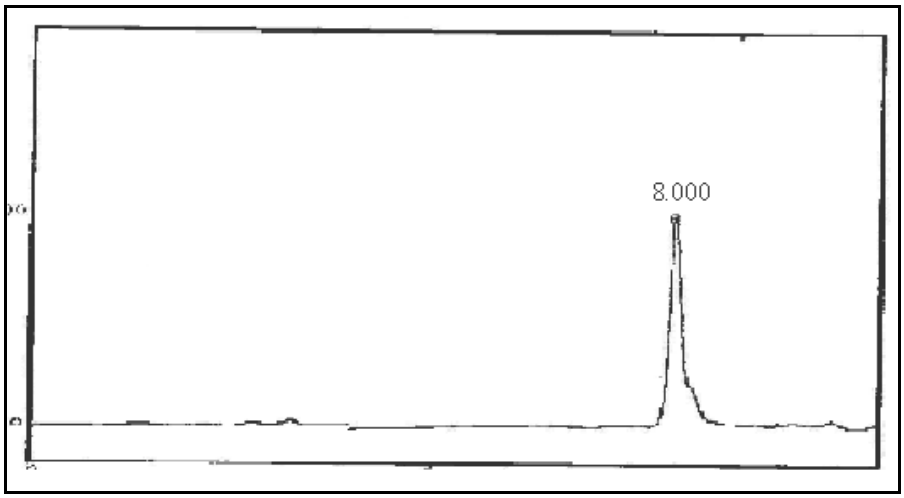
Red mould Rice (RMR) is a *Monascus* fermented rice product used several centuries, RMR is being used for colouring bean curd, meat, wine and other foods. It is also used in East Asia for flavour and preserving foods. *M. purpureus* has also been used, commercially, to produce valuable secondary metabolites viz., pigment (Wong & Koehler, 1981), antihypercholesterolemic agent, monacolin K (lovastatin), hypotensive agent, γ-aminobutyric acid (Su *et al.*, 2003), antioxidant compounds, dimerumic acid (Aniya *et al.*, 1999) and an antibacterial compound, 3-hydoxy- 4-methoxy-benzoic acid (Wu *et al.*, 2000). Lately, the purified pigments are widely used as colorants in processed seafood, sausages and sauce in Asia. RMR contains unsaturated fatty acids that help to reduce serum lipids, total cholesterol and triglyceride levels (Wang *et al.*, 1997; Qin *et al.*, 1999a). RMR also contains phytosterols such as β-sitosterol and campesterol (Heber *et al.*, 1999), known to interfere with cholesterol absorption in the intestine (Moghadasian & Frohlich, 1999). Since RMR posses properties for lowering cholesterol and blood pressure, it has been developed as a health food in human dietary supplements (Manzoni *et al.*, 1999).

Although RMR preparation has been marketed as food supplement in USA, its usage as medicament has not been approved by the Food and Drug Administration (FDA) due to the presence of the mycotoxin citrinin. There are very few reports in the literature on the safety of this fungus for use as food. In the previous chapter, *M. purpureus* produced useful compounds have determined. Since the species of *M. purpureus* produced nephrotoxic mycotoxin, citrinin (Blanc *et al.*, 1995a; Blanc *et al.*, 1995b), the safety of the fungus used in this study was evaluated.

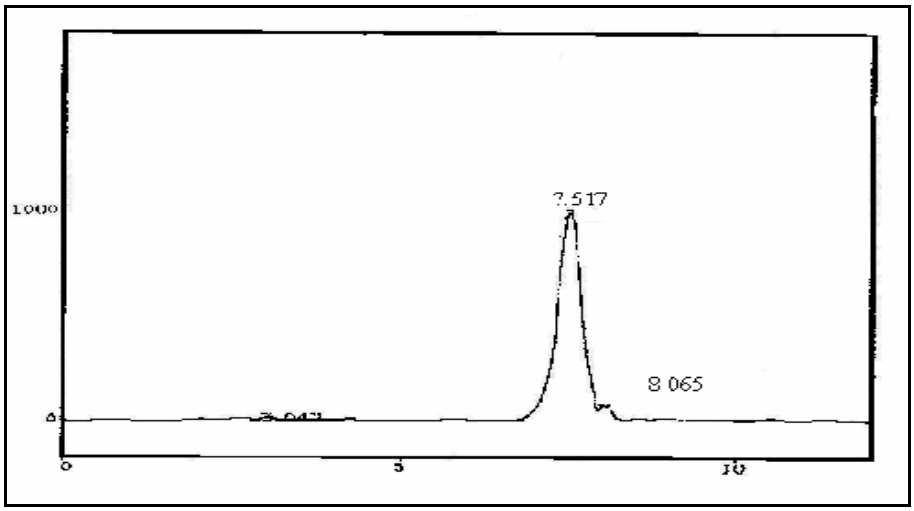
2.1. DETERMINATION OF CITRININ IN *MONASCUS* FERMENTED RICE

Citrinin was estimated in RMR as detailed in Materials and Methods. Presence of citrinin was confirmed by HPLC by comparison of retention time with standard

(Figure 37). Citrinin was eluted based on their corresponding retention time of 8.000 and 8.065 min obtained with the standard. Results showed that *M. purpureus* MTCC 410 fermented red rice contained citrinin at 1.43 mg/kg of fermented rice.



A



B

Figure 37. HPLC elution of (A), Standard citrinin and (B), Citrinin from RMR extract.

Since citrinin has estimated in the RMR, toxicity studies were carried out in rats.

2.2. TOXICOLOGICAL STUDIES ON RMR IN ALBINO RATS

Dietary feeding of RMR in acute and sub-chronic studies was carried out using CFT Wister strain albino rats. The animals were obtained from the stock colony of the CFTRI animal house facility and were kept in separate individual stainless steel cages with screen bottom. Ethical guidelines as laid down by the committee for the purpose of supervision of experiments on animals by the Government of India, Ministry of Social Justice and Empowerment were followed (Materials and Methods).

Rats were fed with commercial semi synthetic diet (M/S Gold Mohar, Lipton India Ltd.). Proximate composition of both commercial diet and RMR is detailed in **Table 10**. The commercial diet contained more protein (22.18%) and less carbohydrate (60.31%) compared to RMR. RMR with a lovastatin and citrinin content were fed at 12.29 mg/g and 0.143 mg/g dry weight.

RMR was incorporated into the diet at levels based on body weight for acute toxicity studies and at different dietary levels for sub-chronic toxicity studies.

Table 10. Proximate composition of commercial rat feed and RMR

	Content (g/100 g dry weight)	
Constituents	**Rat diet**	**RMR**
Crude protein	**22.18**	**11.60**
Crude carbohydrate	**60.31**	**72.10**
Crude fat	**3.38**	**1.58**
Crude fiber	**3.35**	**4.32**
Ash	**6.28**	**0.45**
Moisture	**4.50**	**9.90**
Lovastatin	**-**	**1.229**
Citrinin	**-**	**0.00143**

2.2.1. ACUTE TOXICITY STUDIES

Acute toxicity studies were carried out to evaluate the risk from a single exposure to a substance, typically at a high dose. Acute oral and dermal toxicity studies are frequently designed to express the potency of a substance in terms of a median lethal dose (LD_{50}). The LD_{50} is the dose that is lethal to 50 percent of the laboratory animals in the test. Higher the LD_{50} dose, lower the toxicity. The dose is calculated as mg of the test substance/kg body weight of the tested animals.

Acute toxicity study was carried out using 25 adult male and female (CFT Wister strain) albino rats and divided into five groups, each group with 5 animals. Prior to feeding the rats were fasted overnight. A graded dose of RMR (0, 0.5, 1.0, 2.5 and 5.0 g/kg body weight) was fed through diet on day one only. The experimental details are described in the Materials and Methods.

There were no significant differences with respect to gain in body weight and food intake of rats by feeding different doses of RMR. Relative organ weights of liver, lungs, kidney, heart, testis, adrenal, brain, spleen and ovaries of treated groups were comparable to that of controls groups **(Tables 11 and 12)**. Further, the hematological profile **(Tables 13 and 14)** of treated groups was comparable to that of control group. There was no significant difference between control and RMR fed groups. Histopathological examination of the above vital organs did not show any abnormalities at any of the dosage levels of RMR compared to control group. Results showed that rats fed on diets containing RMR did not develop any clinical signs of toxicity either immediately or during the post treatment even at highest dose of 5.0 g/kg body weight.

Table 11. Relative organ weight of male rats fed with acute doses of RMR

Dosage of RMR (g/kg b.wt)	Relative organ weight (g/100g body weight)							
	Liver	Lungs	Kidney	Heart	Testis	Adrenal	Brain	Spleen
Control	3.46±0.25	0.47±0.03	0.55±0.08	0.25±0.09	1.10±0.11	0.02±0.00	0.58±0.06	0.21±0.02
0.5 %	3.32±0.23	0.48±0.06	0.69±0.14	0.27±0.02	1.13±0.11	0.02±0.00	0.74±0.11	0.19±0.02
1.0 %	2.84±0.70	0.48±0.03	0.62±0.05	0.27±0.03	0.99±0.26	0.01±0.00	0.65±0.03	0.19±0.01
2.5 %	3.33±0.36	0.52±0.05	0.63±0.06	0.31±0.06	1.07±0.12	0.02±0.00	0.69±0.06	0.21±0.04
5.0 %	3.10±0.71	0.52±0.06	0.65±0.09	0.29±0.03	1.16±0.22	0.01±0.00	0.73±0.18	0.19±0.05

Values are mean ± SEM of five animals. No significant difference between control and RMR fed groups ($P < 0.05$)

Table 12. Relative organ weight of female rats fed with acute dose of RMR

Dosage of RMR (g/kg b.wt)	Relative organ weight (g/100g body weight)							
	Liver	Lungs	Kidney	Heart	Ovaries	Adrenal	Brain	Spleen
Control	3.31±1.34	0.67±0.08	0.66±0.09	0.34±0.02	0.06±0.02	0.03±0.00	0.84±0.05	0.22±0.03
0.5 %	3.64±0.17	0.52±0.03	0.67±0.05	0.32±0.03	0.06±0.00	0.02±0.00	0.82±0.05	0.22±0.02
1.0 %	3.46±0.3	0.56±0.20	0.61±0.23	0.25±0.13	0.05±0.04	0.02±0.01	0.75±0.12	0.15±0.03
2.5 %	3.7±0.36	0.61±0.06	0.65±0.04	0.40±0.14	0.05±0.00	0.03±0.01	0.78±0.10	0.24±0.05
5.0 %	3.32±0.36	0.61±0.08	0.60±0.08	0.30±0.03	0.07±0.01	0.03±0.00	0.76±0.10	0.21±0.05

Values are mean ± SEM of five animals. No significant difference between control and RMR fed groups ($P < 0.05$)

Table 13. Hematological profile of male rats fed with RMR for 14 days

Concentration of RMR mixed with diet (w/w)	Hb (g/dl)	RBC (10^6/µl))	WBC (10^3/µl)	PCV (%)	MCH (pg)	MCHC (%)	DC (%)				
							L	P	M	E	B
Control	**12.23±0.11**	**8.3±0.48**	**8900±590.32**	**29.2±0.56**	**15.89±1.26**	**30.10±3.26**	**62.2±0.2**	**30.2±1.3**	**1.6±0.8**	**1.6±0.0**	**-**
2.0 %	**13.26±0.56**	**8.1±0.19**	**8400±980.10**	**23.7±0.98**	**15.23±1.99**	**29.19±2.26**	**60.2±2.9**	**29.8±1.8**	**1.9±0.5**	**1.6±0.0**	**-**
4.0 %	**12.02±0.13**	**8.3±0.12**	**8900±460.42**	**29.7±0.86**	**16.26±1.59**	**30.20±1.46**	**62.3±2.1**	**32.1±2.6**	**1.8±0.3**	**1.5±0.2**	**-**
8.0 %	**12.52±0.21**	**8.2±0.06**	**8300±486.24**	**24.5±0.52**	**16.16±1.20**	**29.56±2.89**	**67.2±2.8**	**30.9±4.6**	**1.6±0.4**	**1.6±0.0**	**-**
12.0 %	**13.02±0.51**	**8.0±0.11**	**8850±562.46**	**25.1±0.49**	**15.11±1.03**	**28.76±5.12**	**69.5±1.9**	**29.4±1.3**	**1.3±0.5**	**1.5±0.0**	**-**

Values are mean ± SEM of four animals. No significant difference between control and RMR fed groups ($p<0.05$).

Hb-Hemoglobin, RBC-Red Blood Cells, WBC-White Blood Cells, PCV-Packed Cell Volume, MCH-Mean Corpuscular Hemoglobin, MCHC-Mean Corpuscular Hemoglobin Concentration, DC-Differential Count, L-Lymphocytes, P-Polymorph Neutrophils, M-Monocyte, E-Eosinophils, B-Basophils.

Table 14. Hematological profile of female rats fed with RMR for 14 days

Concentration of RMR mixed with diet (w/w)	Hb (g/dl)	RBC (10^6/µl)	WBC (10^3/µl)	PCV (%)	MCH (pg)	MCHC (%)	DC (%)				
							L	P	M	E	B
Control	**11.28±0.65**	**8.71±0.03**	**7600±112.46**	**34.69±2.01**	**11.26±1.14**	**38.05±1.79**	**69.3±2.0**	**33.8±4.7**	**1.8±0.2**	**1.9±0.5**	**-**
2.0 %	**10.99±0.43**	**9.86±0.25**	**7300±134.04**	**36.81±1.89**	**13.79±1.32**	**40.13±1.89**	**70.0±2.1**	**32.8±4.2**	**1.6±0.1**	**1.7±0.7**	**-**
4.0 %	**12.48±0.80**	**8.99±1.51**	**7400±216.29**	**33.46±3.85**	**12.95±1.46**	**41.26±1.46**	**69.9±1.0**	**31.7±3.8**	**1.7±1.0**	**1.6±0.0**	**-**
8.0 %	**12.46±0.77**	**8.76±0.12**	**7900±310.26**	**30.53±2.79**	**11.98±1.02**	**42.29±0.19**	**71.0±1.9**	**29.1±3.5**	**1.9±1.0**	**1.5±0.0**	**-**
12.0 %	**11.73±0.72**	**8.46±0.12**	**8300±219.22**	**32.57±4.86**	**11.95±1.26**	**39.56±1.92**	**70.5±1.7**	**28.7±4.4**	**1.6±0.0**	**1.8±0.7**	**-**

Values are mean ± SEM of four animals. No significant difference between control and RMR fed groups ($p<0.05$).

Hb-Hemoglobin, RBC-Red Blood Cells, WBC-White Blood Cells, PCV-Packed Cell Volume, MCH-Mean Corpuscular Hemoglobin, MCHC-Mean Corpuscular Hemoglobin Concentration, DC-Differential Count, L-Lymphocytes, P-Polymorph Neutrophils, M-Monocyte, E-Eosinophils, B-Basophils.

2.2.2. SUB-CHRONIC TOXICITY STUDIES

Sub-chronic toxicity studies were carried out for 14 weeks. Exposure routes are identical to those of acute testing programs (oral, dermal, inhalation). In the previous experiment the acute toxicity studies of RMR in test animals via a specific route of exposure did not indicate the doze causing any risk. That determination was made by examining effects seen over a range of doses and durations of time. In sub-chronic studies, groups of treated animals were given various doses daily for 14 weeks. It was assumed that high doses elicit sub-lethal effects, middle doses evoke only minimal adverse effects and low doses trigger no toxic effects.

Sub-chronic toxicity studies of RMR were carried out using 40 weaned male and female (CFT Wister strain) albino rats, randomly assigned to five groups, with eight animals in each group. Sub-chronic toxicity studies were carried out by dietary feeding rats with different concentration (2-12% w/w of diet) of RMR for 14 weeks to assess the cumulative effect of low doses of lovastatin containing fungal biomass (RMR). At the end of the designated experimental period, the vital organs and physiological parameters were examined to determine differences between control and treated groups.

2.2.3. FOOD INTAKE AND GAIN IN BODY WEIGHT BY RATS

The changes in food intake and body weight have long been accepted as a sensitive indicator of chemically induced changes to organs. In toxicological experiments, comparison of organ weights between control and treated groups have been conventionally used to predict toxic effect of a test (Peters & Boyd, 1966); (Pfeiffer, 1968).

The food intake by the male and female rats was almost similar even though they were fed RMR at different concentrations. This indicated that incorporation of

RMR even at 12% level did not significantly affect food intake in both sexes of treatment groups and were comparable to that of control group **(Figure 38 and 39)**.

The actual food intake, mean gain in body weight and actual intake of biomass (RMR)/kg body weight are presented in **Table 15 and 16**. No difference in food intake, gain in body weight was observed in treated groups of both sexes during the 14 week experimental period **(Figure 40 and 41)**.

These results suggest that feeding of RMR even at 12% did not affect the changes in food intake, gain in body weight and mean body weight.

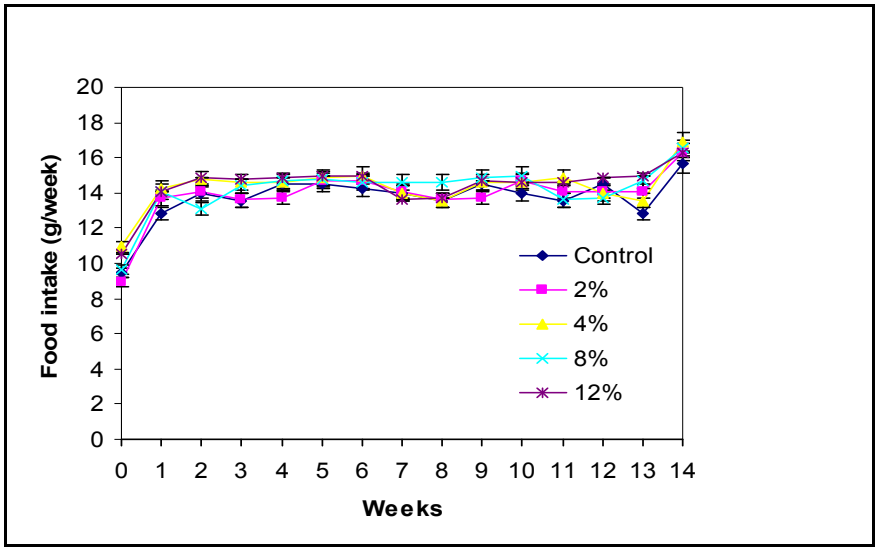


Figure 38. Food intake profile of male rats fed with dietary RMR for 14 weeks. Values are ± SEM of 8 animals. No significant difference between control and RMR fed groups ($p < 0.05$).

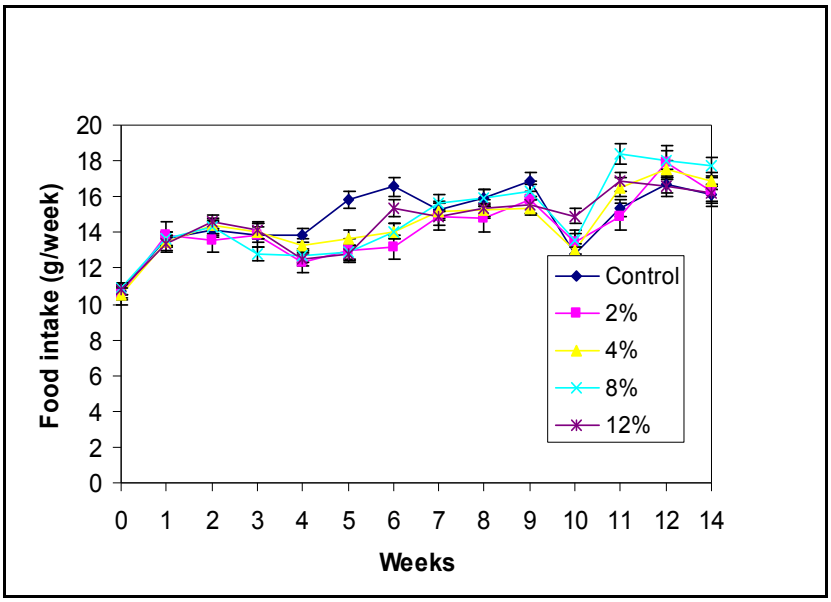


Figure 39. Food intake profile of female rats fed with dietary RMR for 14 weeks. Values are ± SEM of 8 animals. No significant difference between control and RMR fed groups ($p < 0.05$).

Table 15. Food intake, body weight gain and RMR intake by male rats fed with RMR for 14 weeks

Duration of feeding (weeks)	Control		2%			4%			8%			12%		
	Food Intake (g/rat/ day)	Body Weight (g/rat/ week)	Food Intake (g/rat/ day)	Body Weight (g/rat/ week)	RMR intake (g/kg bw/ rat/week)	Food Intake (g/rat/ day)	Body Weight (g/rat/ week)	RMR intake (g/kg bw/ rat/week	Food Intake (g/rat/ day)	Body Weight (g/rat/ week)	RMR intake (g/kg bw/ rat/week	Food Intake (g/rat/ day)	Body Weight (g/rat/ week)	RMR intake (g/kg bw/ rat/week
1	9.47	54.28	8.95	57.14	3.13	10.93	59.12	7.39	9.66	60.00	15.55	10.53	61.5	20.54
2	12.82	74.00	13.73	78.85	3.62	14.29	86.75	6.58	14.08	83.12	13.55	14.07	91.0	18.55
3	13.98	88.28	14.11	90.14	3.03	14.81	97.00	5.53	13.10	93.62	10.87	14.88	101.62	16.14
4	13.55	121.14	13.61	119.85	2.41	14.58	123.25	4.73	14.39	114.25	10.07	14.78	123.12	13.52
5	14.47	146.14	13.75	141.85	2.04	14.57	150.12	3.88	14.65	143.00	8.19	14.84	149.5	11.45
6	14.52	155.28	14.68	154.28	1.86	14.87	159.87	3.50	14.80	155.12	7.17	15.00	155.37	10.26
7	14.23	168.85	14.67	167.57	1.71	15.00	172.75	3.28	14.60	170.87	6.45	15.00	171.12	9.41
8	13.98	191.71	14.11	191.14	1.53	13.98	195.00	2.88	14.58	188.62	6.18	13.61	197.87	8.25
9	13.55	206.85	13.61	208.28	1.29	13.55	211.25	2.48	14.57	205.87	5.24	13.75	207.5	7.25
10	14.47	216.14	13.75	219.14	1.25	14.58	215.25	2.56	14.87	212.37	5.11	14.68	215.5	7.44
11	13.98	232.28	14.68	230.00	1.25	14.57	227.75	2.31	15.00	232.37	4.78	14.58	236.5	6.79
12	13.55	246.00	14.11	246.42	1.20	14.87	259.50	2.33	13.61	262.87	4.14	14.57	262.12	6.67
13	14.47	250.71	14.11	254.28	1.19	13.98	265.62	2.14	13.75	261.87	4.04	14.87	267.00	6.68
14	12.82	263.14	14.11	262.85	1.18	13.55	276.75	1.98	14.68	271.37	4.14	15.00	274.5	6.55

Each value is mean of six rats. No significant difference between control and RMR fed groups ($P < 0.05$).

Table 16. Food intake, body weight gain of and RMR intake by female rats fed with RMR for 14 weeks

Duration of feeding (weeks)	Control		2%			4%			8%			12%		
	Food intake (g/rat/ day)	Body weight (g/rat/ week)	Food intake (g/rat/ day)	Body weight (g/rat/ week)	RMR intake (g/kg bw/ rat/week)	Food intake (g/rat/ day)	Body weight (g/rat/ week)	RMR intake (g/kg bw/ rat/week	Food intake (g/rat/ day)	Body weight (g/rat/ week)	RMR intake (g/kg bw/ rat/week	Food intake (g/rat/ day)	Body weight (g/rat/ week)	RMR intake (g/kg bw/ rat/week
1	10.68	50.25	10.52	53.00	3.96	10.56	55.00	7.68	10.87	57.00	15.25	10.84	59.37	21.91
2	13.61	76.12	13.86	77.50	3.57	13.42	79.00	6.79	13.65	81.62	13.37	13.33	83.50	19.15
3	14.16	94.00	13.52	95.62	2.82	14.38	96.12	5.98	14.28	98.25	11.62	14.57	97.87	17.86
4	13.85	105.12	13.87	103.00	2.69	14.04	108.12	5.19	12.79	110.12	9.29	14.12	112.87	15.01
5	13.84	121.62	12.36	120.12	2.05	13.24	123.37	4.29	12.72	124.75	8.15	12.47	125.5	11.92
6	15.85	134.50	13.01	136.12	1.89	13.67	135.50	3.91	12.87	137.37	7.49	12.84	136.12	11.07
7	16.55	143.75	13.22	147.75	1.77	14.06	146.12	3.79	14.05	148.12	7.58	15.35	147.75	12.30
8	15.25	154.12	14.91	154.37	1.93	15.18	154.12	3.93	15.64	155.25	8.05	14.84	154.50	11.75
9	15.96	163.50	14.81	162.50	1.74	15.23	163.37	3.72	15.96	167.62	7.48	15.35	165.50	11.12
10	16.83	173.12	15.86	171.87	1.84	15.40	172.87	3.78	16.34	173.50	7.12	15.56	176.00	11.52
11	12.81	180.25	13.45	184.62	1.54	13.01	180.87	3.23	13.57	183.75	6.24	14.91	187.12	10.70
12	15.39	186.87	14.89	187.37	1.65	16.53	185.50	4.09	18.36	183.50	8.46	16.85	186.87	11.76
13	16.68	192.87	17.92	191.87	1.98	17.53	192.75	4.30	18.04	196.37	7.91	16.55	194.62	11.37
14	16.14	205.25	16.27	206.37	1.75	16.87	208.87	3.92	17.71	207.25	7.36	16.18	211.25	10.77

Each value is mean of six rats. No significant difference between control and RMR fed groups ($P < 0.05$).

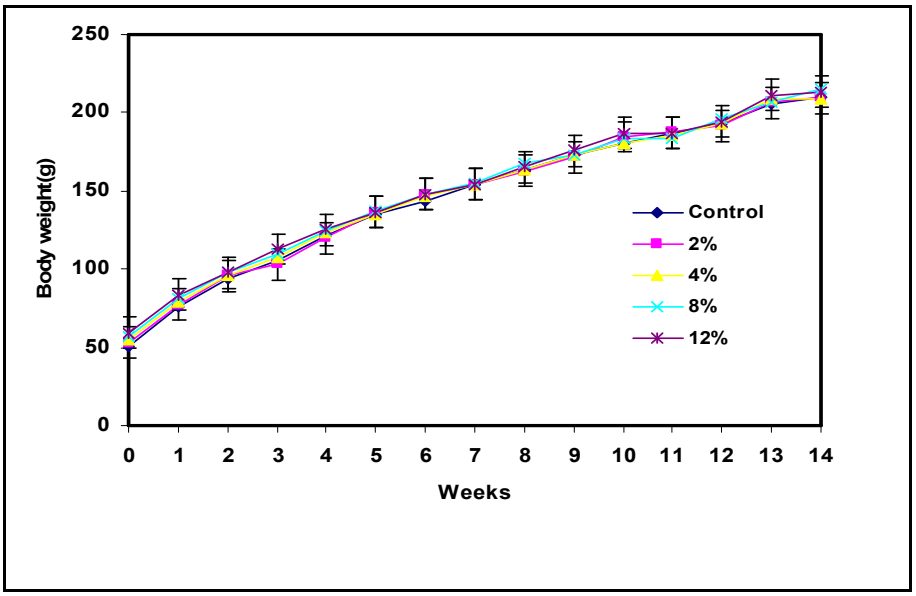


Figure 40. Mean absolute body weight of female rats fed with dietary RMR for 14 weeks. Values are ± SEM of 8 animals. No significant difference between control and RMR fed groups ($p < 0.05$).

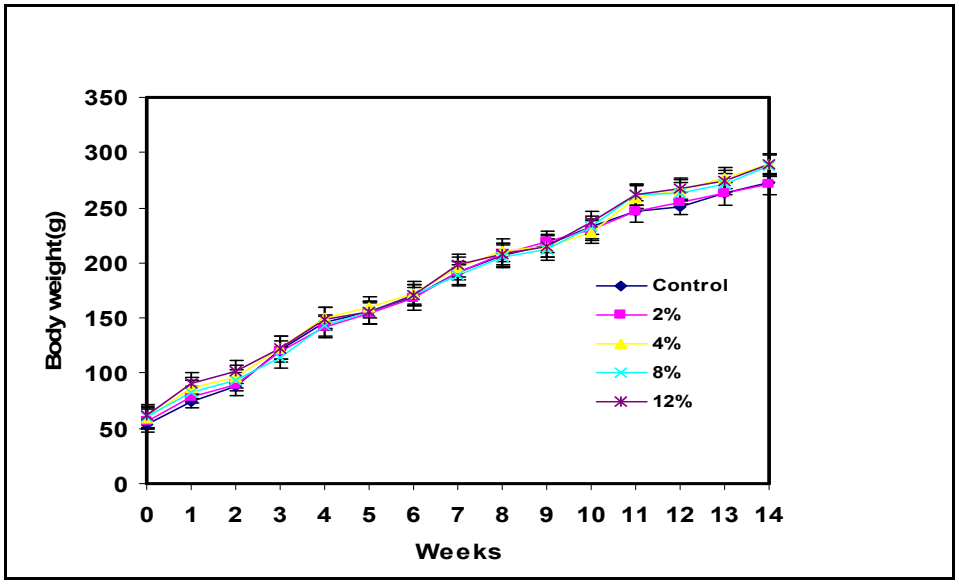


Figure 41. Mean absolute body weight of male rats fed with dietary RMR for 14 weeks. Values are ± SEM of 8 animals. No significant difference between control and RMR groups ($p < 0.05$).

2.1.4. RELATIVE ORGAN WEIGHTS OF MALE AND FEMALE RATS FED WITH RMR

During routine toxicological studies, the organ weights were measured to see the endocrine effect. Analysis of organ weight in toxicology studies is an important endpoint for the identification of potentially harmful effects of RMR. Organ weight can be the most sensitive indicator that showed the effect of an experimental compound, as significant differences in organ weight between treated and untreated (control) animals may occur in the absence of any morphological changes.

After feeding RMR to the rats for 14 week, gain in relative organ weight was measured in male and female rats. The details are described in Materials and Methods.

There were no significant differences in the relative organ weights of various vital organs in RMR treated rats as compared to that of the control rats **(Tables 17 and 18)**. These studies indicated that, the RMR fed to rats did not show any adverse effect on the organs.

Table 17. Effect of feeding of RMR for 14 weeks on relative organ weight (g/100 g body weight) of male rats

Concentration of RMR mixed with diet (w/w)	Relative organ weight (g/100g body weight)							
	Liver	Lungs	Kidney	Heart	Testis	Adrenal	Brain	Spleen
Control	2.97±0.17	0.51±0.06	0.77±0.09	0.28±0.05	1.17±0.20	0.03±0.01	0.67±0.15	0.23±0.04
2.0 %	2.98±0.19	0.41±0.03	0.64±0.04	0.30±0.01	1.05±0.12	0.03±0.01	0.63±0.07	0.23±0.04
4.0 %	2.92±0.25	0.37±0.01	0.60±0.07	0.29±0.05	1.01±0.07	0.02±0.01	0.59±0.01	0.18±0.06
8.0 %	2.96±0.12	0.41±0.02	0.63±0.04	0.29±0.02	1.04±0.06	0.01±0.00	0.59±0.06	0.20±0.02
12.0 %	2.94±0.16	0.44±0.07	0.64±0.04	0.30±0.01	1.11±0.10	0.02±0.00	0.58±0.02	0.23±0.02

Values are mean ± SEM of six animals. No significant difference between control and RMR fed groups.

Table 18. Effect of feeding of RMR for 14 weeks on relative organ weight (g/100 g body weight) of female rats

Concentration of RMR mixed with diet (w/w)	Relative organ weight (g/100g body weight)							
	Liver	Lungs	Kidney	Heart	Testis	Adrenal	Brain	Spleen
Control	**2.81±0.19**	**0.52±0.03**	**0.75±0.05**	**0.30±0.01**	**0.06±0.01**	**0.04±0.01**	**0.88±0.10**	**0.20±0.03**
2.0 %	**2.76±0.22**	**0.50±0.10**	**0.66±0.09**	**0.35±0.10**	**0.05±0.00**	**0.03±0.01**	**0.85±0.08**	**0.21±0.03**
4.0 %	**3.00±0.28**	**0.56±0.10**	**0.77±0.12**	**0.33±0.03**	**0.06±0.00**	**0.03±0.00**	**0.87±0.14**	**0.23±0.02**
8.0 %	**3.06±0.14**	**0.59±0.11**	**0.71±0.05**	**0.33±0.02**	**0.08±0.05**	**0.04±0.03**	**0.77±0.21**	**0.25±0.03**
12.0 %	**2.98±0.23**	**0.49±0.06**	**0.68±0.03**	**0.32±0.02**	**0.06±0.01**	**0.02±0.00**	**0.89±0.07**	**0.24±0.04**

Values are mean ± SEM of six animals. No significant difference between control and RMR fed groups.

2.1.5. HEMATOLOGICAL PROFILE

After autopsy, blood samples were collected from four rats of each group of the experimental and control groups and examined for haematological profiles like Hemoglobin Concentration (Hb), Red Blood Cells (RBC), White Blood Cells (WBC), Packed Cell Volume (PCV), Mean Corpuscular Hemoglobin (MCH), Mean Corpuscular Hemoglobin Concentration (MCHC), Differential Count (DC), Lymphocytes (L), Polymorph Neutrophils (P), Monocyte (M), Eosinophils (E), Basophils (B). MCH was calculated from hemoglobin and erythrocytes counts, whereas MCHC was determined from hemoglobin concentration and packed cell volume values. Data on the various hematological parameters are presented in **Table 19 and 20**.

No significant alteration was observed between control and treated groups.

Table 19. Hematological profile of male rats fed with RMR for 14 weeks

Conc. of RMR mixed with diet (w/w)	Hb (g/dl)	RBC (10^6/µl))	WBC (10^3/µl)	PCV (%)	MCH (pg)	MCHC (%)	DC (%)				
							L	P	M	E	B
Control	**15.47±0.15**	**9.6±0.65**	**9500±660.80**	**38.00±0.70**	**16.11±2.64**	**40.71±5.04**	**70.2±2.6**	**28.5±2.2**	**1.7±1.1**	**1±0.0**	**-**
2.0 %	**15.42±0.60**	**9.2±0.20**	**9400±1080.12**	**38.75±1.03**	**16.76±2.0**	**39.79±3.31**	**68.2±3.7**	**28.7±2.6**	**2.0±1.4**	**1±0.0**	**-**
4.0 %	**16.05±0.30**	**9.4±0.23**	**9500±1707.82**	**39.50±0.86**	**17.07±1.09**	**40.63±1.09**	**68.5±2.9**	**31.2±4.2**	**2.0±0.8**	**1.5±0.6**	**-**
8.0 %	**15.72±0.23**	**9.0±0.38**	**9900±238.04**	**39.50±0.86**	**17.46±2.00**	**39.79±2.23**	**70.2±2.6**	**30.5±6.6**	**1.5±0.5**	**1±0.0**	**-**
12.0 %	**15.25±0.71**	**9.4±0.38**	**9850±694.62**	**39.50±0.86**	**16.22±0.96**	**38.60±8.18**	**69.5±3.3**	**28.2±3.6**	**1.5±0.7**	**1±0.0**	**-**

Values are mean ± SEM of four animals. No significant difference between control and RMR fed groups (p<0.05).

Hb-Hemoglobin, RBC-Red Blood Cells, WBC-White Blood Cells, PCV-Packed Cell Volume, MCH-Mean Corpuscular Hemoglobin, MCHC-Mean Corpuscular Hemoglobin Concentration, DC-Differential Count, L-Lymphocytes, P-Polymorph Neutrophils, M-Monocyte, E-Eosinophils, B-Basophils.

Table 20. Hematological profile of female rats fed with RMR for 14 weeks

Concentration of RMR mixed with diet (w/w)	Hb (g/dl)	RBC (10^6/µl)	WBC (10^3/µl)	PCV (%)	MCH (pg)	MCHC (%)	DC (%)				
							L	P	M	E	B
Control	**13.32±0.74**	**9.8±0.38**	**7900±191.48**	**37.75±1.49**	**13.59±1.04**	**35.28±2.61**	**67.0±2.0**	**31.2±6.7**	**1.6±0.6**	**2.0±0.7**	**-**
2.0 %	**14.7±0.30**	**10.2±0.20**	**7500±104.14**	**36.5±1.19**	**14.41±0.32**	**40.27±2.58**	**71.0±2.4**	**32.2±6.7**	**1.3±0.6**	**1.5±0.7**	**-**
4.0 %	**14.75±0.39**	**10±0.51**	**7700±387.29**	**34.25±0.85**	**14.75±0.87**	**43.06±2.26**	**69.5±1.3**	**27.7±1.7**	**2.0±1.0**	**1.0±0.0**	**-**
8.0 %	**13.52±0.27**	**9.2±0.23**	**8000±424.26**	**35.25±1.03**	**14.69±1.12**	**38.29±1.48**	**70.7±2.5**	**27.5±2.5**	**2.0±1.0**	**1.0±0.0**	**-**
12.0 %	**13.95±0.29**	**9.6±0.32**	**8200±316.22**	**39.5±0.86**	**14.23±1.06**	**35.31±0.92**	**70.0±2.4**	**28.5±2.4**	**1.0±0.0**	**2.0±0.7**	**-**

Values are mean ± SEM of four animals. No significant difference between control and RMR fed groups ($p<0.05$).

Hb-Hemoglobin, RBC-Red Blood Cells, WBC-White Blood Cells, PCV-Packed Cell Volume, MCH-Mean Corpuscular Hemoglobin, MCHC-Mean Corpuscular Hemoglobin Concentration, DC-Differential Count, L-Lymphocytes, P-Polymorph Neutrophils, M-Monocyte, E-Eosinophils, B-Basophils.

2.1.6. HISTOPATHOLOGICAL EXAMINATION

The organs fixed with 10% neutral buffered formalin were embedded in paraffin, sectioned, stained with hematoxylin and eosin and examined under a microscope as mentioned in Materials and Methods.

Microscopic examination showed histological picture of each vital organ. There were no treatment-related lesions observed in the tissues examined from animals at terminal sacrifice. Like in the control animals, there were no adverse histopathology deviations in spleens, livers or kidneys of animals of any treated group.

Livers of treated animals showed no shrunken hepatocytes or congestions in portal tracts and sinusoids. Lungs showed organized alveolar spaces and no thickening of inter alveolar septa or a cellular infiltration was observed. Kidney was also normal and did not present any glomerular or vascular congestion. Adrenals showed normal layer of cells in both cortex and medulla.

Other vital organs like heart and brain also showed normal structure. Normal appearance of heart muscle was observed. Brain showed normal cellular architecture both in cerebrum and cerebellar regions. Histological examination of ovaries in the treatment groups revealed different stages of follicular development. Ovarian tissue showed different stages of follicular development with mature corpus luteum. No abnormalities were observed in germinal epithelium, stages of follicular development, maturation and corpus luteum. Spleen showed normal architecture with well defined follicles containing germinal centers. Testis was with normal somniferous tubules showing different stages of germinal cells, with spermatozoa.

Histopathological investigations revealed that the rats treated with RMR did not show any incidence of organ toxicity in this study.

2.1.7. CLINICAL CHEMISTRY

Clinical chemistry involves the estimation of marker enzymes like LDH, SGPT, SGOT and ALP. Elevated levels of LDH are found in pathologic situations like myocardial infraction, liver diseases, renal disease, and certain forms of anemia, malignant diseases and progressive muscular dystrophy (Henry, 1979). Leakage of liver enzyme LDH is very commonly used for measuring cytotoxicity of test compounds. Increased SGOT and SGPT levels were observed in connection with damages of heart, skeletal muscle as well as of liver parenchyma (Moss & Henderson, 1999). Pathological increased ALP levels are associated with hepatobiliary and bone diseases.

The changes in the activity of these clinical enzymes were measured in the rats fed with RMR. The above serum enzymes are analyzed by using the enzymatic kits as mentioned in the Materials and Methods.

The results on the enzyme activities of test and control samples are represented in **Table 21 and 22**. No marked alteration in any of the specific activities of enzymes in RMR fed rats was recorded.

The record of biochemical parameters in treatment groups of rats were non significantly different in comparison with the control group indicating that RMR has no adverse effect on liver and kidney functioning.

Table 21. Effect of feeding RMR for 14 weeks on serum enzymes of male rats

Concentration of RMR mixed with diet (w/w)	ALP	SGOT (ALAT)	SGPT (ASAT)	LDH	Creatinine	Urea
Control	**191.91±25.17**	**134.26±14.07**	**31.51±1.99**	**1467.93±140.02**	**0.57±0.11**	**41.92±8.17**
2%	**181.90±28.46**	**133.77±23.88**	**30.54±2.09**	**1382.26±145.70**	**0.53±0.05**	**39.21±6.86**
4%	**187.86±10.29**	**133.70±22.27**	**29.81±4.10**	**1322.75±122.39**	**0.56±0.05**	**38.62±4.45**
8%	**187.74±10.64**	**124.99±33.37**	**31.00±3.24**	**1390.03±122.89**	**0.55±0.15**	**41.22±6.73**
12%	**184.50±40.98**	**123.17±19.28**	**30.08±2.83**	**1345.41±73.44**	**0.56±0.08**	**40.17±7.84**

Values are mean ± SEM of five animals. LDH, Lactate Dehydrogenase (U/L); ALP, Alkaline Phosphatase (U/L); ALAT, Alanine Amino Transferase (U/L); ASAT, Asparate Amino Transferase (U/L). No significant difference between control and RMR fed groups ($P < 0.05$).

Table 22. Effect of feeding of RMR for 14 weeks on serum enzymes of female rats

Concentration of RMR mixed with diet (w/w)	ALP	SGOT (ALAT)	SGPT (ASAT)	LDH	Creatinine	Urea
Control	**136.84 ± 0.25**	**125.17±35.82**	**33.89±4.84**	**1224.39 ±47.36**	**0.58±0.06**	**49.10±6.53**
2%	**130.22±20.78**	**123.35±28.79**	**33.39±7.26**	**1158.84 ±22.91**	**0.54±0.11**	**46.68±7.06**
4%	**123.97±18.04**	**113.04±22.27**	**28.83±6.29**	**1183.16±112.33**	**0.57±0.03**	**41.96±7.93**
8%	**124.65±8.23**	**119.16±18.54**	**30.97±5.16**	**1155.61±38.95**	**0.52±0.14**	**43.62±6.53**
12%	**128.53±10.30**	**124.17±18.72**	**32.18±5.74**	**1168.18±45.59**	**0.57±0.03**	**43.49±5.56**

Values are mean ± SEM of five animals. LDH, Lactate Dehydrogenase (U/L); ALP, Alkaline Phosphatase (U/L); ALAT, Alanine Amino Transferase (U/L); ASAT, Asparate Amino Transferase (U/L). No significant difference between control and RMR fed groups ($P < 0.05$).

2.1.8. TC AND TG LEVELS IN SERUM AND LIVER OF RATS FED WITH RMR

The total cholesterol and triacylglycerol content in liver and serum were analyzed to see the effect of RMR. Effect of RMR on cholesterol and triacylglycerol levels in serum and liver is described in **Tables 23 and 24**. A dose dependent decrease in cholesterol and triacylglycerol in liver and serum was observed in both male and female rats fed with RMR.

The decrease in serum cholesterol levels ranged from 5.43% to 15.83% in male rats and 11.42% to 22.0% in female rats. The decrease in serum triacylglycerol levels ranged from 12.61% to 21.61% and 11.4% to 26.15% in male and female rats respectively among different groups fed with RMR for 14 weeks. In addition, the decrease in liver cholesterol level ranged from 14.6% to 38.3% in male rats and 9.0% to 30.6% in female rats and decrease in serum triacylglycerol levels ranged from 10.8% to 25.14% and 10.93% to 23.63% in male and female rats respectively among different groups fed with RMR for 14 weeks.

These results indicated that RMR significantly decreases the cholesterol and triacylglycerol levels in liver and serum of experimental rats.

Table 23. Effect of feeding of RMR for 14 weeks on serum cholesterol of male and female rats

Concentration of RMR mixed with diet (w/w)	Cholesterol (mg/dl)		Triacylglycerol (mg/dl)	
	Male rats	Female rats	Male rats	Female rats
Control	74.56±18.99	72.24±4.68	139.47±15.19	61.29±13.91
2%	70.51±15.78	63.99±6.29	121.87±8.23	54.45±12.27
4%	66.47±11.46	64.36±9.25	121.01±18.82	54.32±11.62
8%	64.96±19.11	61.10±9.31	120.34±7.39	52.51±8.69
12%	62.75±15.23	56.34±7.61	109.33±18.62	45.26±6.22

Values are mean ±S.E.M. of five rats in each group. Mean values within each column with different superscripts are significantly different ($p \leq 0.05$) by Duncan's test.

Table 24. Effect of feeding RMR for 14 weeks on liver cholesterol of male and female rats

Concentration of RMR mixed with diet (w/w)	Cholesterol (mg/g)		Triacylglycerol (mg/g)	
	Male rats	Female rats	Male rats	Female rats
Control	**60.77±21.95**	**55.79±18.28**	**66.8±5.25**	**67.7±5.80**
2%	**51.92±18.34**	**50.67±16.82**	**59.6±4.27**	**60.3±5.83**
4%	**46.13±13.84**	**44.82±11.36**	**56.1±6.69**	**57.8±4.32**
8%	**44.82±11.36**	**42.61±16.49**	**52.7±5.27**	**55.1±7.18**
12%	**37.49±7.86**	**38.71±5.39**	**50.0±9.22**	**51.7±6.52**

Values are mean ±S.E.M. of five rats in each group. Mean values within each column with different superscripts are significantly different ($p \leq 0.05$) by Duncan's test.

These results demonsrate the safety of RMR since it did not induce acute or sub-chronic toxic effects in rats. No treatment related histopathological changes were observed between the control and the RMR fed rats even at highest dosage of 12% for 14 weeks. Further, the RMR was well tolerated even at 12% dietary level as evidenced by absence of any adverse effects on growth, body weight gain, organ weight, hematological parameters or serum enzyme levels. Serum cholesterol and triacylglycerol levels were significantly lower than those in the control group. This suggested the hypolipidemic effect of RMR.

2.2 ISOLATION AND CHARACTERIZATION OF BIOACTIVE COMPOUND FROM *M. PURPUREUS* MTCC 410

Monascus anka, *M. ruber, M. purpureus* are the common speices used to prepare RMR to obtain a mixture of pigments of polyketide origin (Tseng *et al.*, 2000). Recently, RMR has become a popular dietary supplement due to many of its bioactive constituents. It is well-known that *Monascus* can produce the bioactive secondary metabolites such as γ-aminobutyric acid (GABA), that importance in neurotransmitting, hypotensive, and diuretic functions (Kono & Himeno, 2000). Moreover, mycelial extracts from *Monascus* were reported to be antimutagenic (Izawa *et al.*, 1997), antibiotic, embryotoxic and teratogenic (Nozaki *et al.*, 1991). However, there have been very few reports focusing on the antitumor effects of *Monascus* metabolites. In general, a compound that shows direct cytotoxic effects on tumor cells, and immunomodulatory activity may possess potency for antitumor application. Ankaflavin has shown selective cytotoxicity to cancer cell lines by an apoptosis-related mechanism and a relatively low toxicity to normal fibroblasts (Su *et al.*, 2005). It also has shown immunosuppressive activity on mouse T splenocytes (Martinkova *et al.*, 1999).

Normally wide range of natural pigments like, *Monascus* pigments (red, orange and yellow), carotenoids, anthocyanins, flavonoids and anthroquinone are used as color additives and different methods have been described to analyze these for use in foods. Among these, thin layer chromatography (TLC), gas chromatography (GC), high-performance liquid chromatography (HPLC) with a visible light absorbance

detector or a photodiode array detector and HPLC-mass spectrometry (LC-MS) are the most powerful analytical separation methods (Toshiro & Shigeru, 2000). There were few reports on the bioactive functions of these pigments.

Though, the above properties are attributed to ankaflavin, there are no reports regarding the antioxidant activity of this compound. Hence, ankaflavin was purified and its antioxidant activity was estimated in this section.

2.2.1 PURIFICATION OF ANKAFLAVIN (PIGMENT) FROM RMR

To evaluate the bioactivity of ankaflavin present in RMR, fourteen day old *M. purpureus* fermented rice was powdered to 60-80 mesh and the total pigments extracted using methanol. Five gram methanol extract was subjected to silica gel column chromatograph and step-wise elution in a linear gradient of hexane, chloroform and ethyl acetate (100:0; 100; 25:75; 50:50; 75:25 v/v) was carried out. About 13 fractions, each measuring approximately 100 ml was collected depending upon colour intensity of the eluting fractions (**Figure 42**).

Figure 42. Silica gel column chromatography for the separation of ankaflavin (pigment).

The fractions were concentrated by flash evaporation and fractions of same R_f value pooled together after TLC. The fraction showing higher DPPH activity was further purified as above.

Of the 8 fractions collected, concentrated and evaluated for DPPH radical scavenging activity, the fractions eluted with chloroform and chloroform: ethyl acetate mixture scavenged more DPPH. These fractions further separated on a silica gel column and eluted with a linear gradient of hexane, chloroform and ethyl acetate. The two fine fractions showing DPPH activity were eluted. These fractions were concentrated to dryness and dissolved in chloroform for HPLC analysis. The detailed procedure is described in Materials and Methods.

A single peak (**Figure 43** showing a retention time of 13.5 mins was alone eluted. This showed the purity of the compound.

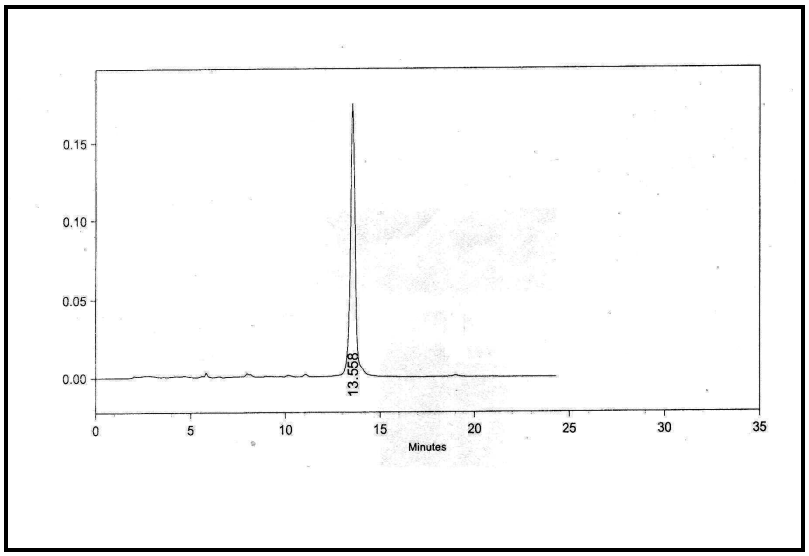


Figure 43. HPLC profile of the pure compound

2.2.2. NMR CHARACTERIZATION OF THE BIOACTIVE COMPOUND

The purified compound was further characterized by structural analysis. The compound was subjected to UV-Visible spectrum, MS, and NMR. ^{1}H and ^{13}C NMR were recorded for the purified pigment as mentioned in Materials and Methods.

^{1}H NMR spectrum assigned hydrogen atoms in the molecule. The assignment strategies involved the analysis of 1-D ^{1}H NMR (**Figure 44**). The assignments of proton signals are shown in **Table 25.** Likewise the data from ^{13}C 1-D NMR (**Figure 45**) and ^{13}C- ^{1}H NMR HSQCT spectra (**Figure 46**) were simultaneously analyzed in order to assign individual carbons to the molecule (**Table 25**). The selective direct coupling of carbon and hydrogen correlation was determined by HMQC (**Figure 47**).

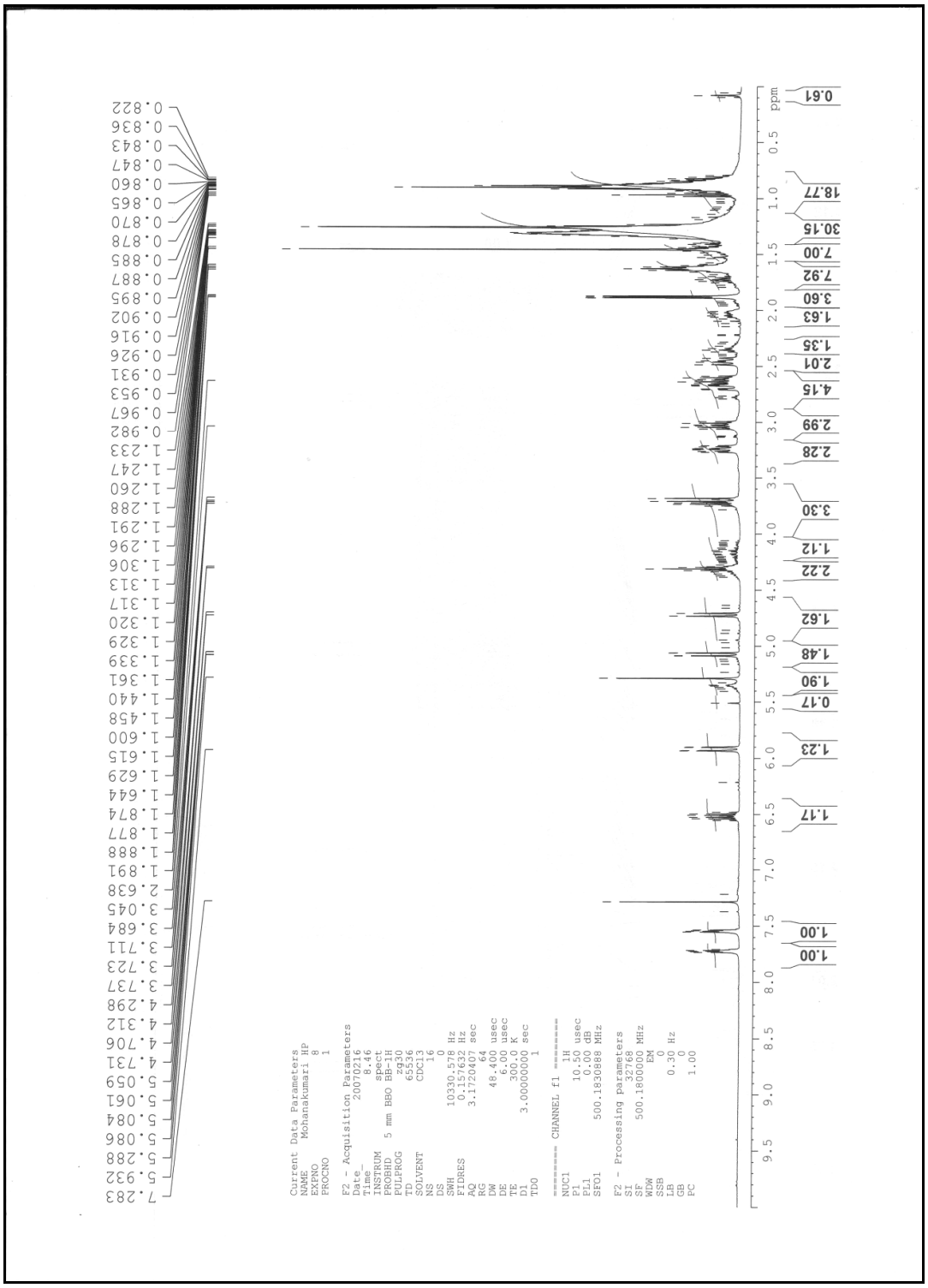


Figure 44. Proton Nuclear Magnetic Resonance (^{1}H NMR) Spectroscopy of the pure compound

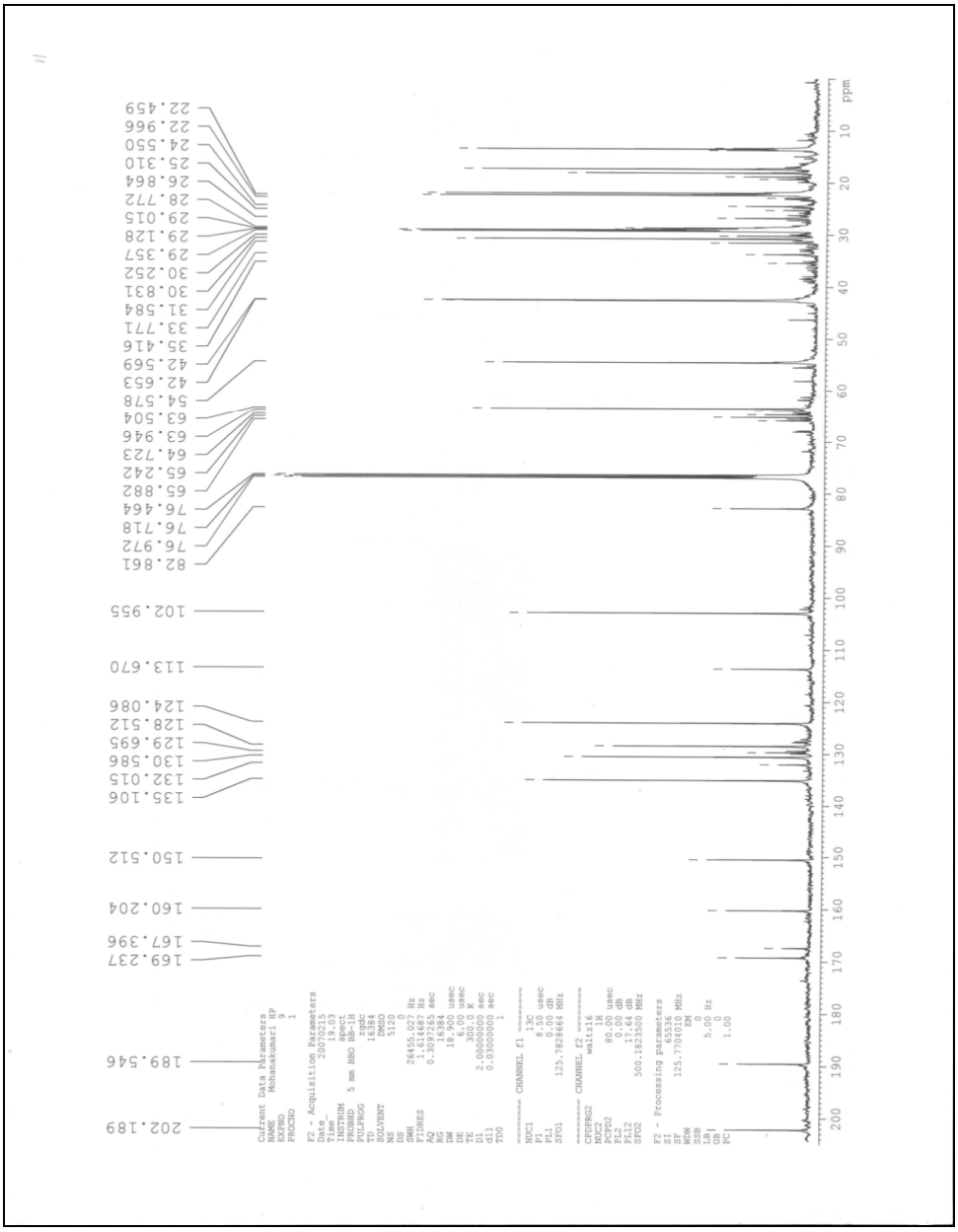


Figure 45.Carbon-13 Nuclear Magnetic Resonance (^{13}C NMR) Spectroscopy of the pure compound

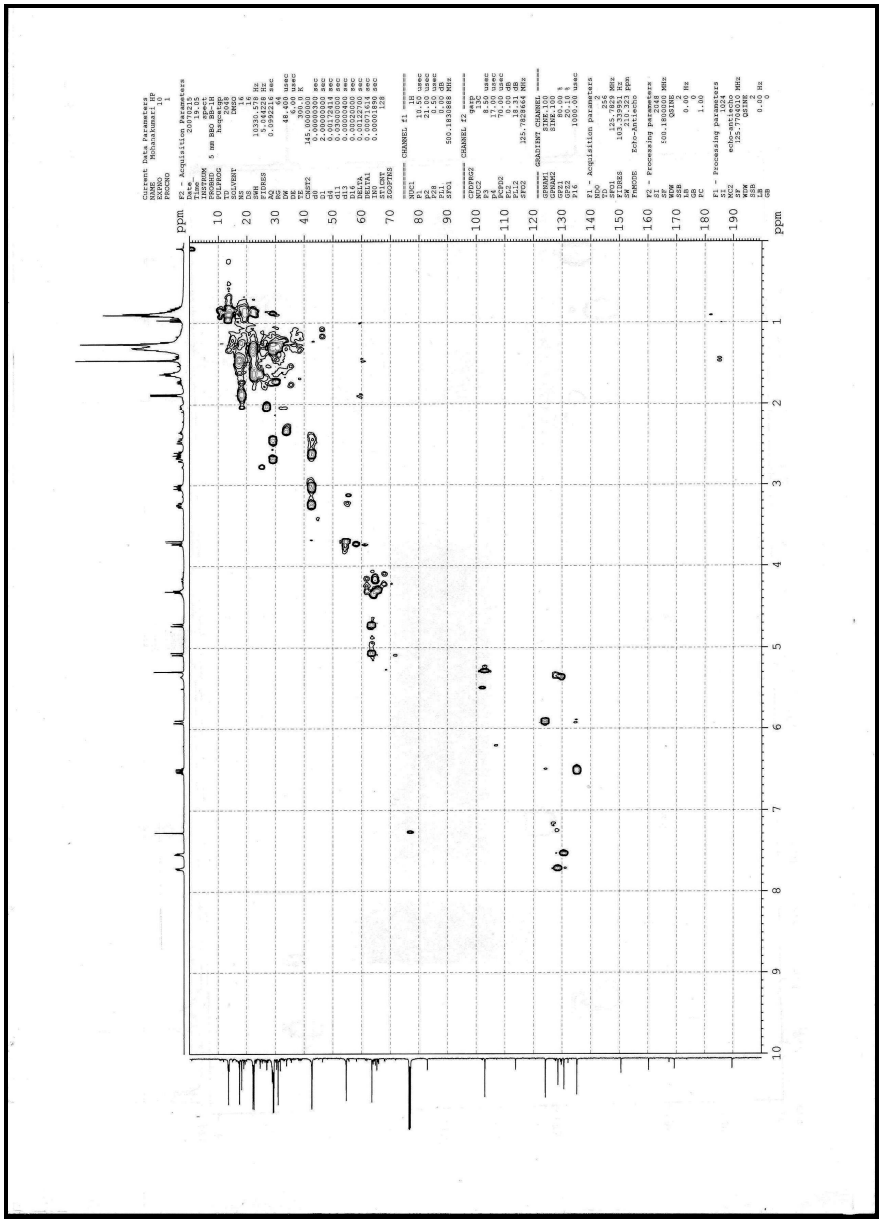


Figure 46. Two Dimensional Heteronuclear Single Quantum Coherence (2D-HSQC) spectrum of the pure compound

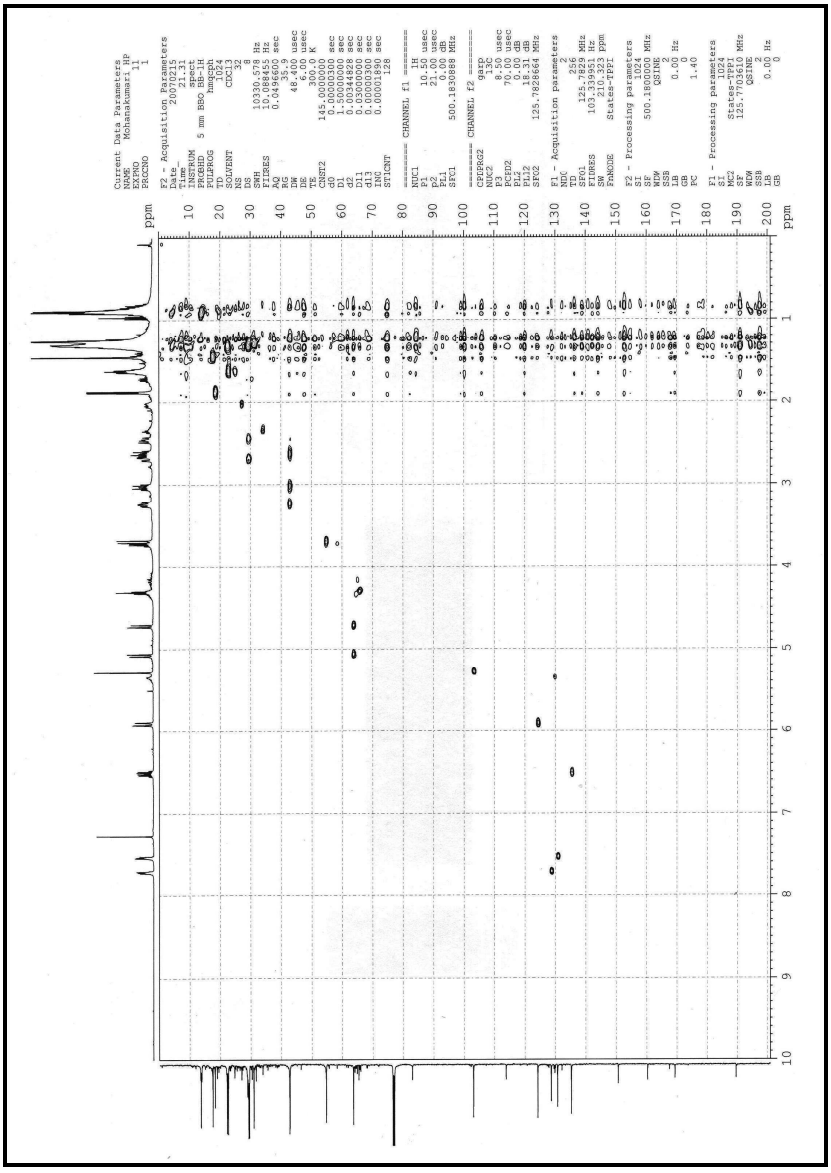


Figure 47. Two Dimensional Heteronuclear Multiple Quantum Coherence (2D-HMQC) spectrum of the pure compound

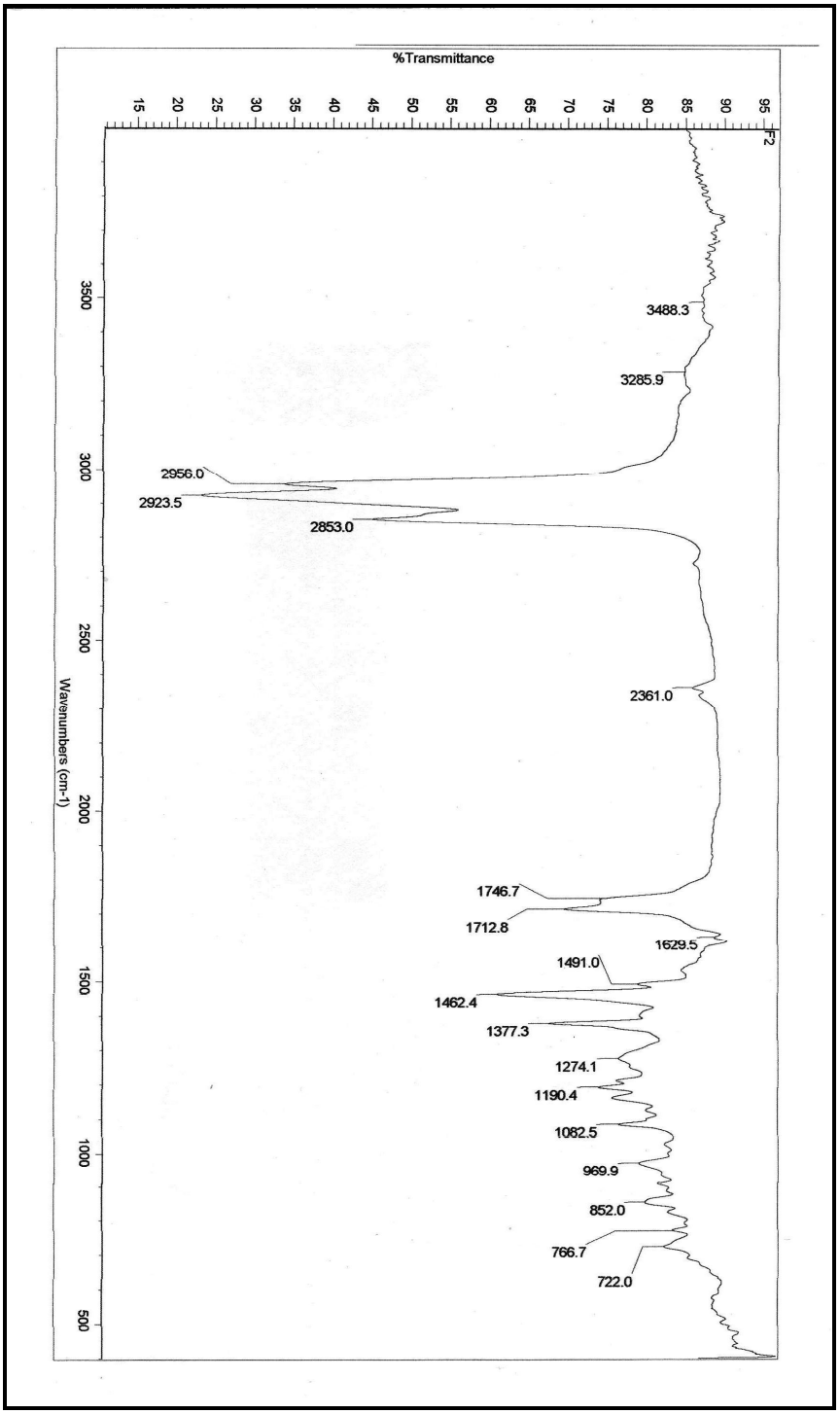


Figure 48. IR spectrum of the pure compound

Table 25. 2D-HSQCT NMR spectral data of the pure compound

Group	^{1}H NMR δ_{ppm} (J,Hz)	^{13}C NMR δ_{ppm}
1-CH_3	1.85 (d, 6.5)	18.1
2-CH	5.90 (m, 6.5, 15.8)	130.5
3-CH	6.48 (d, 15.8)	124.3
4-C	-	169.5
5-CH	5.28 (s)	103.2
6-CH_2	4.31 (m, 12.7)	63.5
7-CH	3.25 (d, 8.3)	42.5
8-C	-	83.1
9-CO	-	189.5
10-C	-	135.2
11-C	-	150.5
12-CH_2	2.65 (m, 5.0, 16.7)	33.5
13-CH	3.72 (d, 5.0)	54.5
14-CO	-	160.2
15-CH_3	1.45 (s)	17.2
16-CO	-	202.5
17-CH_2	2.45 (t, 6.5)	35.5
18- CH_2	1.29	23.1
19-CH_2	1.30 (m)	28.5
20-CH_2	1.28 (m)	29.2
21-CH_2	1.26 (m)	31.5
22-CH_2	1.23 (m)	22.1
23-CH_3	0.87 (t, 7.0)	13.5

IR data (**Figure 48**) showed OH stretching at 2854-2955 cm^{-1}, carbonyl stretching at 1717.6 cm^{-1} and C=C stretching at 1462 cm^{-1}. UV-visible spectrum (**Figure 49**) of the compound with absorption at 383 nm indicated π-π* transition of the olefinic group. Spectroscopic determination of the mass of a compound relies on bombarding molecules with high energy electrons and converting them to molecular ions. The ions accelerated in an electric field are separated based on their mass to charge ratio (*m/e*) by a spectrometer for estimation of molecular mass of the compound. Since the compound of molecular mass 427 resolved as a major peak (**Figure 50)**, this fraction was identified as the compound of interest.

Mass spectrum data supported the identification of major compound as ankaflavin.

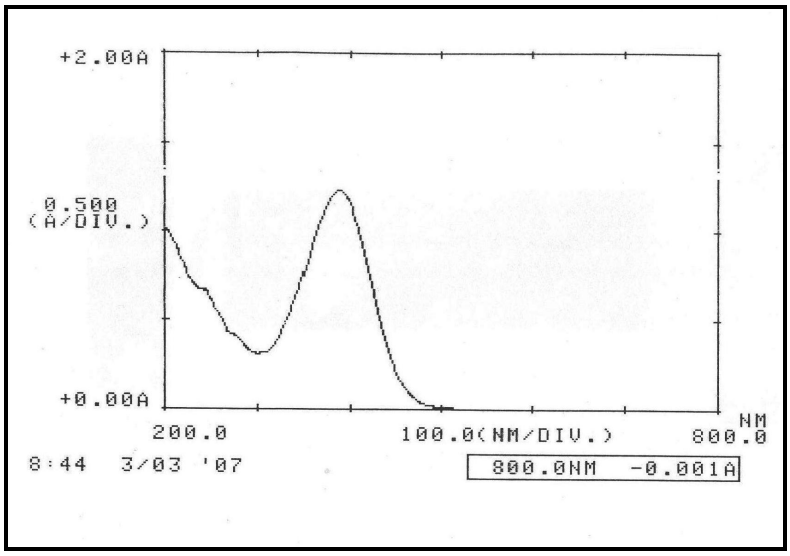
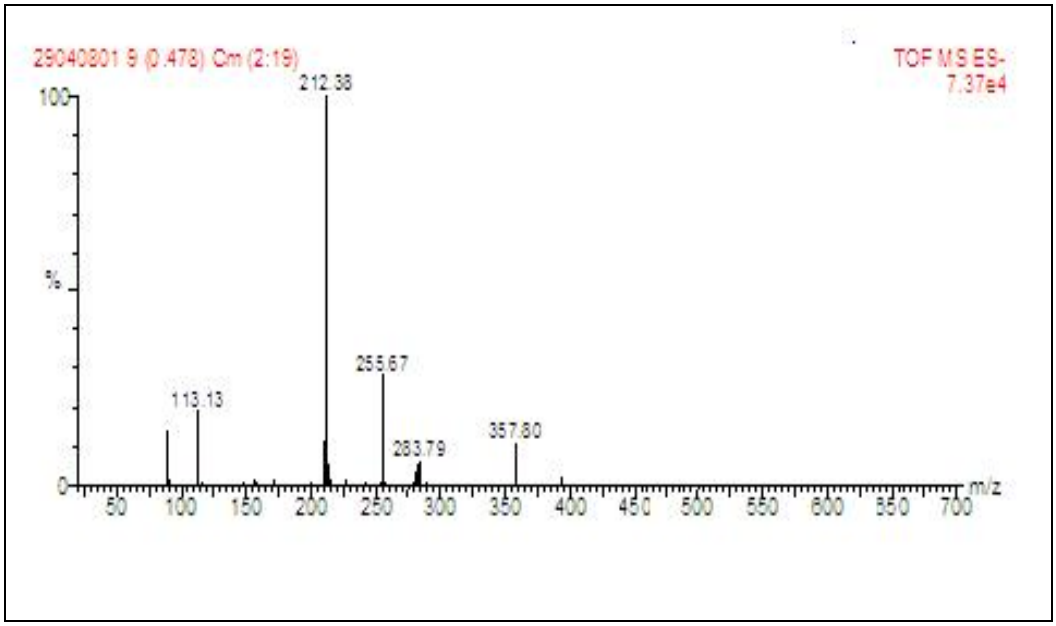


Figure 49. UV-Visible spectrum of the pure compound



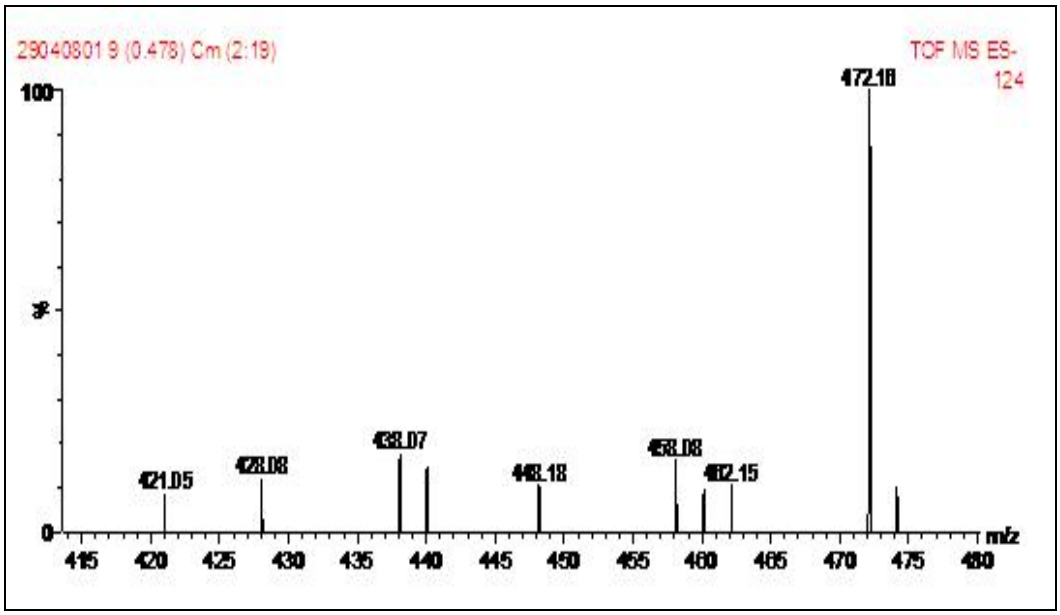


Figure 50. Mass spectrum of ankaflavin

Based on the above data, the compound was characterized as ankaflavin the structure of which is shown in **Figure 51.** The melting point and optical rotation of ankaflavin were 116 °C and +0.095 respectively.

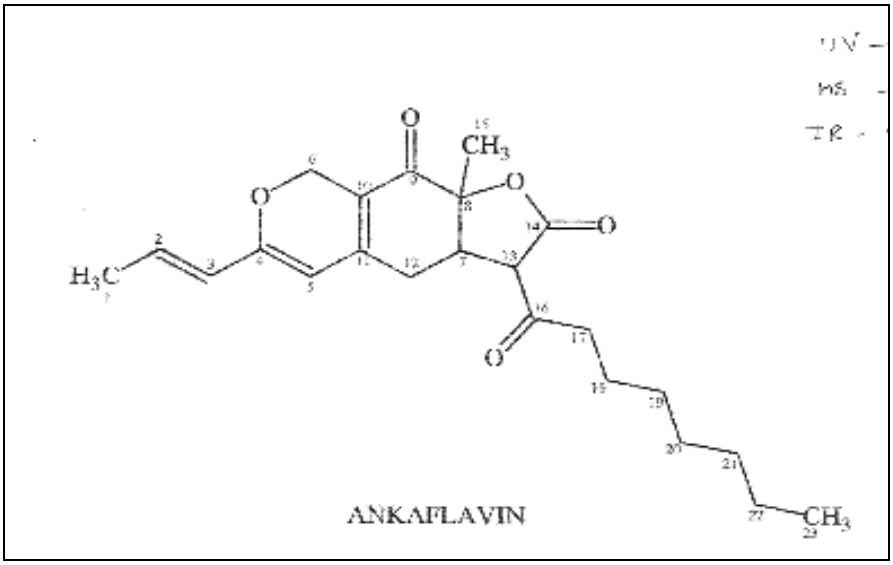


Figure 51. Structure of ankaflavin

2.2.3. DETERMINATION OF ANTIOXIDANT ACTIVITY OF ANKAFLAVIN

A compound can exert its antioxidant activity by scavenging radicals, decomposing peroxides or chelating metal ions. The antioxidant activity of both crude methanol extract and purified compound was carried out by *in vitro* assays for DPPH radical scavenging, inhibition of ascorbate autoxidation, reducing activity and lipid peroxidation **(Table 26)**.

The purified samples dissolved in ethanol were used for the assay. Fourty µg of ankaflavin was used for the assay procedure. All the experiments were carried out in triplicates by maintaining appropriate blanks and controls. The details are described in Materials and Methods.

The phenolic content of methanol extract of RMR was 141.32±0.01 µg ml^{-1} and expressed as mg gallic acid equivalent/100g dry weight. The IC_{50} value to scavenge DPPH radical for methanol extract, ankaflavin and BHA were found to be 100.78, 50.01 and 18.96 µg ml^{-1} respectively **(Table 26)**.

Inhibition of ascorbate autoxidation and reducing activity by crude methanol extract of RMR was found to be more compared to pure ankaflavin and BHA. The DPPH and lipid peroxidation inhibition activity of ankaflavin was better compared to methanol extract. BHA showed lipid peroxidation inhibition activity of 38.02±1.53 µg/ml **(Table 26).**

Table 26. Antioxidant activty of RMR methanol extract and ankaflavin

	Phenolics mg/100g	**DPPH scavenging (IC_{50})**	**Inhibition of ascorbate autoxidation (%)**	**Reducing (equivalent cysteine, µM)**	**Lipid peroxidation (IC_{50})**
Methanol extract	**141.32±0.01**	**100.78±2.66**	**21.10±0.95**	**1.35±0.05**	**47.02±1.91**
Ankaflavin	**79.92±0.03**	**50.01±1.56**	**15.32±1.02**	**0.95±026**	**15.39±1.01**
BHA	**-**	**18.96±1.28**	**9.76±1.45**	**0.65±0.11**	**38.02±1.53**

Ankaflavin isolated from the solid-state cultures of *M. purpureus* MTCC 410 was a potent antioxidant molecule.

2.3. EFFECT OF *MONASCUS* PIGMENT ON *LACTOBACILLUS* BACTERIA

Probiotics are live microbial food supplements which benefit the health of consumers by maintaining or improving their intestinal microbial balance. Due to their perceived health benefits, probiotic bacteria have been increasingly included in yoghurts and fermented milks during the past two decades. The use of probiotic bacterial cultures stimulates the growth of preferred microorganisms, eliminates out potentially harmful bacteria, and reinforces body's natural defense mechanisms. Before a probiotic can benefit human health it must fulfill several criteria: It must have good technological properties so that it can be manufactured and incorporated into food products without losing viability and functionality or creating unpleasant

flavours or textures. It must survive passage through the upper gastrointestinal (GI) tract and arrive alive at its site of action and must be able to function in the gut environment.

The literature on the fungus *Monascus* has documented the use of its colour in foods. *Monascus* sp grown culture substrates appears red in colour due to rubropunctamine and monascorubramine pigment. The applications of *Monascus* colours for sausage, hams, fish paste, surimi and tomato ketchup have been described (Laurent Dufosse´ *et al.*, 2005). The pharmaceutical applications of polyketides (azophilones) and monacolins produced by *Monascus* were evidenced by their antioxidant, antibacterial, anti-inflammatory properties and ability to reduce body cholesterol (Akihisa *et al.*, 2005a). Amino acid derivatives of azaphilones possesses antibacterial properties (Kim et al. 2006) and ankaflavin is known to induce apoptosis in human cancer cell lines, HepG2 and A549 (Su et al., 2005). Other active ingredients in RMR include sterols (β-sitosterol, campesterol, stigmasterol, sapogenin), isoflavones and monounsaturated fatty acids. In addition to their cholesterol-lowering effect, *Monascus* fermented products also have antioxidant activities (Aniya et al., 1999), however, these activities are different from those of many plant-derived foods (Liu & Ng, 2000). *M. purpureus* is also known to produce both glucosidic (genistin and daidzin) and aglyconic (genistein and daidzein) isoflavones.

In order to improve the nutraceutical value of *Monascus* fermented products, the antioxidant activities was increased by supplementation of methanol extract of *M. purpureus* with Lactic acid bacteria (LAB).

2.3.1. EFFECT OF *M. PURPUREUS* EXTRACT ON VIABILITY OF LAB

To increase the nutraceutical values of *M. purpureus* using LAB, it is necessary to evaluate the effect of *M. purpureus* pigment on viable count of LAB. Pigments from *M. purpureus* fermented rice were extracted with methanol. Its effect on LAB viable cell counts was determined, in duplicate, using the pour plate method and MRS agar medium (Materials and Methods).

The comparative growth of the five isolates of LAB and its combination with *M. purpureus* pigment at 24 h at 37° C is shown in **Table 27.** Among the LAB cultures, the variations was observed in viable count of *L. casei* (9.8 log10 CFU/ml) followed by *L. plantarum* (9.5 log10 CFU/ml) and *L. helviticus* (8.6 log10 CFU/ml). After 24 h incubation, no significant difference in viable counts of LAB was observed. Instead, there was an increase in the viable count of *L. helviticus* (8.8 log10 CFU/ml) and *L. plantarum* (9.7 log10 CFU/ml) compared to control.

Table 27. Viability of LAB in combination with *Monascus* pigment (Averages of duplicate analyses).

Isolates	**Colony Forming Unit (CFU)**	
	LAB	**Pigment +LAB**
L. acidophilus	**$7.60X10^9$**	**$7.59X10^9$**
L. bulgaricus	**$5.15X10^9$**	**$5.16X10^9$**
L. casei	**$9.8\ X10^9$**	**$9.78\ X10^9$**
L. helviticus	**$8.6\ X10^9$**	**$8.8\ X10^9$**
L. plantarum	**$9.5X10^9$**	**$9.7X10^9$**

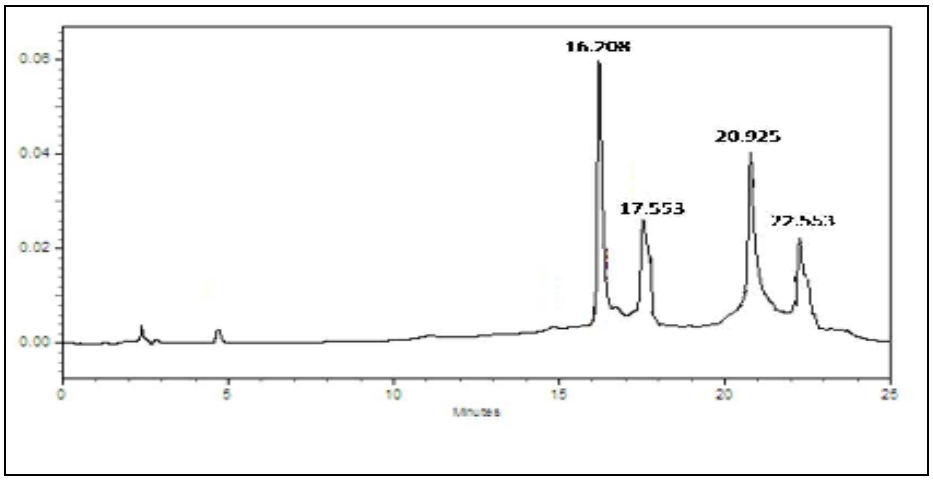
The data suggest that *M. purpureus* pigment is not antimicrobial to LAB.

2.3.2. ESTIMATION OF ISOFLAVONES IN *M. PURPUREUS* EXTRACT

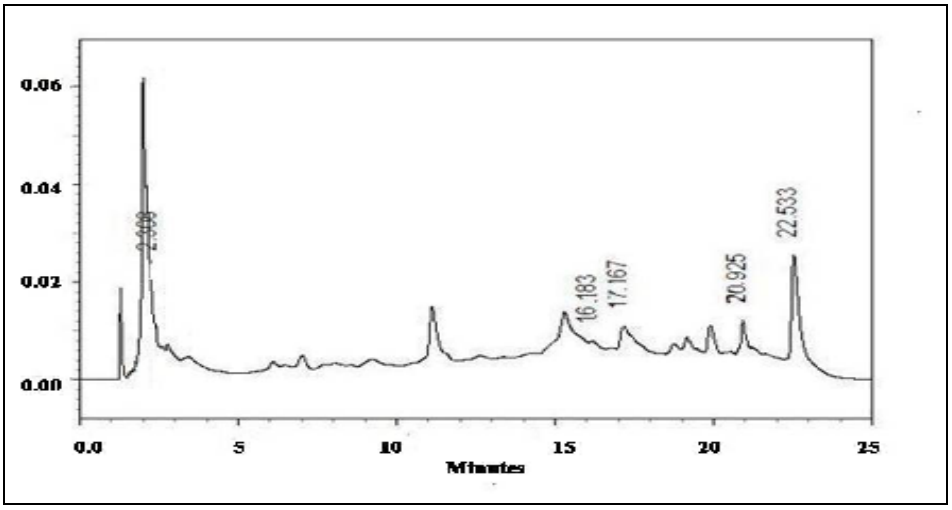
In previous experiment, the *M. purpureus* pigments did not show any adverse effect on viable count of LAB. The isoflavone isomers were eluted according to their polarity and hydrophobic interaction with the reversed-phase HPLC column. The elusion profile of glucosidic (genistin and daidzin) and aglyconic isoflavones (genistein and daidzein) in standard and RMR are shown in **Figure 52**. The

concentrations of each isoflavones were quantified by HPLC using standard isoflavones.

The concentrations of glucosidic isoflavones (genistin and daidzin) and aglyconic isoflavones (genistein, daidzein) in *M. purpureus* pigment were 0.37 and 20.39 mg/100 ml respectively.



A



B

Figure 52. HPLC profile of isoflavones standard (A) and that in RMR extract (B).

2.3.3. ANTIOXIDANT ACTIVITIES OF LAB CULTURE GROWN WITH *MONASCUS* PIGMENT

Various studies indicate that isoflavones have antioxidant, anticarcinogenic, and osteoporosis-preventing characteristics. Depending upon the method of antioxidant assays, various forms of isoflavones have been shown to possess antioxidant activities.

The previous experiment showed the presence of islflavones in *M. purpureus* pigment contains isoflavones. The antioxidant activity of methanol extract of *M. purpureus* and also the antioxidant activity of LAB cultivated in presence of the above *M. purpureus* extract were studied.

The DPPH scavenging activity, inhibition of ascorbate autoxidation, reducing activity of *M. purpureus* pigment and LAB are shown in **Table 28**. The increase in antioxidative activity of LAB cultured with *M. purpureus* pigment was observed.

Higher DPPH scavenging activity, inhibition of ascorbate and reducing activity were observed in cultures of *L. casei*, *L. plantarum* and *L. helviticus* with 41.68 %, 10.01 and 0.83 μM grown with pigments respectively.

The increase in the antioxidant capacity may be due to the aglyconic form of genistein and daidzein of *M. purpureus*. These aglyconic forms are formed by the catalytic action of β-glucosidase on glucosidic form of isoflavnes during fermentation. The results indicated *M. purpureus* pigments to induce the antioxidant activity in LABs.

Table 28. Antioxidant activities of LAB grown in the presence of *M. purpureus* pigment

Cultures	Antioxidant activity		
	DPPH scavenging (%)	**Inhibition of ascorbate autoxidation (%)**	**Reducing (equivalent cysteine, µM)**
***M. purpureus* pigment**	**35.81**	**7.31**	**0.65**
Pigment + *L. acidophilus*	**39.08**	**9.98**	**0.70**
Pigment + *L. bulgaricus*	**36.19**	**8.65**	**0.73**
Pigment + *L. casei*	**41.68**	**8.18**	**0.77**
Pigment + *L. helviticus*	**36.90**	**9.80**	**0.83**
Pigment + *L. plantarum*	**38. 35**	**10.01**	**0.75**

CHAPTER 3

HYPOLIPIDEMIC AND ANTIOXIDANT ACTIVITY OF *MONASCUS PURPUREUS*

INTRODUCTION

Secondary metabolites produced by the filamentous fungus *M. purpureus,* during fermentation, include pigments (red, orange and yellow), dihydromonacolins, monacolin K (lovastatin)- an inhibitor of HMG-CoA reductase (Endo, 1979), γ-aminobutyric acid (GABA)- an antihypertensive metabolite, dimerumic acid an antioxidant (Aniya *et al.*, 2000) and metabolites with antibacterial, antitumor and immunosuppressive properties (Martinkova *et al.*, 1999). Since the metabolites can lower cholesterol level and blood pressure, they have found application as functional health food and as dietary supplement (Manzoni *et al.*, 1999).

In recent years, a high fat diet (HFD) has been implicated to atherosclerosis a cardiovascular disease (Libby, 2002). Hypertriglyceridemia/hyperlipidemia is considered as the major risk factor for atherosclerosis and cardiovascular diseases. Management of hyperlipidemia with healthy and less atherogenic diet, less stressful life style and physical activity are successful for certain patients, but a majority of patients need therapeutical intervention to effectively lower hyperlipidemia. Atherosclerosis is a process in which fat, cholesterol, cellular waste products, calcium and other substances are deposited inside the artery (Ross, 1993). The process leads to an inflammatory disease via a sequential process of initiation, progression and rupture of lipid-rich atherosclerotic plaques (Libby, 2002). Cholesterol is a key substance causing coronary artery disease (CAD). The cholesterol level in the blood can be expressed by the atherogenic index (AI) and is a risk factor for atherosclerosis and heart disease. Therefore, lowering the cholesterol levels has become an important concern for patients with cardiovascular diseases. Management of hyperlipidemia with diet and exercise is successful in certain patients, but for a significant number of patients, pharmacological intervention is required to effectively lower cholesterol. Lipid lowering agents that inhibit 3-hydroxy-3-methylglutaryl coenzyme A (HMG-

CoA) reductase (EC 1.1.1.34) are prominent among the drugs of choice for treating hyperlipidemia. Therefore, activity regulation of HMG-CoA reductase can control the cholesterol content in the body. The available anti-hyperlipidemic drugs lowers circulating cholesterol levels either by prevention of cholesterol absorption, increasing hepatic uptake of low density lipoproteins (LDL) or inhibition of cholesterol synthesis. One principle mechanism to lower cholesterol is to increase LDL receptor numbers by competitively inhibiting HMG-CoA reductase. The HMG-CoA reductase inhibitors significantly reduce cholesterol in animal and human subjects. Reduction is due to the structural similarity of acid form of the statins to HMG-CoA, the natural substrate of HMG-CoA reductase (Istvan & Deisenhofer, 2001).

Statins, a class of fungal secondary metabolites, have become the focus of great attention due to their ability to influence the *de novo* synthesis of endogenous cholesterol. Lovastatin has a methylbutyryl side-chain at 8-α and a methyl group in 6-α of the naphthalene ring which later present as the corresponding β-hydroxy acid in the pharmaceutical active drug. While pravastatin 6-hydroxy sodium salt is an analogue of mevastatin (Manzoni & Rollini, 2002). Lovastatin is a well known inhibitor of HMG-CoA reductase produced by *Aspergillus terreus, M. purpureus* and other fungi. Hence these fungi are widely used for the hypercholesterolemia drug, for use in reducing plasma cholesterol levels in humans (Alberts *et al.*, 1980). Statins that inhibit HMG-CoA reductase are widely used drugs to control hyperlipidemia (Alberts, 1990; Blankenhorn *et al.*, 1993). Moreover, plant phytosterols, saponins, fungal metabolites such as mevinolin, monacolin K are being investigated for their antihyperlipidemic and antiatherosclerotic properties (Kroon *et al.*, 1982; Ikeda & Sugano, 1998; Harwood *et al.*, 1993).

M. purpureus and its mutants produced pigments, statin, fatty acids, sterols and the role of amidase enzymes in pigment production have also been described earlier (Dhale *et al.*, 2009). In the earlier toxicological studies, RMR obtained under solid state fermentation of *M. purpureus* did not show any adverse effect (Mohan Kumari *et al.*, 2009). Interestingly, rats fed with RMR for 14 weeks significantly

reduced the cholesterol and triacylglycerol levels in both serum and liver **(Expteriment.2.1.8)**. Based on this data, the comparative long term and short term effects of *M. purpureus* MTCC 410 on hyperlipidemia induced rats was designed. This study describes the functional food characteristics like, hypolipidemic, antioxidant molecules and antioxidant enzymes.

3.1. HYPOLIPIDEMIC PROPERTY OF *M. PURPUREUS* RMR

To understand the health-related properties ascribed to RMR, a complete study of the metabolites of RMR as a hypolipidemic agent was carried out. *M. purpureus* MTCC 410 produced 12.29 ± 0.39 mg of statin, 4.47±0.3 mg of sterol and significant amount of fatty acids per gram of dry substrate.

RMR also contained phytosterols such as beta-sitosterol and campesterol (Heber *et al.*, 1999), which are known to interfere with cholesterol absorption in the intestine (Moghadasian & Frohlich, 1999). The combination of such dietary sterols with statin drugs has in fact been suggested as a more effective means of lowering cholesterol than statin alone (Plat & Mensink, 2001). RMR possesses properties for lowering cholesterol level and blood pressure. Hence developed as a health food in human dietary supplements (Manzoni *et al.*, 1999) requires the determination of citrinin concentration. Citrinin levels produced by *M. purpureus* MTCC 410 when analysed it was found to be 1.427 mg/kg in RMR (Mohan Kumari *et al.*, 2009). This was much lower than the prescribed for acute nephrotoxicity, which is 18.4 g/kg substrate (Krejci *et al.*, 1996).

The production of more quantity of monacolin, sterols and low quantity of citrinin suggested the possible application of *M. purpureus* RMR to evaluate its hypolipidemic property.

3.1.1 GROUPING AND EXPERIMENTAL DIET

It is well documented that the reduction of serum cholesterol levels can reduce morbidity and mortality both in patients with coronary heart disease. Reducing serum cholesterol is also the primary prevention in healthy humans at high risk for coronary heart disease. In an earlier experiment (acute and sub-chronic toxicological studies) of RMR did not show any adverse effect in rats but significantly reduced the cholesterol and triacylglycerol levels in both serum and liver at 14 weeks (Mohan Kumari et al., 2009). Hence the effect of diet enriched with RMR on rats was studied.

Sixty four male albino rats (CFT-Wistar strain) weighing 150-180 g bred in animal house facility at the Central Food Technological Research Institute, Mysore, India were used as experimental animals. Experimental diets were fed to the animals in accordance with American Institute of Nutrition (AIN)-76 diet formulation (American Institute of Nutrition 1977), with slight modification **(Table 29)**. The details are described in Materials and Methods.

Table 29. Composition of the experimental diet (g kg^{-1}diet)

Diet composition	Normal diet	HFD	HFD+8% RMR	HFD+12% RMR	HFD+16% RMR	HFD+lovastatin (g/kg)
Corn starch	**500**	**488.5**	**408.5**	**368.5**	**328.5**	**488**
Cellulose	**50**	**50**	**50**	**50**	**50**	**50**
Casein (fat free)	**200**	**200**	**200**	**200**	**200**	**200**
Borasugar	**100**	**100**	**100**	**100**	**100**	**100**
Groundnut oil	**100**	**100**	**100**	**100**	**100**	**100**
Mineral mix[a]	**35**	**35**	**35**	**35**	**35**	**35**
Vitamin mix[b]	**10**	**10**	**10**	**10**	**10**	**10**
DL-Methionine	**3**	**3**	**3**	**3**	**3**	**3**
Choline chloride	**2**	**2**	**2**	**2**	**2**	**2**
Cholesterol	**-**	**10**	**10**	**10**	**10**	**10**
Bile acid	**-**	**1.5**	**1.5**	**1.5**	**1.5**	**1.5**
Lovastatin	**-**	**-**	**-**	**-**	**-**	**0.080**

[a] Bernheart tommerali salt mixture.

[b] AIN-76A vitamin mix.

3.1.2 FOOD INTAKE AND GAIN IN BODY WEIGHT BY RATS

Comparison of the organ weights of animals fed with RMR to that of the normally fed animals is often complicated by differences in body weights between groups. Hence the ratio of the organ weight to body weight (to account for differences in body weight) and the ratio of the organ weight to the brain weight (which represents a surrogate measure for lean body mass, which is not usually affected by xenobiotics) were used to determine the effect.

The gain in relative body weight was measured in rats fed with *M. purpureus* fermented rice for seven and fourteen week.

Effects of RMR supplementation to HFD on food intake and gain in body weight are measured (**Figure 53 and 54)**. No significant differences in average food intake were observed between control and RMR fed groups. The average gain in body weight of RMR fed groups was found to be lower compared to control group at initial periods of feeding (1-7 weeks), however, no significant change was observed between 8-14 weeks of feeding.

These results indicated the daily food intake and gain in body weight of rats were normal and did not differ among various experimental groups.

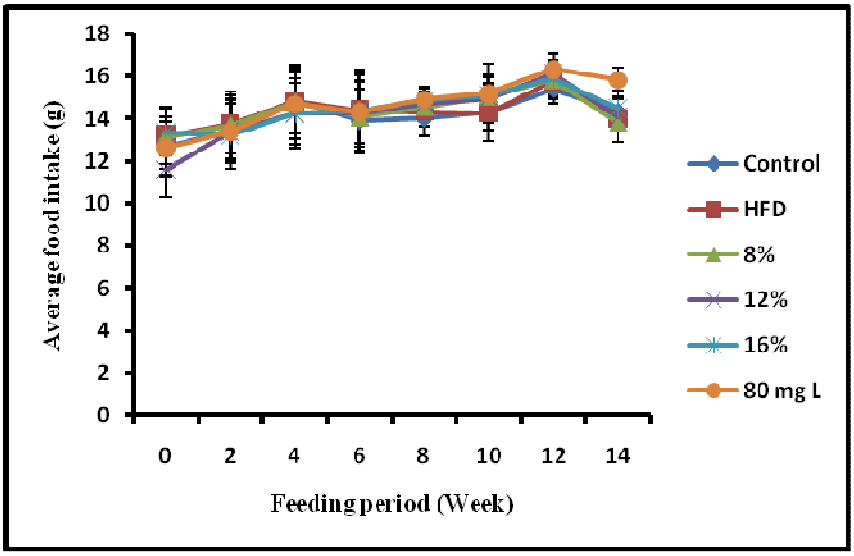


Figure 53. Food intake profile of male rats fed with dietary RMR. Values are ± SEM of 8 animals. No significant difference between control and *M. purpureus* RMR fed groups ($p < 0.05$).

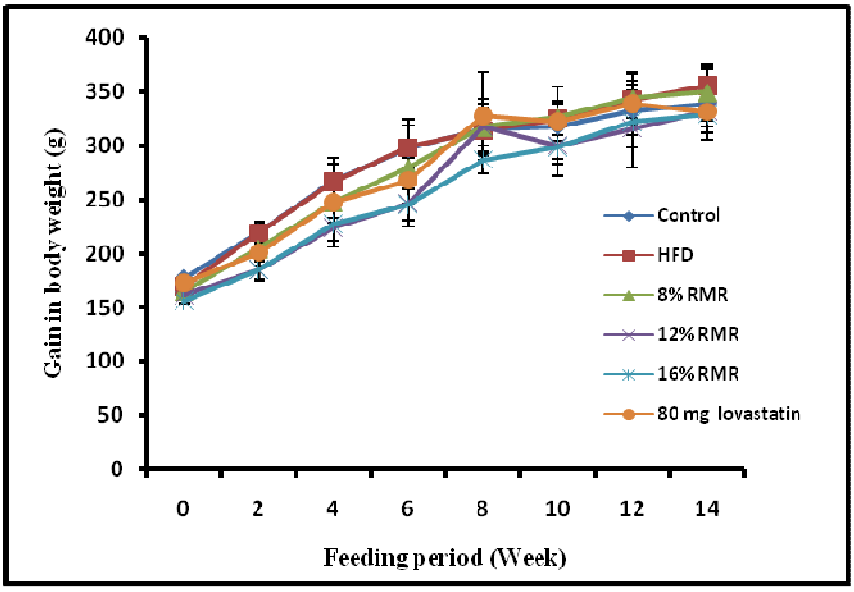


Figure 54. Mean absolute body weight of female rats fed with dietary RMR. Values are ± SEM of 8 animals. No significant difference between control and *M. purpureus* RMR fed groups ($p < 0.05$).

3.1.3 RELATIVE ORGAN WEIGHTS OF MALE RATS FED WITH RMR

During the routine toxicological studies the organ weight was measured to see the endocrine effect. Analysis of organ weight in toxicology studies is an important endpoint for identification of potentially harmful effects of chemicals. Organ weight can be the most sensitive indicator of an effect of an experimental compound, as significant differences in organ weight between treated and untreated (control) animals may occur in the absence of any morphological changes.

The gain in relative organ weight was measured in rats fed with *M. purpureus* fermented rice for seven and fourteen weeks. The details are described in Materials and Methods.

There were no marked differences in mean relative organ weights of various vital organs except in liver of HFD and RMR fed groups compared to control group. These changes in liver weight are ascribed to HFD. The average liver weight of HFD

fed rats increased by 60.56% over that of control group and addition of RMR reduced liver weights ranging from 16.16, 14.65 and 14.26 g in rats fed HFD supplemented with 8, 12 and 16% RMR, respectively during 14 week study **(Tables 30 and 31)**.

Similarly, the average relative liver weight of HFD fed rats increased by 48.8% over that of control group and addition of RMR reduced liver weights ranging from 4.30 to 3.43 g in rats fed HFD supplemented with 8 to 16% RMR **(Tables 32 and 33)**.

These results indicated that daily food intake and gain in body weight of rats were normal and did not differ among various experimental groups.

Table 30. Effect of feeding RMR for 7 weeks on organ weight of male rats

Dosage of RMR (g/kg b.wt)	Organ weight (g/100g body weight)							
	Liver	Lungs	Kidney	Heart	Testis	Adrenal	Brain	Spleen
Control	9.68±1.08	1.26±0.15	2.28±0.18	0.84±0.05	2.82±0.37	0.09±0.04	1.83±0.15	0.70±0.06
HFD	14.02±0.40	1.22±0.14	2.30±0.06	0.88±0.06	2.62±0.16	0.05±0.03	1.95±0.07	0.64±0.14
HFD+8%	10.46±0.42	1.22±0.09	2.23±0.09	0.85±0.04	2.33±0.41	0.05±0.02	1.65±0.24	0.72±0.11
HFD+12%	8.90±0.61	1.21±0.18	2.28±0.02	0.78±0.07	2.87±0.23	0.07±0.03	1.48±0.15	0.73±0.16
HFD+16%	8.27±0.84	1.20±0.04	2.29±0.06	0.81±0.04	2.75±0.15	0.07±0.01	1.44±0.18	0.69±0.10
HFD+80 mg lovastatin	8.57±0.42	1.22±0.06	2.28±0.02	0.82±0.09	2.59±0.29	0.07±0.01	1.43±0.09	0.74±0.02

Values are mean ± SEM of five animals. No significant difference between control and RMR fed groups ($P < 0.05$)

Table 31. Effect of feeding RMR for 14 weeks on organ weight of male rats

Dosage of RMR (g/kg/b.wt)	Organ weight (g/100g body weight)							
	Liver	Lungs	Kidney	Heart	Testis	Adrenal	Brain	Spleen
Control	**11.31±2.18**	**1.27±0.04**	**2.27±0.05**	**0.76±0.07**	**2.23±0.16**	**0.09±0.04**	**1.78±0.06**	**0.80±0.06**
HFD	**18.16±1.11**	**1.27±0.05**	**2.31±0.08**	**0.78±0.01**	**2.21±0.08**	**0.08±0.01**	**1.79±0.09**	**0.80±0.04**
HFD+8%	**16.16±1.67**	**1.26±0.07**	**2.29±0.06**	**0.75±0.08**	**2.19±0.11**	**0.09±0.01**	**1.75±0.06**	**0.78±0.04**
HFD+12%	**14.65±0.74**	**1.27±007**	**2.25±0.09**	**0.78±0.10**	**2.25±0.14**	**0.09±0.04**	**1.74±0.08**	**0.81±0.06**
HFD+16%	**14.26±0.80**	**1.23±0.07**	**2.25±0.04**	**0.71±0.24**	**2.22±0.10**	**0.09±0.01**	**1.75±0.08**	**0.80±0.06**
HFD+80 mg lovastatin	**14.45±0.86**	**1.26±0.11**	**2.27±0.03**	**0.72±0.21**	**2.25±0.11**	**0.09±0.01**	**1.75±0.03**	**0.81±0.03**

Values are mean ± SEM of five animals. No significant difference between control and RMR fed groups ($P < 0.05$)

Table 32. Effect of feeding RMR for 7 weeks on relative weight of male rats

Concentration of RMR mixed with diet (w/w)	Relative organ weight (g/100g body weight)							
	Liver	Lungs	Kidney	Heart	Testis	Adrenal	Brain	Spleen
Control	**3.08±0.16[a]**	**0.50±0.07**	**0.74±0.07**	**0.32±0.03**	**0.89±0.14**	**0.03±0.01**	**0.62±0.06**	**0.26±0.03**
HFD	**4.30±0.23[c]**	**0.50±0.05**	**0.72±0.02**	**0.30±0.02**	**0.87±0.06**	**0.02±0.01**	**0.60±0.15**	**0.25±0.04**
HFD+8%	**3.76±0.25[b]**	**0.50±0.03**	**0.73±0.05**	**0.31±0.02**	**0.88±0.15**	**0.02±0.01**	**0.59±0.07**	**0.26±0.05**
HFD+12%	**3.55±0.39[b]**	**0.49±0.04**	**0.73±0.04**	**0.31±0.03**	**0.88±0.08**	**0.03±0.01**	**0.60±0.06**	**0.23±0.05**
HFD+16%	**3.43±0.30[ab]**	**0.50±0.02**	**0.74±0.04**	**0.30±0.02**	**0.87±0.18**	**0.03±0.00**	**0.58±0.09**	**0.24±0.05**
HFD+80 mg lovastatin	**3.49±0.25[b]**	**0.50±0.03**	**0.73±0.03**	**0.30±0.02**	**0.89±0.08**	**0.02±0.00**	**0.61±0.10**	**0.26±0.01**

Values are mean of five rats. No significant difference in relative organ weights between control, HFD and HFD + RMR fed groups ($p<0.05$) except liver. Mean values within first column (liver) with different superscripts are significantly different ($p<0.05$).

Table 33. Effect of feeding RMR for 14 weeks on relative organ weight of male rats

Concentration of RMR mixed with diet (w/w)	Relative organ weight (g/100g body weight)							
	Liver	Lungs	Kidney	Heart	Testis	Adrenal	Brain	Spleen
Control	3.44±0.74[a]	0.38±0.06	0.69±0.13	0.26±0.06	0.86±0.17	0.03±0.02	0.54±0.10	0.25±0.07
HFD	5.12±0.45[c]	0.36±0.02	0.65±0.05	0.25±0.02	0.81±0.05	0.02±0.00	0.50±0.03	0.23±0.02
HFD+8%	4.64±0.59[b]	0.36±0.03	0.66±0.06	0.25±0.02	0.82±0.06	0.02±0.00	0.50±0.05	0.23±0.02
HFD+12%	4.42±0.09[b]	0.38±0.03	0.68±0.05	0.26±0.03	0.85±0.05	0.03±0.01	0.53±0.03	0.25±0.02
HFD+16%	4.34±0.15[b]	0.38±0.03	0.69±0.03	0.26±0.03	0.84±0.03	0.03±0.00	0.53±0.03	0.25±0.02
HFD+80 mg lovastatin	4.37±0.25[b]	0.38±0.03	0.69±0.02	0.26±0.02	0.85±0.01	0.03±0.00	0.53±0.01	0.24±0.01

Values are mean of five rats. No significant difference in relative organ weight between control, HFD and HFD+RMR fed groups ($p<0.05$). Mean values within first column (liver) with different superscripts are significantly different ($p<0.05$).

3.1.4 ANALYSIS OF SERUM CLINICAL ENZYMES IN RATS FED WITH RMR

For this study, blood samples were routinely collected during 7 and 14 weeks to monitor clinical pathological parameters. The clinical enzymes such as LDH, ALP, SGOT, and SGPT were assayed to examine the pathological parameter of the chemicals.

Details and procedure followed for toxicological evaluation are described in Materials and Methods.

Effects of RMR on serum clinical enzymes viz., LDH, ALP, SGOT, SGPT are shown in **Tables 34 and 35**. No significant increase in these enzyme levels was observed in RMR supplemented groups compared to HFD group and control group.

The creatinine and urea concentration were also quantified during this study. No significant difference was seen in control and experimental groups **(Table 34 and 35)**.

Thus the animals fed with RMR showed no pathological dysfunction.

Table 34. Effect of feeding RMR for 7 weeks on clinical enzymes of male rats

Concentration of RMR (w/w)	ALP	SGOT	SGPT	LDH	Creatinine	Urea
Control	**221.91±25.17**	**174.85±8.36**	**51.20±6.68**	**777.03±38.32**	**0.84±0.10**	**27.45±11.46**
HFD	**238.90±28.46**	**173.28±9.80**	**52.98±15.87**	**745.26±116.28**	**0.83±0.18**	**28.44±8.99**
HFD+8%	**221.86±10.29**	**170.49±6.95**	**50.12±14.89**	**760.20±111.15**	**0.81±0.35**	**27.80±8.25**
HFD+12%	**228.74±10.64**	**172.79±14.74**	**50.29±10.38**	**740.45±101.48**	**0.82±0.18**	**27.73±8.99**
HFD+16%	**218.50±40.98**	**174.22±9.11**	**53.19±7.17**	**795.13±190.95**	**0.84±0.13**	**28.06±7.65**
HFD+80 mg lovastatin	**233.29±10.26**	**172.65±13.89**	**52.59±6.05**	**751.88±110.51**	**0.81±0.36**	**28.52±5.86**

Values are mean ± SEM of five animals. LDH, lactate dehydrogenase (U/L); ALP, alkaline phosphatase (U/L); ALAT, alanine amino transferase (U/L); ASAT, asparate amino transferase (U/L). No significant difference between control, HFD and HFD + RMR fed groups ($P < 0.05$).

Table 35. Effect of feeding RMR for 14 weeks on clinical enzymes of male rats

Concentration of RMR in diet (w/w)	ALP	SGOT	SGPT	LDH	Creatinine	Urea
Control	**236.84±29.00**	**157.05±12.65**	**65.16±11.75**	**963.03±38.32**	**0.53±0.17**	**56.82±13.99**
HFD	**230.22±20.78**	**155.97±17.87**	**63.45±15.30**	**965.26±116.28**	**0.51±0.23**	**58.42±24.09**
HFD+8%	**223.97±18.04**	**156.25±15.83**	**63.06±19.55**	**960.20±111.15**	**0.55±0.18**	**54.82±11.26**
HFD+12%	**224.65±8.23**	**151.85±16.55**	**60.76±6.66**	**940.45±101.48**	**0.59±0.10**	**56.89±20.15**
HFD+16%	**228.53±10.30**	**156.39±3.60**	**65.75±8.47**	**975.13±190.95**	**0.56±0.15**	**55.82±15.47**
HFD+80 mg lovastatin	**236.36±10.59**	**155.03±18.29**	**61.67±9.38**	**951.88±110.51**	**0.55±0.12**	**57.82±9.14**

Values are mean ± SEM of five animals. LDH, lactate dehydrogenase (U/L); ALP, alkaline phosphatase (U/L); ALAT, alanine amino transferase (U/L); ASAT, asparate amino transferase (U/L). No significant difference between control, HFD and HFD + and RMR fed groups ($P < 0.05$).

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3.1.5. TC AND TG LEVELS IN SERUM AND LIVER OF RATS FED WITH RMR

Liver is the only organ which can substantially influence net excretion of cholesterol from the body either directly as free cholesterol into the bile or after conversion of cholesterol to bile acids. Thus, this conversion is a significant regulatory step in determining the overall cholesterol balance. High cholesterol diet leading to hyperlipidemia is regarded as an important factor in the development of ischemic heart disease, and the focus so far has been mainly on the systemic and coronary vascular effects of cholesterol (Angelin, 1991). Triacylglycerol are esters of glycerol with three fatty acids. Measurement of TG is used in screening of the lipid status to detect atherosclerotic risks and in monitoring of lipid lowering measures.

Immediaitely after obtaining the animals, the basal cholesterol and triacylglycerol were estimated. The RMR supplemented with cholesterol and bile acids was fed to the rats for seven and fourteen weeks. Changes in TC and TG levels were measured in both serum and liver. The details are described in Materials and Methods.

Changes in TC and TG levels in serum and liver of HFD fed group and HFD supplemented with different levels of RMR are described in **Tables 36 and 37**. After 7 weeks, HFD fed group showed 2.24 and 1.97 fold increase in serum and liver cholesterol levels respectively. Similarly, HFD induced 1.54 and 1.97 fold increase in serum and liver triacylglycerol respectively. RMR supplementation in HFD resulted in a dose dependent decrease in cholesterol and triacylglycerol levels both in serum and liver compared to HFD group at 7 and 14 weeks.

Cholesterol and triacylglycerol levels significantly decreased by 44.46 and 45.30% respectively in serum and 48.45 and 42.08% in liver of rats fed with 16% RMR for 7 weeks compared to HFD group. However, at the end of 14 weeks, the decrease in cholesterol and triacylglycerol levels in serum and liver of RMR fed groups was not relatively higher than that of rats fed with RMR for 7 weeks.

Table 36. Effect of RMR on serum TC and TG levels in experimental rats

Concentration of RMR in diet (w/w)	0th week		7th week		14th week	
	Cholesterol (mg dl^{-1})	Triacylglycerol (mg dl^{-1})	Cholesterol (mg dl^{-1})	Triacylglycerol (mg dl^{-1})	Cholesterol (mg dl^{-1})	Triacylglycerol (mg dl^{-1})
Control	**32.21±5.36**	**84.96±22.92**	**66.24±4.52^{a}**	**94.00±25.36ab**	**99.90±13.80^{bc}**	**99.07±11.43^{a}**
HFD	-	-	**150.02±17.29^{e}**	**145.54±38.43 ^{c}**	**165.81±13.56 ^{e}**	**151.53±13.02 ^{d}**
HFD+8%	-	-	**126.96±6.37^{d}**	**110.25±11.41 ^{b}**	**121.90±6.48 ^{d}**	**113.77±12.09 ^{b}**
HFD+12%	-	-	**96.97±9.78 ^{c}**	**94.29±13.80 ^{ab}**	**105.20±5.38 ^{cd}**	**103.07±4.76 ^{bc}**
HFD+16%	-	-	**82.78±7.62 ^{a}**	**79.60±11.27 ^{a}**	**83.91±24.37 ^{ab}**	**86.35±6.11 ^{ab}**
HFD+80 mg lovastatin	-	-	**80.25±5.42 ^{ab}**	**76.56±5.25 ^{a}**	**77.85±2.69 ^{a}**	**84.70±1.90 ^{a}**

Control, normal diet (without cholesterol); HFD- High Fat Diet (containing 1% cholesterol and 0.15% bile salts); HFD+8 % RMR(8 g/100g RMR and High Fat Diet); HFD+12 % RMR(12 g/100g RMR and High Fat Diet), HFD+16 % RMR(16 g/100g RMR and High Fat Diet) HFD + 80 mg lovastatin. Data are presented as means±SD (n=5). Mean values within each column with different superscripts are significantly different ($p<0.05$)

Table 37. Effect of RMR on liver TC and TG levels in experimental rats

Concentration of RMR in diet (w/w)	0th week		7th week		14th week	
	Cholesterol (mg dl^{-1})	Triacylglycerol (mg dl^{-1})	Cholesterol (mg dl^{-1})	Triacylglycerol (mg dl^{-1})	Cholesterol (mg dl^{-1})	Triglyceride (mg dl^{-1})
Control	89.51±15.24	67.29±7.66	96.29±16.40 ^{a}	75.24±8.57 ^{a}	62.96±18.35 ^{a}	76.37±5.24 ^{a}
HFD	-	-	189.93±8.53 ^{d}	148.79±58.92^{b}	152.19±26.34 ^{d}	125.38±14.93 ^{b}
HFD+8 %	-	-	163.76±14.10 ^{c}	107.05±44.53 ^{ab}	114.67±5.40 ^{c}	115.97±18.78 ^{b}
HFD+12 %	-	-	133.20±22.56 ^{b}	96.78±42.44 ^{a}	96.72±22.32 ^{bc}	95.05±19.50 ^{a}
HFD+16 %	-	-	97.89±9.70 ^{ab}	86.17±15.57 ^{a}	83.04±22.68 ^{ab}	80.88±10.99 ^{a}
HFD+80 mg lovastatin	-	-	92.50±11.76 ^{a}	85.12±12.17 ^{a}	79.75±14.23 ^{ab}	79.80±8.06 ^{a}

Control, normal diet (without cholesterol); HFD- High Fat Diet (containing 1% cholesterol and 0.15% bile salts); HFD+8 % RMR(8 g/100g RMR and High Fat Diet); HFD+12 % RMR(12 g/100g RMR and High Fat Diet), HFD+16 % RMR(16 g/100g RMR and High Fat Diet) HFD+80 mg lovastatin. Data are presented as means±SD (n=5). Mean values within each column with different superscripts are significantly different ($p<0.05$).

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3.1.6. SERUM HDL-C AND LDL-C LEVELS IN RATS FED WITH RMR

In plasma, cholesterol is transported via lipoproteins, LDL (bad lipoprotein) and HDL (good lipoprotein). LDL provides cholesterol to those tissues that need it. HDL removes excessive cholesterol from peripheral tissues back to the liver and plays a major role in maintaining cholesterol homeostasis in the plasma. LDL-C contributes to atherosclerotic plaque formation within the arterial intima and is strongly associated with coronary heart disease. HDL-C has a protective effect impending plaque formation and shows an inverse relationship to CHD prevalence. Therefore, lowering the level of cholesterol in one's diet has become an important concern for patients with cardiovascular diseases.

The RMR supplemented with cholesterol and bile acids was fed to the rats for seven and fourteen weeks. The changes in HDL-C, LDL-C and atherogenic indices were measured as described in Materials and Methods.

The effect of RMR on serum HDL-C and LDL-C levels are described in **Table 38**. HFD induced 3.62 fold increase in LDL-C level and 4.12 fold in LDL/HDL ratio compared to the control group at the end of 7^{th} week. At the end of 14^{th} week, LDL-C level increased by 5.14 fold in HFD fed group. LDL-C levels decreased by 66.81% and HDL-C level increased by 46.63% in serum of rats fed with 16% RMR for 7 weeks. Supplementation of 16% RMR in the diet for 14 weeks significantly decreased LDL-C by 3.37 fold compared to HFD control group.

RMR reduced liver weight and also fatty infiltration suggesting inhibition of deposition of lipids in liver.

Table 38. Effect of RMR on serum HDL and LDL levels of HFD fed male rats

Conc. of RMR in diet (w/w)	7th week				14th week			
	HDL-C (mg dl^{-1})	LDL-C (mg dl^{-1})	LDL-C/ HDL-C	TC/HDL-C	HDL-C (mg dl^{-1})	LDL-C (mg dl^{-1})	LDL-C/ HDL-C	TC/HDL-C
Control	33.30±8.26 ^{a}	32.94±2.77 ^{b}	0.99±0.34 ^{b}	1.99	46.96±3.71^{ab}	28.48±2.10^{a}	0.61±0.57^{b}	1.61
HFD	29.27±8.33^{ab}	119.29±11.07^{a}	4.08±1.33^{a}	5.08	40.20±2.71 ^{a}	146.61±5.05^{c}	3.65±1.86^{a}	4.65
HFD+8%	33.48±4.07^{c}	92.48±5.25^{b}	2.76±1.29^{b}	4.00	48.51±2.85^{ab}	92.30±1.90^{b}	1.90±0.67^{b}	2.90
HFD+12%	37.50±3.88^{bc}	58.37±5.18 ^{a}	1.56±1.34^{a}	2.61	51.12±1.93^{ab}	66.08±1.51^{b}	1.29±0.78^{a}	2.31
HFD+16%	42.92±0.71^{a}	39.59±1.19 ^{b}	0.92±0.54^{bc}	1.95	54.43±1.84 ^{b}	43.48±1.45^{ab}	0.80±0.79^{b}	1.81
HFD+80 mg lovastatin	42.28±2.35 ^{a}	38.12±2.14 ^{a}	0.90±0.91 ^{c}	1.90	55.28±0.43 ^{b}	44.77±0.89^{ab}	0.81±2.08^{b}	1.84

Control, normal diet (without cholesterol); HFD- High Fat Diet (containing 1% cholesterol and 0.15% bile salts); HFD + 8 % RMR; HFD + 12 % RMR, HFD + 16 % RMR; HFD + 80 mg lovastatin. Data are presented as mean ± SD (n=5). Mean values within each column with different superscripts are significantly different ($p<0.05$) LDL-C low-density lipoprotein cholesterol, HDL-C high-density lipoprotein cholesterol.

3.1.7. 3-HYDROXY-3-METHYLGLUTARYL-CoA REDUCTASE ACTIVITY

HMG-CoA reductase (3-hydroxy-3-methylglutaryl coenzyme A, EC 1.1.1.34) is involved in the conversion of HMG-CoA to mevalonate. It is known to be a key rate-limiting step in cholesterol biosynthesis. Therefore, activity of HMG-CoA reductase and its regulation can control the cholesterol content in the body.

In this experiment, the HFD and RMR were fed to the rats to evaluate the effect of RMR on induced hyperlipidemia. HMG CoA reductase activity in control and HFD supplemented RMR groups were measured as detailed in Materials and Methods.

High cholesterol feeding decreased HMG-CoA reductase activity by 56.45% in HFD fed rats (**Figure 55**). RMR fed groups resulted in 22 to 54% inhibition of HMG-CoA reductase activity in liver. RMR supplementation with HFD resulted in a dose dependent inhibition of HMG-CoA reductase activity in liver compared to HFD fed group for 14 weeks. HMG-CoA reductase activity decreased by 22 to 54% in RMR fed groups compared to HFD fed group.

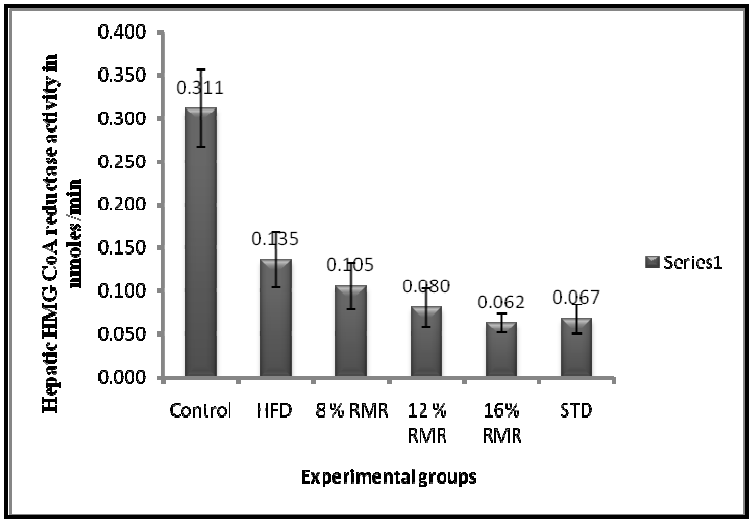

Figure 55. Hepatic HMG-CoA reductase activity in rats fed with control and experimental diets for 14 weeks. Values are means with the standard errors of the means, shown by vertical bars. Mean values with different vertical bars were significantly different ($p<0.05$).

3.1.8. MICROSCOPIC EXAMINATION OF LIVER

The liver is an important organ in the body for metabolism and it does a number of functions that include glycogen storage, decomposition of red blood cells, plasma protein synthesis, harmone production, and detoxification. It also regulates a wide variety of high-volume biochemical reactions requiring highly specialized tissue, including the synthesis and breakdown of small and complex molecules, many of which are necessary for normal vital functions.

The livers of the rats fed with HFD and HFD supplemented with RMR were examined microscopically, after 7 and 14 weeks, to evaluate the changes. The details are described in Materials and Methods.

Gross examination of the vital organs during autopsy did not show any abnormalities that could be attributed to biomass feeding in both sexes of rats. Microscopic examination of liver (**Figure 56**) did not show any damage to liver tissue of RMR fed groups like in the control group and HFD fed groups. Liver sections of group III, IV, V supplemented with RMR showed relatively less fatty infiltration compared to that of group II fed with HFD. Group VI fed with lovastatin also showed similar picture to that of RMR fed groups.

No significant changes were observed in serum clinical enzymes, LDH, SGOT, SGPT and ALP levels, of RMR and HFD fed groups. RMR supplementation in HFD did not induce any histopathological alterations in liver as that of HFD fed group. 16% RMR had significantly reduced total cholesterol in serum and liver and was comparable to 80 mg lovastatin. The atherogenic effect of the RMR significantly decreased LDL-C level in serum. The HDL value and HDL-C to LDL-C ratio increased in rats fed with RMR.

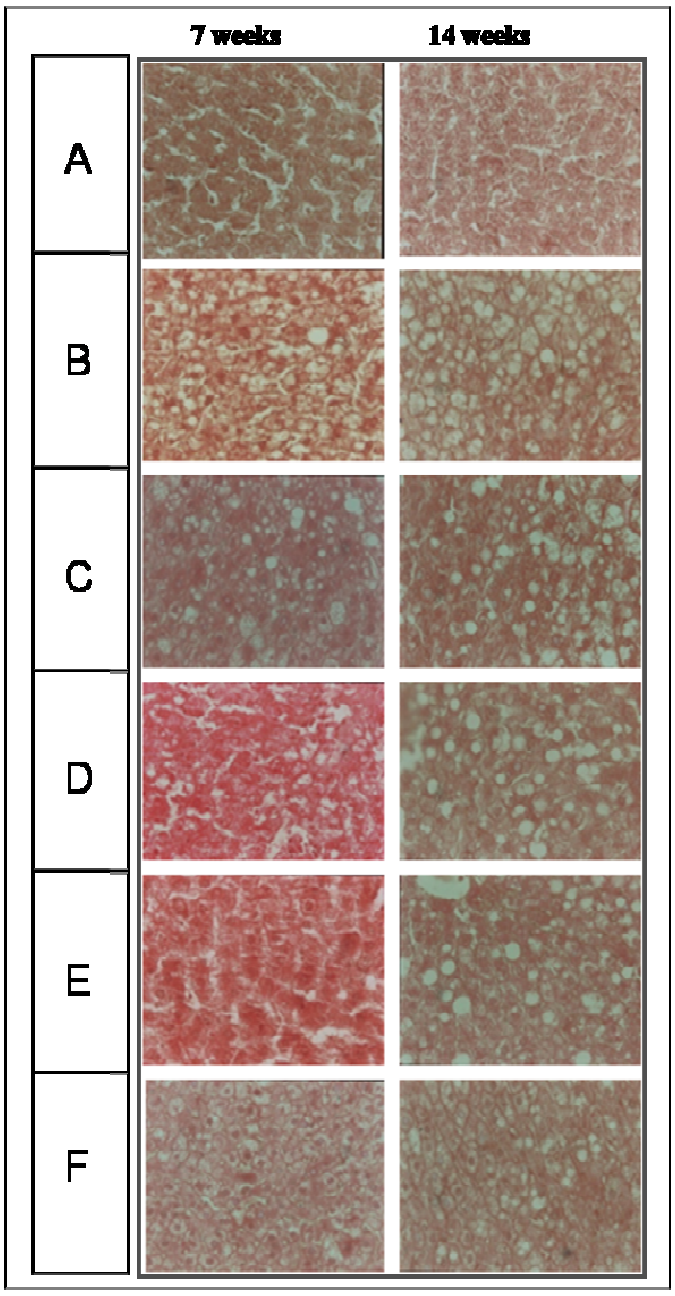

Figure 56. Histopathological features of liver from experimental rats (400X). A.Control group; B. HFD group; C. HFD+8% RMR group; D. HFD+12% RMR; E. HFD+16% RMR; F. HFD+standard lovastatin.

The data suggested that RMR can be used as an efficient hypolipidemic food supplement without any adverse effects and possess health benefits molecules related to regulation of blood lipids.

3.2. ANTIOXIDANT PROPERTY OF RMR

The increase in rate of mortality and morbidity in developed and developing countries is due to sedentary life style and high fat diet (HFD). Oxidative stress in hyperlipidemia is thought to be a factor in the development of atherosclerotic plaques (Volkovova *et al.*, 2006). It is due to accumulation of oxysterol and lipid peroxide in arteries generated due to oxidation of fatty acids by free radicals and this leads to coronary heart disease (Rosenson, 2004a). In hyperlipidemia, cell membranes and extracellular matrix can change their lipid composition and are more prone to radical generation (Kasiske *et al.*, 1990). There is few evidence that, antioxidants can be preventive and therapeutic agents. Hence, there has been an nterest in the application of antioxidants to treat of human diseases (Vaya & Aviram, 2001).

Dimerumic acid and dihydromonacolin-MV isolated form *Monascus* sp. have strong antioxidant activity (Aniya *et al.*, 1999; Dhale *et al.*, 2007b against DPPH and lipid peroxidation. The antioxidant that can scavenge the DPPH radical is expected to inhibit lipid peroxidation (Matsubara et al., 1991). Rice fermented using *Monascus* sp has demonstrated significant hypolipidemic effects in hyperlipidemia hamster model rats (Lee, 2006). Hence the antioxidant property of RMR was evaluated by using the stable free radical DPPH known to abstract the labile hydrogen atom of chemical compounds (Ratty *et al.*, 1988). The details are described in Material and Methods.

3.2.1. DPPH RADICAL SCAVENGING ACTIVITY OF RMR

DPPH is a stable free radical and accepts an electron or hydrogen radical to become a stable diamagnetic molecule. DPPH reacts with antioxidant compounds that can donate hydrogen and gets reduced. The DPPH radical has been used for the

determination of antioxidant activity of extracts and compounds. The intensity of the yellow colour depends on the amount and nature of radical scavenger present in the sample or standard.

The metabolite extracted from fermented rice with solvents was evaporated to dryness by flash evaporation. After lyophilization it was solubilazed in ethanol and used for the assay. The detailed protocol of DPPH radical scavenging activity of RMR has been described in Materials and Methods.

Table 39. DPPH radical scavenging activity of RMR

Solvents used for extraction	**DPPH radical scavenging activity (%)**
Hexane	**12.56±0.53**
Chloroform	**8.56±1.96**
Methanol	**46.23±1.06**

The RMR was initially defatted with hexane and chloroform. These extracts did not show any significant activity against DPPH radical. Highest DPPH free radical scavenging activity was estimated with methanol extract **(Table 39)**. The methanol extract of RMR scavenged 50% DPPH radical at 100.78±2.66 µg/ml concentration.

3.2.2. EFFECT OF RMR ON SERUM ANTIOXIDANT MOLECULES AND LIPID PEROXIDES

In the experiment above, DPPH scavenging activity of RMR showed that the DPPH radical scavenging can have the ability to inhibit liver lipid peroxidation. Accordingly, it was expected that RMR can protect the tissue and cells against ROS in oxidatively stressed rats fed with HFD. Hence, the effect of RMR preparation on

antioxidant enzyme and molecules in oxidatively stressed rats fed on HFD was evaluated.

The rats were fed with HFD and HFD supplemented with RMR for 14 weeks. The serum collected was for estimated for antioxidants and lipid peroxides.The details are described in Materials and Methods.

The effect of RMR on antioxidant molecules and lipid peroxides in the serum of rats fed on HFD for 14 weeks is shown in **Table 40**. Total thiol content of serum in rats fed on HFD increased two fold compared to control group. While its concentration reduced in rats fed with HFD supplemented with RMR (8, 12 and 16%), the RMR supplementation used in different concentration (8, 12 and 16%) decreased total thiol concentration significantly in serum ranging from 20.3-46.9%.

Significant decrease in serum glutathione concentrations was observed in rats fed with HFD compared to control rats (24 µg/dl and 32 µg/dl) respectively. The increase in serum glutathione concentration was more significant in rats fed with HFD supplemented with RMR. The increase in serum glutathione concentration (28.1-67.6%) in RMR fed rats was dose dependent.

The concentration of ascorbic acid decreased significantly in rats fed on HFD. But it increased significantly in rats fed with 16% RMR. The increase in the ascorbic acid concentration ranged from 31.3 to 74.9%.

The oxidation of serum lipid was higher (99µmol/dl) in rats fed with HFD. While, the oxidation of lipids (lipid peroxides) was reduced in rats fed with RMR, the reduction in the formation of lipid peroxide in serum ranged from 27.1-51.7% in the respective diet groups III-V.

Table 40. Effect of RMR on serum antioxidants and lipid peroxides

Concentration of RMR in diet (w/w)	Total thiols (mmol/dl)	Glutathione (µg/dl)	Ascorbic acid (mg/dl)	Lipid peroxides (µmol/dl)
Control	**0.32 ± 0.01^{a}**	**32.09 ± 1.86^{b}**	**3.18 ± 0.10^{d}**	**68.0 ± 5.13^{c}**
HFD	**0.64 ± 0.03^{d}**	**24.80 ± 1.34^{a}**	**2.11 ± 0.12^{a}**	**99.3 ± 4.92^{e}**
HFD+8%	**0.51 ± 0.02^{c}**	**31.76± 1.49^{b}**	**2.77 ± 0.03^{b}**	**72.39± 5.13^{d}**
HFD+12%	**0.43 ± 0.03^{b}**	**36.04± 1.15^{c}**	**3.04 ± 0.08^{c}**	**61.90± 6.01^{b}**
HFD+16%	**0.34 ± 0.02^{a}**	**41.56± 1.38^{d}**	**3.69 ± 0.05^{e}**	**46.11± 5.38^{a}**
HFD+80 mg lovastatin	**0.33 ± 0.02^{a}**	**42.16± 1.74^{d}**	**3.72 ± 0.08^{e}**	**47.99± 5.13^{a}**

Values are mean ±S.E.M of five rats in each group. Mean values within each column with different superscripts are significantly different Duncan's test at $p<0.05$.

3.2.3. ANTIOXIDANTS AND LIPID PEROXIDES IN LIVER

The effect of RMR on antioxidants and lipid peroxides in the liver of control and experimental rats are shown in **Table 41**. The rats fed with HFD exhibited a significant increase in total thiols (1.96 fold) and lipid peroxide compared to control group. The rats fed with HFD showed lipid peroxide concentration of 10.90 µmol/dl and glutathione concentration of 15.59 µg/dl.

Administration of RMR and lovastatin reduced the levels of total thiols and lipid peroxide significantly in rat's liver. The decrease in total thiols and lipid peroxides was observed in rats fed with HFD supplemented with RMR. HFD supplemented with 16% RMR showed only 46.1µmol of lipid peroxides and glutathione concentration of 41.56 µg/dl. The decrease in total thiol and lipid peroxides concentrations ranged from 25.3-58.4% and 21.2-43.2%, respectively. However liver glutathione and ascorbic acid contents increased by 35.5-74.6% and 36.4-82.2%.

Table 41. Effect of RMR on liver antioxidants and lipid peroxides

Concentration of RMR in diet (w/w)	Total thiols (mmol/mg protein)	Glutathione (µg/ mg protein)	Ascorbic acid (µg/ mg protein)	Lipid peroxides (nmol/ mg protein)
Control	**1.49 ± 0.06^{b}**	**21.19 ± 1.31^{b}**	**2.09 ± 0.09^{c}**	**7.98 ± 0.32^{d}**
HFD	**2.93 ± 0.08^{e}**	**15.59 ± 0.70^{a}**	**1.29 ± 0.03^{a}**	**10.90± 0.49^{f}**
HFD+8%	**2.19 ± 0.09^{d}**	**21.13 ± 0.67^{b}**	**1.76 ± 0.03^{b}**	**8.59 ± 0.33^{e}**
HFD+12%	**1.69 ± 0.05^{c}**	**24.12 ± 0.97^{c}**	**2.08 ± 0.05^{c}**	**7.01± 0.51^{c}**
HFD+16%	**1.22 ± 0.07^{a}**	**27.22 ± 0.91^{d}**	**2.35 ± 0.04^{d}**	**6.19± 0.43^{b}**
HFD+80 mg lovastatin	**1.19 ± 0.06^{a}**	**27.59 ± 0.86^{e}**	**2.38 ± 0.07^{d}**	**6.13± 0.53^{a}**

Values are mean ±S.E.M of five rats in each group. Mean values within each column with different superscripts are significantly different Duncan's test at $p<0.05$.

3.2.4. ANTIOXIDANT ENZYMES IN SERUM

Antioxidant enzymes neutralize many types of disease-causing free radicals and rids the body of harmful effects. The antioxidant enzymes include glutathione reductase (GR), glutathione peroxidase (GPx), superoxide dismutase (SOD), and catalase (CAT).

In the previous experiment estimated values of antioxidants and lipid peroxides in the serum and liver of rats fed with RMR were described. The antioxidant enzymes play important role in oxidatively stressed rats to neutralize the free radicals. Hence, rats were fed with HFD and HFD supplemented with RMR for 14 week. The serum collected was analysed for antioxidant enzymez (Materials and Methods).

The effect of RMR on antioxidant enzymes (GR, GPx, SOD and CAT) in the serum of rats fed on HFD is shown in **Table 42**. The enzyme activities decreased significantly in rats fed with HFD and the administration of RMR increased the enzyme activities. The increase in GR enzyme activity was 39.8, 60.5 and 88.7% in serum of rats fed on HFD supplemented with 8, 12 and 16% RMR respectively. Feeding RMR showed significant increase in the activity of GPx in rats. The increased enzyme activity (31.8, 57.9 and 85.3%) was observed in group III to V compared to the group I and II. Similarly, RMR enhanced serum CAT (21.5, 37.9 and 48.0%) and SOD (11.8, 29.8 and 41.7%) activity in rats fed on HFD in group III to V.

Table 42. Effect of RMR on serum antioxidant enzymes

Conc. of RMR in diet (w/w)	GR (mmol/min/ml)	GPx (mmol/min/ml)	Catalase (mmol/min/dl)	SOD (mmol/min/dl)
Control	**32.40 ± 1.86^{c}**	**29.53 ± 1.43^{c}**	**193.57 ± 7.95^{d}**	**9.03 ± 0. 75^{f}**
HFD	**21.19 ± 1.34^{a}**	**19.96 ± 0.60^{a}**	**139.26 ± 8.23^{a}**	**5.61 ± 0. 82^{a}**
HFD+8%	**29.62 ± 1.49^{b}**	**26.31 ± 0.76^{b}**	**169.21 ± 7.95^{b}**	**6.27 ± 0.64^{b}**
HFD+12%	**34.01 ± 1.15^{d}**	**31.51 ± 0.93^{d}**	**192.03± 9.57^{c}**	**7.28 ± 0.76^{c}**
HFD+16%	**39.98 ± 1.38^{e}**	**36.99 ± 0.46^{e}**	**206.12±10.02^{f}**	**7.95 ± 0.90^{d}**
HFD+80 mg lovastatin	**41.01 ± 1.74^{f}**	**37.05 ± 1.07^{e}**	**201.99 ± 9.11^{e}**	**8.01 ± 0.85^{e}**

Values are mean ±S.E.M of five rats in each group. Mean values within each column with different superscripts are significantly different Duncan's test at $p<0.05$.

3.2.5. ANTIOXIDANT ENZYMES OF LIVER

The liver is a central player in the whole body energy homeostasis by its ability to metabolize glucose and fatty acids. The liver is also able to store significant quantities of lipids in conditions associated with prolonged excess energy consumption or impaired fatty acid metabolism. During oxidative stress, the lipid oxidizes to form lipid peroxides.

The rats fed with HFD and RMR were assayed for the production of antioxidant enzymes. The detailed protocol for antioxidant enzyme assay is described in Materials and Methods.

The effect of RMR on antioxidant enzymes (GR, GPx, SOD and CAT) in liver of rats fed with HFD is shown in **Table 43**. The activities of the enzymes decreased significantly in rats fed with HFD and the administration of RMR increased the activities of these enzymes. Similarly, a significant increase in the levels of liver antioxidant enzymes; GR (35.6-92.3%), Gpx (41.3-88.0%), CAT (13.8-41.4%) and SOD (11.3-46.8%) was observed in the liver of rats fed with RMR supplemented HFD. There was no significant difference in the activity of antioxidant enzymes in liver of group fed with 16% RMR supplemented and 80 mg lovastatin fed rats. The percentage increase in GR, GPx, CAT and SOD activity was 52, 53, 70 and 68% respectively (compared to HFD fed group).

Table 43. Effect of RMR on liver antioxidant enzymes

Conc. of RMR in diet (w/w)	GR (µmol/min/mg protein)	GPx (mmol/min/mg protein)	Catalase (mmol/min/mg protein)	SOD(mmol/min/mg protein)
Control	**26.01 ± 1.01^{d}**	**35.09 ± 1.13^{c}**	**191.57 ± 12.02^{f}**	**8.26 ± 0.57^{d}**
HFD	**15.38 ± 0.70^{a}**	**21.17 ± 0.90^{a}**	**131.01 ± 11.22^{a}**	**5.92 ± 0.59^{a}**
HFD+8%	**20.86 ± 0.97^{b}**	**29.92 ± 0.76^{b}**	**149.06 ± 10.95^{b}**	**6.59 ± 0.44^{b}**
HFD+12%	**25.08 ± 0.97^{c}**	**35.17 ± 0.93^{d}**	**169.17± 12.57^{c}**	**7.75 ± 0.54^{c}**
HFD+16%	**29.58 ± 0.91^{e}**	**39.81 ± 0.86^{e}**	**185.25 ± 10.29^{d}**	**8.69 ± 0.49^{f}**
HFD+80 mg lovastatin	**30.04 ± 0.86^{f}**	**40.02 ± 1.07^{f}**	**186.03 ± 10.89^{e}**	**8.61 ± 0.55^{e}**

Values are mean ±S.E.M of five rats in each group. Mean values within each column with different superscripts are significantly different Duncan's test at $p<0.05$.

DISCUSSION

Rice is one of the most important cereal crops cultivated extensively in warm climate regions of Asia. It is a staple food consumed throughout the world particularly in Asian countries. Commercially, more than 2000 varieties of rice are grown throughout the world. For centuries, China has developed methods to convert agricultural commodities into functional foods by fermenting them with micro-organisms like *Lactobacillus, Monascus, Penicillium,* etc (Ma *et al.*, 2000). In this regard fermentation of rice with the red fungus *Monascus*, is important since the product is microbial color. The red color of *Monascus* fermented rice often refered to as red mould rice or RMR due to rubropunctamine and monascorubramine pigments. For products, steamed rice is fermented with food fungus *Monascus* and the colour extracted is added to wines and other fermented food products like red soybean cheese to enhance colour.

RMR is consumed in China because it is used to enhance the colour and flavour of the food and also used as traditional medicine for digestive and vascular functions (Ma *et al.*, 2000). Available literature demonstrate the ability of RMR to lower lipid levels in animal models and humans due to the presence of HMG-CoA reductase that inhibit cholesterol synthesis (Li *et al.*, 1998; Heber *et al.*, 1999; Journoud & Jones, 2004).

Many researchers suggested that products of *Monascus* also have several multifunctional compounds. The results described in this thesis substantiate the above.

METABOLITES OF *M. PURPUREUS*

M. purpureus MTCC 410 produces polyketides, statins (hydroxyl and lactone form of lovastatin and pravastatin), sterols and significant amount of saturated (palmitic and stearic) and unsaturated fatty acids (oleic, linoleic and linolenic) both in shake flask and solid-state fermentation. Except sterols, solid-state fermentation of the

fungus and mutants resulted in the yield of more polyketides **(Figure 14)**, statins **(Figure 20)** and fatty acids **(Figure 25)**. In relation to pigment production, mutant CFR 410 -11 was more efficient compared to the wild type and CFR 410-22.

Production of higher concentration of metabolites in solid-state fermentation can be attributed to better hyphal adhesion and penetration offered by the microstructure of the rice grain (Johns & Stuart, 1991). The hyphal growth is a combination of apical extension of hyphal tips and the generation of new hyphal tips through branching. The hyphal mode of fungal growth is also tolerant to low water activity and high osmotic pressure conditions. Thus the conditions apparently made the fungus competitive for efficient bioconversion of solid substrates to produce these products of interest in higher concentration. Due to hyphal mode of growth, efficient colonization of solid substrates also occurred for the utilization of available nutrients (Raimbault, 1998).

The hydrolytic enzymes excreted by the hyphal tip, allow penetration into solid substrates and increase the accessibility of all available nutrients within the particles. The hyphal growth allowed a close contact between hyphae and substrate surface there by, close contact of the precursor made their entry into the mycelium to promote biosynthesis and fungal metabolic activities (Raimbault, 1998). Free access of atmospheric oxygen to the substrate appearanty resulted in higher growth rate for larger biomass in solid state fermentation than in shake flasks.

OPTIMIZATION

Production of statin by *M. purpureus* was influenced by the carbon and nitrogen sources. Since there appeared a direct relationship linked with its formation and biomass formed.

It may be that the induction of key enzymes involved in statin production is closely influenced by the carbon sources. Maltose served as a better carbon source

probably by relieving the carbon catabolite inhibition caused by glucose ((Miyake *et al.*, 2006a)).

Production of statin is associated with the nitrogen limited growth when excess carbon can be channeled for secondary metabolite synthesis. In view of these results, statin yield could be improved when carbon is not limiting but growth has been arrested by nitrogen limitation (Su *et al.*, 2003).

The broth pH could play a crucial role in the behaviour of secondary metabolite production as well as cell growth of fungus because of enzyme activation or degeneration (Buckland *et al.*, 1989). The effect of pH for lower concentration of statin may be due to production of primary metabolite itaconic acid at acidic pH, which is known to decrease biomass and statin (Riscaltadi *et al.*, 2002). Acidic condition can also affect the formation of key enzymes necessary for statin production.

The temperature shift was crucial for the statin production. Temperature below 30°C caused the pH to decrease due to the cells metabolizing the sugar into acids resulting in acidification of fermentation broth. At the ideal temperature utilization of nitrogen sources to form ammonia (Long-Shan *et al.*, 2005) occurred for biomass production.

Our study showed increased sterol content and decreased statin content in shake flask grown *M. purpureus*. Only small quantities of statins were estimated in these cultures. Sterols, like the statins, are synthesized from acetyl CoA precursor. Hence higher concentration of statins in solid state fermentation compared to shake flask cultures may be due to the diversion of precursors for the synthesis of statins rather than sterols.

AMIDASE ACTIVITIES

Pigments quantities of *M. purpureus* and mutants grown in 4% rice flour suggested that more pigment synthesis occurred in CFR 410-11 due to increased

amidase production by the fungus. More pigment was quantitied in CFR 410-11 compared to *M. purpureus* MTCC 410 and CFR 410-22 **(Table 8).**

Though, pigmentation is a characteristic property of *M. purpureus,* many strains characterized have shown varied pigment production (Blanc *et al.*, 1999a; Broder & Koehler, 1980; Campoy *et al.*, 2006; Chen & Johns, 1993a; Hamdi *et al.*, 1997; Krairak *et al.*, 2000). 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 & Demain, 1991b; Schneweis *et al.*, 2001; Suh & Shin, 2000; Tseng *et al.*, 2000). A mutant of *M. purpureus* isolated by temperature selection after UV treatment demonstrated a characteristic albino phenotypy on PDA plates (**Figure 11C**). Correspondingly, when fermenting rice flour, this mutant produced a low quantity of the pigment (**Table 8**). In the case of the mutant CFR 410-11(**Figure 11B**), the pigment production was higher due to the effect of mutation leading membrane causing higher growth rate.

Identification of L-asparaginase and L-glutaminase activities in the extracellular fluids of *M. purpureus* suggested their role in red pigment production (**Table 7**). These amidase upon synthesis, cause 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 with the amino acids (L-asparagine and L-glutamine) caused a Schiff's base reaction. These results evidently indicated that, monascorubramine and rubropunctamine (red pigments) are the derivatives of monascorubrine and rubropunctatin (orange pigment) respectively.

L-asparagine and L-glutamine were used as sole carbon and nitrogen source to validate their effect in pigment production. The spores were cultured separately, in the medium containing L-asparagine and L-glutamine. The pigment production in these medium suggested the release of NH_3 due to hydrolysis of the amino acids presents in the medium (Schiff's base reaction of orange pigment). $NaNO_3$ used as control

medium, did not produce any pigment (**Table 9**). The results were further confirmed by paper chromatography. The culture medium with L-asparagine and L-glutamine showed L-aspartic acid and L-glutamic acid spots as the hydrolyzed products. Thus NH_3 released by the action of amidase, reacted with orange pigment to produce red pigment.

M. purpureus produce extracellular carboxypeptidase that releases free amino acids from carboxyl terminal of peptides or proteins (Liu *et al.*, 2004). Even though, hyper secretion of acid protease was observed in MTCC 410-22, it was unable to produce pigment. Apparently, higher acid protease activity of MTCC 410-22 repressed the polyketide synthase enzyme (Bond & Butler, 1987) like in the case of MTCC 410-11 that produced more pigment but exhibited low acid protease activity (Dhale, 2007c).The differences in amidase and acid protease activities observed in *M. purpureus* and mutants require further this study to understand the mechanism. However, production of L-asparaginase and L-glutaminase enzymes by the fungus suggested its use for the studies concerning their effects on tumors.

STATINS OF *M. PURPUREUS* AND MUTANTS

High shear damaging of mycelial filament, low pH, high temperature and low oxygen supply are considered as unfavourable conditions for production of lovastatin in shake flask fermentation. Low yield of statin in shake flask cultures (**Figure 18**) may be due to heat generation and pH deviation which result in the activation of unfavorable enzymes that decompose the molecular structure of lovastatin (Kumar *et al.*, 2000). Heat generation lead to serious moisture loss, low yield or loss of fungal activity and the products remain in the mycelium due to their low solubility in the acidic medium. During shake flask cultivation it was observed that lumping of mycelial biomass lead to low oxygen transfer due to increased viscosity and thus affected the biosynthesis of lovastatin (Kumar *et al.*, 2000).

Mutants produced less statin compared to wild type (**Figure 19**). Regardless of monacolin biosynthesis, the mutants showed some important biochemical differences,

which lead to speculations on the nature of mutation. The change in inheritance is probably the cause since alteration of several other important characteristics of the fungi like reduction of growth rate and metabolic activity reduced the typical secondary metabolite (Rasheva *et al.*, 2003).

Low yield of lovastatin in mutant may be due to their selection at elevated temperature (40° C) that affected the biosynthetic pathway of statin production. This suggests that production of statin is possibly dependent on temperature (Juzlova *et al.*, 1996a; Negishi *et al.*, 1986). During lovastatin biosynthesis, lots of intermediate monacolins like monacolin X, M and J are synthesized (Endo *et al.*, 1985a; Endo *et al.*, 1986a). The final product lovastatin formed by the chemical modification of monacolin X, the α-methyl - β- ketobutyryl ester of monacolin J (Endo *et al.*, 1986a). Higher quantities of monacolin k (lovastatin) in parent compared to that in mutants may be due to the mutational effect on the biosynthetic enzymes involved in intermediate reaction of monacolin K biosynthesis. Accumulation of Monacolin X in mutants resulted in low lovastatin yield (Endo *et al.*, 1986a).

In monacolin biosynthesis, lovastatin nonaketide synthase cyclizes one acetate and eight malonate molecules to form dihydromonacolin L (Auclair *et al.*, 2001). A 3α-hydroxy-3, 5-dihydromonacolin L and its dehydrated form monacolin L arise spontaneously from dihydromonacolin L. Hydroxylation of monacolin L at C8 by molecular oxygen result in the formation of monacolin J (Endo *et al.*, 1985b; Endo *et al.*, 1985a). In hyper pigment mutant (CFR 410-11), monacolin J is converted to dehydromonacolin-MV2. It is differed from monacolin-J in one additional OH group at C3 carbon atom. The UV λ max at 329 nm that evidenced double bond between C5 and C8 in dehydromonacolin-MV2 suggested its origin from monacolin J by oxidation at position C8. Synthesis of structural variant of monacolin J by the mutant *M. purpureus* apparently reasoned an aberration in the biosynthetic pathway induced by mutation (Dhale, 2007c). Accumulation of monacolin X or dehydromonacolin-MV2 may be reasoned for reduced content of lovastatin in mutants.

SAFETY EVALUATION OF *M. PURPUREUS* RMR

Monascus species are reported to produce citrinin (mycotoxin) as fermentation byproduct along with pigments and monacolins. Citrinin has been considered to have hepato-nephrotoxic properties (Lockard *et al.*, 1980; Kogika *et al.*, 1993), causes functional and structural kidney damage and alterations in liver metabolism (Da Lozzo *et al.*, 1998). The safety of *M. purpureus* MTCC 410 RMR was assessed by conducting toxicological studies in albino rats. Acute and sub-chronic toxicity studies were conducted on both sexes of albino rats.

PRODUCTION OF CITRININ

Monascus sp. grown on rice are reported to produce citrinin levels up to about 2.5 g/kg dry matter, while in shake flask cultures its production is approximately 56 mg/kg dry matter (Eckert *et al.*, 2005). It inhibits several enzymes linked to the respiratory chain of the kidney cortex and liver mitochondria, as well as malate and glutamate dehydrogenases and the ATP-synthetase complex (Da Lozzo *et al.*, 1998). Citrinin was found to be nephrotoxic and teratogenic in chronic toxicity studies in rats at a dosage of 50 mg/kg body weight/day after 60 weeks in the test animals (Eckert *et al.*, 2005). In *M. purpureus* MTCC 410 used in this investigation only 1.427 mg citrinin per kg RMR was determined (**Figure 38**), much lower than the acute nephrotoxic level of 18.4 g/kg (Krejci *et al.*, 1996).

ACUTE TOXICITY STUDY

Feeding acute doses of RMR at 0.5, 1.0, 2.5 and 5.0 g/kg body weight to rats did not cause any symptoms of toxicity or mortality. Rats fed on diets containing *M. purpureus* RMR did not develop any clinical signs of toxicity either immediately or during the post treatment even at highest dose of 5.0 g/kg body weight. Feeding RMR at different levels did not cause any alteration in relative weight in both male and female rats (**Tables 11 and 12**). Further, the hematological profile **(Table 13 and 14)**

of treated groups was comparable to that of control. No histological alterations were observed at any of the dosage levels **(Figure 28).**

SUB-CHRONIC TOXICITY STUDY

Similarly in sub-chronic study, dietary feeding of RMR at 2.0%, 4.0%, 8.0% and 12.0% level (w/w) for 14 weeks did not produce any significant changes in food intake **(Figure 29 and 30)** or gain in body weight **(Figure 31 and 32)** of the experimental rats compared to control rats. There were no significant differences in the relative weight of vital organs **(Tables 12 and 13)**, hematological parameters (**Table 17 and 18)**, macroscopic and microscopic changes in vital organs and serum clinical enzyme levels (**Table 21 and 22)** between the experimental and control groups. Moreover, the rats fed with RMR showed a significant reduction in cholesterol and triacylglycerol levels (**Tables 23 and 24)** in both serum and liver. The results showed that toxicity studies with RMR *M. purpureus* did not cause any toxic effects in albino rats.

M. purpureus fermented rice did not induce acute or sub-chronic toxic effects in rats. No treatment related histopathological changes were observed even at highest dosage of 12% for 14 weeks. Further, the RMR was well tolerated even at 12% dietary level as evidenced by absence of any adverse effects on growth, body weight gain, organ weight, hematological parameters or serum enzyme levels. In addition, the serum cholesterol and triacylglycerol levels significantly dropped in RMR treated rats than the control group suggesting its hypocholesterolemic characteristic.

ANTIOXIDANT ENZYMES AND MOLECULES

The antioxidant property of *M. purpureus* 410 RMR was evaluated using stable free radical DPPH known to abstract the labile hydrogen atom of chemical compounds (Ratty *et al.*, 1988). The antioxidant that can scavenge the DPPH radical is expected to inhibit lipid peroxidation (Matsubara *et al.*, 1991). In this study, DPPH

scavenging activity of RMR confirmed that the mold extracts with DPPH radical scavenging ability could also inhibit liver lipid peroxidation. Accordingly, as expected RMR protected the tissue and cell against ROS in oxidatively stressed rats fed on HFD.

HFD can bring remarkable modifications in the antioxidant defense mechanism against the process of lipid peroxidation. Membrane lipids are particularly susceptible to oxidation not only because of their high polyunsaturated fatty acid content, but also due to their association with enzymatic and non enzymatic systems that are able to generate free radical species. Due to ROS on membrane lipoproteins and polyunsaturated fatty acids by-products (malondialdehyde, lipid hydroperoxide and conjugated dienes) are formed and these can be estimated by TBARS methods (Ohkawa *et al.*, 1979). The increased TBARS level in serum indicated free radical generation due to oxidative stress conditions in rats fed on HFD. Higher concentration of TBARS in rats fed on HFD can be attributed to a depressed antioxidant defense system. However, higher activity of antioxidant enzymes in rats fed on HFD supplemented with RMR (**Table 42 and 43**) significantly scavenged ROS. These results suggested that RMR supplemented HFD diet induced antioxidant enzyme activity in rats fed on HFD.

Monacolins, known as statins, isolated from *Monascus* sp. were found to protect body organs against oxidation of LDL and inhibit HMG-CoA reductase (Istvan & Deisenhofer, 2001). The statins play important role in atherosclerosis as antioxidant by preventing the oxidation of LDL during oxidative stress. The coronary heart disease (CHD) do not always have elevated levels of LDL but, the normal LDL levels with high levels of stress are more prone to CHD (Rosenson, 2004b). Increased oxidation of serum lipid and LDL-cholesterol in serum and its entry into artery leads to the formation of atherosclerotic lesion. The formation of atherosclerotic plaque in macrophages due to foam cells is prone to damage (Osterud & Bjorklid, 2003); (Rosenson, 2004a). Glutathione is the main antioxidant compound in our body that protect against oxidative damage to organs. The rats fed on HFD showed lipid peroxide concentration of 99.3µmol/dl and glutathione concentration of 24.8µg/dl.

Contradictory to this, HFD supplemented with 16% RMR showed only 46.1µmol of lipid peroxides and glutathione concentration of 41.56µg/dl. These showed that RMR supplementation induced production of serum glutathione and protected the body organ against the oxidative stress.

The crucial components of the antioxidant defense system in the body are cellular antioxidant enzymes (CAT, SOD, GR and GPx) which are involved in the reduction of ROS and peroxides produced in living organism. These enzymes are also involved in the detoxification of certain compounds of exogenous origin and play a primary role in the maintenance of a balanced redox status (Alı, 2003). The oxidation of liver lipid was comparatively less than the serum lipid. The liver being the main detoxifying organ and possesses a high metabolic rate and it is subjected to many insults potentially causative of oxidative stress.

Ingestion of RMR along with a HFD in rats caused a significant increase in the activities of antioxidant enzymes, suggesting that RMR can protect the tissues from lipid peroxidation by their antioxidant ability and consequent reduction in lipid peroxidation. The decreased concentration of GSH in rats fed on HFD can be reasoned for enhanced oxidation or its consumption by electrophilic compounds like lipoperoxidation aldehydes. Similarly, ascorbic acid concentration was reduced due to increased utilization to trap the ROS or due to a decrease in GSH concentration as the GSH is involved in the recycling of ascorbic acid. Consumption of RMR by rats enhanced production of these in the serum and liver of rats fed on HFD. The increased concentration of lipid peroxides in rats fed on HFD demonstrated induced oxidative stress in the body. It is hypothesized that reduction of such oxidative stress may slow down or prevent onset of atherosclerotic process. The long-term feeding of RMR significantly reduced the lipid peroxides concentration in rats fed on HFD.

The above results suggested the importance of RMR to reduce cholesterol levels and oxidative stress related atherosclerosis. Even though the cells and tissues

have evolved both enzymatic and non-enzymatic antioxidant systems to combat the oxidative stress caused by ROS, the endogenous antioxidants were not sufficient under extreme oxidative stress conditions. Hence to prevent the diseases, a balanced intake of antioxidant to scavenge ROS is important.

It can be concluded from this study that, anti-athrosclerotic influence of RMR is not only due to monacolins, but also due to the induced antioxidant enzymes to scavenge ROS produced during stress conditions.

ANTIOXIDANT PROPERTIES OF ANKAFLAVIN

Based on ^{1}H (proton), ^{13}C (carbon) spectra **(Figure 35 to 38)** and 2D-HSQCT NMR spectral data **(Table 25)**, antioxidant compound was identified as ankaflavin **(Figure 42)**. It was further confirmed by IR **(Figure 39)**, MS **(Figure 40)** and UV visible spectrum **(Figure 41).** A compound can exert its antioxidant activity by scavenging radicals, decomposing peroxides or chelating metal ions. Ankaflavin isolated from the solid-state cultures of *M. purpureus* MTCC 410 is a DPPH radical scavenger known to abstract the liable hydrogen atom.

The antioxidant ankaflavin reacted with DPPH (a stable free radical) and converted it to α, α -diphenyl-β-hydrazine. The intensity of discolouration indicated the potentiality of compounds to donate electrons. The activity of ankaflavin was attributed to its ability to donate electron(s) to the free radicals (Shimada *et al.*, 1992). On the other hand antioxidants are believed to intercept the free radical chain of oxidation and to donate hydrogen from the phenolic hydroxyl groups, there by forming a stable end product, does not further initiate or propagate oxidation reactions (Sherwin, 1978).

Ankaflavin strongly inhibited ascorbate autoxidation, peroxidation of lipids *in vitro* and reducing activity **(Table 26)**. The peroxidation of lipid inhibition activity was due to hydrogen(s) donating capacity to lipid peroxyl radicals and hydroxyl

radicals to terminate the chain reaction. However, lipid peroxidation was induced by metal ion such as Fe^{+2}. This indicated that lipid peroxidation inhibition and hydroxyl radical scavenging activity was due to metal chelation or chain termination of peroxyl and hydroxyl radical formation reaction. The reducing powers are generally associated with the presence of reductones**.** Antioxidant action of reductones is based on the breaking of the free-radical chain by donating hydrogen atom. The reductones also react with certain precursors of peroxide, thus preventing peroxide formation.

VIABILITY OF *LACTOBACILLUS* CULTURES IN RMR EXTRACTS

Antibacterial property of *Monascus* sp was first mentioned by (Wong & Bau, 1977). Effectiveness of monascidin-A against *Bacillus, Streptococcus* and *Pseudomonas* was been reported. Amino acid derivatives of *Monascus* pigments produced during fermentation possessed antimicrobial activities. Derivatives with L-Phe, D-Phe, L-Tyr, and D-Tyr exhibited high activities against Gram +ve and Gram -ve bacteria. Derivatives with L-Asp, D-Asp, L-Tyr, and D-Tyr were effective against the filamentous fungi *Aspergillus niger, Penicillium citrinum, and Candida albicans* (Kim *et al.*, 2006).

The *Lactobacillus* strain has been shown to cleave β-glycosidic isoflavones during fermentation of milk supplemented with soy-germ powder. The interactions between the *Lactobacillus* strain and soygerm powder suggested that combining both can exhibit advantageous probiotic effect. Lactic acid bacteria including *Lactobacilli* and *Bifidobacteria* are the most common bacterial species considered as potential probiotics.

M. purpureus extracts have shown the presence of isoflavones (**Figure 53**). The elusion profile of glucosidic (genistin and daidzin) and aglyconic isoflavones (genistein and daidzein) in *M. purpureus* RMR and its combination with LAB cultures are shown in **Figure 16**. Among the five LAB cultures, the highest viable count was observed in *Lactobacillus casei* (9.8 log10 CFU/ml) followed by *Lactobacillus plantarum* (9.5 log10 CFU/ml) and *Lactobacillus helviticus* (8.6 log10

CFU/ml). No significant differences in viable counts of LAB grown with *M. purpureus* pigment were observed. Instead, there was an increase in the viable count of *Lactobacillus helviticus* (8.8 log10 CFU/ml) and *Lactobacillus plantarum* (9.7 log10 CFU/ml) compared to control (**Table 27**). The results indicate RMR extract induced the growth of LAB cultures. The culture filtrates of *Lactobacillus* also showed antioxidant activity against DPPH radicals (**Table 28**). These studies indicate the use of RMR extracts along with probiotic culture for application for the development of fermented products.

HYPOLIPEDIMIC PROPERTIES OF RMR

Hyperlipidemia is considered a major risk factor for atherosclerosis and cardiovascular diseases. Hyperlipidemia can be managed in certain patients with healthy and less atherogenic diet, less stressful life style and physical activity. But a majority of patients need therapeutical intervention to effectively lower hyperlipidemia. Statins, are widely used to inhibit HMG-CoA reductase involved in the biosynthesis of cholesterol to control hyperlipidemia (Alberts, 1990); (Blankenhorn *et al.*, 1993). Plant phytosterols, saponins, fungal metabolites such as mevinolin, monacolin K are also being investigated for their antihyperlipidemic and antiatherosclerotic properties (Kroon *et al.*, 1982; Ikeda & Sugano, 1998; Harwood *et al.*, 1993).

This study showed use of *M. purpureus* MTCC 410 for use as food supplement to lower blood-lipid levels and monacolins were identified as the main active constituents. The short term and long term effect of feeding RMR and monitoring hyperlipidemia induced in male albino rats showed decreased cholesterol and triacylglycerol content in both serum and liver (**Table 36 and 37**). Consumption of RMR by rats, at a dose of 16% for 7 weeks, reduced cholesterol levels by 44.46 and 48.45% in serum and liver respectively and the triacylglycerols content was reduced to 45.30 and 42.08% in serum and liver compared to HFD fed group. LDL-cholesterol levels were decreased by 66.81% while HDL increased by 46.63% in sera of 16.0% RMR fed rats for 7 weeks. The hypolipedimic effect was comparatively significant when compred with similar reports of other strains of *Monascus* sp (Lee *et*

al., 2006). This study indicated that RMR was very effective in reducing cholesterol and triacylglycerol and LDL-C levels in hyperlipidemic rats.

The liver weight of the rats fed with HFD was significantly higher than the rats fed with RMR supplementation **(Table 31 and 32)**. This was because the fat metabolism in rats was reduced while it was converted to cholesterol in control rats fed with HFD alone. These results indicated ditary significance of RMR to inhibit cholesterol synthesis in rats fed with HFD. The higher HMG CoA reductase activity assay **(Figure 46)** confirmed the above result. These findings are in concurrence with reported decreased lipid deposition in liver and atheroma in aorta of rabbits fed with RMR (Li *et al.*, 1998).

The rats fed with control diet showed increased HMG-CoA reductase activity compared to rats fed with HFD and HFD supplemented with RMR **(Figure 46)**. Decreased HMG-CoA reductase activity in rats fed with HFD can be reasoned for feedback inhibition due to external supply of cholesterol (**Table 22**) and decreased HMG-CoA reductase activity in rats supplemented with RMR diet due to its inhibition by monacolins present in RMR. Thus, the significant hypolipidemic effect of *M purpureus* MTCC 410 RMR observed in this study can be attributed to the production of monacolins (2.45 g kg^{-1}).

Even though, HMG-CoA reductase activity observed was less in rats fed with HFD, the cholesterol content observed was high in both serum and liver. The data have indicated the hypolipidemic characteristics of RMR. The RMR fed to the rats along with HFD effectively inhibited the HMG-CoA reductase enzyme. In other hand, the controlled rats fed on normal diet biosynthesized cholesterol to maintain membrane integrity. Further, the cholesterol lowering effect of RMR in this study was comparable to therapeutic drug lovastatin. Lovastatin is reported to suppress cholesterol synthesis and also increase the activity of LDL-receptors and thus reduce total cholesterol and LDL levels in the body.

Adverse effects of statins on muscle, such as myopathy and rhabdomyolysis can occur when doses higher than those are consumed. The standard dose (atorvastatin 10-20, fluvastatin 40-80, lovastatin 40, pravastatin 40, rosuvastatin 10, and simvastatin 20-40 mg) refer to the commonly prescribed daily doses which typically reduce LDL-C by 30-45% **(**Armitage, 2007**)**. The RMR supplementation and 80 mg of lovastatin reduced more than 68 % of LDL-C in rats compared to only HFD fed rats (**Table 29**). In this study we did not observe any significant changes in relative organ weight, serum clinical enzymes like, LDH, SGOT, SGPT, ALP levels between RMR and HFD fed groups. RMR supplementation in HFD did not induce any histopathological alterations in liver compared to that of HFD fed group. The acute and sub-chronic toxicological studies of *M. purpureus* MTCC 410 RMR did not show any adverse effect in rats (Mohan Kumari *et al.*, 2009). The atherogenic effect of the RMR significantly decreased LDL-C level in serum. The HDL value and HDL-C to LDL-C ratio increased in rats fed on RMR. The data suggested that *M. purpureus* MTCC 410 RMR can be considered as an efficient hypolipidemic food supplement without any adverse effects.

SUMMARY

Monascus purpureus, used as a food additive, coloring and flavoring agent in foods and beverages in ancient China. Production of secondary metabolites like polyketides, statins, sterol and fatty acids during solid-state and shake flask culture by *M. purpureus* and its mutants through different fermentation conditions were studied. Mutant CFR 410-11 over produced polyketide pigments (1148 OD Units) compared to *M. purpureus* MTCC 410 (604 OD Units). The mutant CFR 410-22 showed low quantity of polyketide pigments (473 OD Units) compared to MTCC 410 in solid-state cultures.

A comparative study made by TLC and HPLC revealed that statin produced by *M. purpureus* and its mutants were similar to the commercial statin with R_f value and retention time. Solid-state cultures produces highest amount of statin compared to shake flask cultures. More sterols are produced in shake flask cultures compared to solid-state. Significant amount of fatty acids production in solid-state than in shake flask cultures were confirmed by GC.

Among the metabolites of *M. purpureus,* lovastatin (Monacolin K) potent inhibitor of 3-hydroxy-3-methylglutaryl-CoA reductase (HMG-CoA) is found to be very important and have been proven to be cholesterol lowering drug. Cultural condition has a significant influence on the yield of statin. Maltose (5.09 mg/g) and peptone (5.53 mg/g) as carbon and nitrogen source with pH 5.0 (5.79 mg/g) and 28° C (5.99 mg/g) is the optimum condition for statin production.

L-asparaginase (0.103 IU) and L-glutaminase (0.139 IU) activity was comparatively more in CFR 410-11 than MTCC 410 and CFR 410-22. The mutant CFR 410-11 secreted more red pigment cultured on rice (2.248 OD Units) and in broth (0.841 OD Units). Significant difference in amidase activities in MTCC 410 and its mutant CFR 410-11 and CFR 410-22 revealed the importance of these enzymes in pigment production.

Safety of RMR was assessed by conducting acute and sub-chronic toxicological studies on both sexes of albino rats. Feeding acute doses of RMR did not cause any symptoms of toxicity or mortality. Similarly, dietary feeding of RMR did not produce any significant changes in food intake or gain in body weight of the experimental rats compared to control rats. No significant differences in the relative weight of vital organs, hematological parameters, macroscopic and microscopic changes in vital organs and serum clinical enzyme levels between the experimental and control groups.

RMR containing lovastatin at a concentration of 11.51g/ kg reduced total cholesterol (TC), triglyceride (TG), lipoprotein cholesterol (LDL) in high fat diet (HFD) fed Wistar rats. There was no significant difference with regard to the food intake, gain in body weight and organ weights of rats in different dietary groups. RMR significantly lowered serum and hepatic cholesterol and triacylglycerol levels. LDL-C levels decreased by 66.28 and 70.12%, while HDL-C increased by 44.45 and 34.58% in serum of 16% RMR fed groups for 7 and 14 weeks, respectively. The atherogenic indices (LDL-C/HDL-C and TC/HDL-C) of 16% RMR for 14 weeks were reduced by 77.80 and 61.05%, respectively. RMR fed groups resulted in 22 to 54% inhibition of HMG-CoA reductase activity in liver. Further, the hypolipidemic effect of RMR was comparable to therapeutic drug lovastatin. In addition, histological examinations of liver of hyperlipidemic rats showed decreased lipid accumulation in red mould rice powder fed rats.

RMR effectively scavenged 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radicals (IC_{50}=100µg/ml). The consumption of RMR with HFD showed increased enzyme activity (glutathione reductase, glutathione peroxidase, superoxide dismutase and catalase) and increased levels of non-enzymic (total thiols, glutathione and ascorbic acid) antioxidants in rats. Lipid peroxidation was significantly inhibited in rats (46µmol/dl) fed on RMR compared to rats fed on HFD only (99µmol/dl). The treatment of 16% RMR is equivalent to 80mg of lovastatin. This study has confirmed that, consumption of RMR can induce antioxidant enzymes and molecules to scavenge the reactive oxygen species (ROS) released due to oxidative stress in rats fed on HFD.

REFERENCES

Aebi, H. (1984). Catalase in vitro. *Methods Enzymology* **105**, 121-126.

Akihisa, T., Tokuda, H., Yasukawa, K., Ukiya, M., Kiyota, A., Sakamoto, N., Suzuki, T., Tanabe, N. & Nishino, H. (2005a). Azaphilones, furanoisophthalides, and amino acids from the extracts of *Monascus pilosus*-fermented rice (red-mold rice) and their chemopreventive effects. *Journal of Agricultural and Food Chemistry* **53**, 562-565.

Akihisa, T., Tokudab, H., Ukiyaa, M. & other authors (2005b). Anti-Tumor-Initiating effects of monascin, an azaphilonoid pigment from the extract of *Monascus pilosus* fermented rice (Red-Mold Rice). *Chemistry & Biodiversity* **2**, 1305-1309.

Alberts, A. W., Chen, J., Kuron, G. & other authors (1980). Mevinolin: a highly potent competitive inhibitor of hydroxymethylglutaryl-coenzyme a reductase and a cholesterol-lowering agent. *Proceedings of Naturall Academic Science USA* **77**, 3957-3960.

Alberts, A. W. (1990). Lovastatin and simvastatin inhibitors of HMG-CoA and cholesterol biosynthesis. *Cardiology* **77**, 12-21.

Alı, M. (2003). Effect of grape antioxidant dietary fiber on the total antioxidant capacity and the activity of liver antioxidant enzymes in rats. *Nutrition Research* **23**, 1251-1267.

Angelin, B. (1991). Regulation of Hepatic Cholesterol Metabolism in Man. *Annals of Medicine* **23**, 177-180.

Aniya, Y., Yokomakura, T., Yonamine, M., Shimada, K., Nagamine, T., Shimabukuro, M. & Gibo, H. (1999). Screening of antioxidant action of various molds and protection of *Monascus* anka against experimentally induced liver injuries of rats. *General Pharmacology* **32**, 225-231.

Aniya, Y., Ohtani, I. I., Higa, T., Miyagi, C., Gibo, H., Shimabukuro, M., Nakanishi, H. & Taira, J. (2000). Dimerumic acid as an antioxidant of the mold, *Monascus* anka. *Free Radical and Biological Medicine* **28**, 999-1004.

AOAC (2000). *Association of Official Analytical Chemists (AOAC)*.

Auclair, K., Sutherland, A., Kennedy, J., Witter, D. J., Van den Heever, J. P., Hutchinson, C. R. & Vederas, C. J. (2001). Lovastatin nonaketide synthase catalyze an intramolecular Diels-Alder reaction of a substrate analogue. *Journal of American Chemical Society* **122**, 11519-11520.

Barker, B. T. P. (1903). Morphology and development of the ascocarp in *Monascus*. *Annals of Botany* **17**, 167-236.

Bau, C. A. (1996). Physiological properties of *Monascus*. *Journal of Chinese Fermentation* **2**, 11-20.

Beutler, E., Duron, O. & Kelly, B. M. (1963). Improved method for the determination of blood glutathione. *Journal of Laboratory and Clinical Medicine* **61**, 882-888.

Bharucha, C., Meyer, H., Mody, A. & Carman, R. H. (1976). *Hematology, In: Handbook of medical laboratory technique*. Mysore,: Wesley Press.

Blanc, J. P., Laussac, P. J., Bars, L. J. & other authors (1995a). Characterization of monacidin A from *Monascus* as citrinin. *International Journal of Food Microbiology* **27**, 201-213.

Blanc, J. P., Loret, M. O. & Goma, G. (1999a). Pigments and citrinine production during cultures of *Monascus* in liquid and solid media. *Advances in Solid State Fermentation* **Chapter 32**, 393-406.

Blanc, P. J., Loret, M. O. & Goma, G. (1995b). Production of citrinin by various species of *Monascus*. *Biotechnology Letters* **17**, 291-294.

Blankenhorn, D. H., Azen, S. P., Kramsch, D. M. & other authors (1993). MARS Research Group. Coronary angiographic changes with lovastatin therapy: The Monitored Atherosclerosis Regression Study (MARS). *Annals of Internal Medicine* **119**, 969-976.

Blein, S., Hawrot, E. & Barlow, P. (2000). The metabotropic GABA receptor: molecular insights and their functional consequences. *Cell Molecule and Life Science* **57**, 635-650.

Blois, M. S. (1958). Antioxidant determinations by the use of a stable free radical. *Nature* **181**, 1199-1200.

Bond, J. S. & Butler, P. E. (1987). Intracellular proteases. *Annual Review of Biochemistry* **56**, 333-364.

Bousvaros, A., Guandalini, S., Baldassano, R. N. & other authors (2005). A randomized, double-blind trial of Lactobacillus GG versus placebo in addition to standard maintenance therapy for children with Crohn's disease. *Inflammatory Bowel Disease* **11**, 833-839.

Braun, S. & Vecht-Lifshitz, S. E. (1991). Mycelial morphology and metabolite production. *Trends in Biotechnology* **9**, 63-68.

Broder, C. U. & Koehler, E. P. (1980). Pigment produced by *Monascus purpureus* with regard to quality and quantity. *Journal of Food Science* **45**, 567-569.

Bu'Lock, J. D. (1961). Intermediary metabolism and antibiotic synthesis. *Advanced Applied Microbiology* **3**, 293-342.

Buckland, B., Gbewnyo, K., Hallada, T., Kaplan, L. & Masurekar, P. (1989). Production of lovastatin, an inhibitor of cholesterol accumulation in human. In: Demain, A.L,, Somkuti, G.A., Hunter-Cevera, J.C., Rossmoore, H.W., editors Novel microbial products for medicine and agriculture. . *New York: Elsevier* **161-169**.

Campoy, S., Rumbero, A., Martín, J. F. & Liras, P. (2006). Characterization of an hyperpigmenting mutant of *Monascus purpureus* IB1: identification of two novel pigment chemical structures. *Applied Microbiology and Biotechnology* **70**, 488-496.

Carels, M. & Shepherd, D. (1977). The effect of different nitrogen sources on pigment production and sporulation of *Monascus* species in submerged, shaken culture. *Canadian Journal of Microbiology* **23**, 1360-1372.

Castilho, L. R., Polato, C. M. S., Baruque, E. A., Sant'Anna Jr, G. L. & Freire, D. M. G. (2000). Economic analysis of lipase production by Penicillium restrictum in solid-state and submerged fermentations. *Biochemical Engineering Journal* **4**, 239-247.

Chairote, E., Chairote, G., Niamsup, H. & Lumyong, S. (2008). The presence and the content of Monacolins in Red Yeast rice prepared from Thai glutinous rice *World Journal of Microbiology and Biotechnology* **24**, 3039-3047.

Chang, Y., Huang, J., Lee, C., Shih, I. & Tzeng, Y. (2002). Use of response surface methodology to optimize culture medium for production of lovaststin by *Monascus ruber*. *Enzyme and Microbial Technology* **30**, 889-894.

Chen, C.-C. & Liu, I.-M. (2006). Release of acetylcholine by Hon-Chi to raise insulin secretion in Wistar rats. *Neuroscience Letters* **404**, 117-121.

Chen, C. H., Hu, H. Y., Cho, Y. C. & Hsu, W. H. (2006). Screening of compactinresistant microorganisms capable of converting compactin to pravastatin. *Current Microbiology* **53**, 108-112.

Chen, F. S. & Hu, X. Q. (2005). Study on red fermented rice with high concentration of monacolin K and low concentration of citrinin. *International Journal of Food Microbiology* **103**, 331-337.

Chen, M. H. & Johns, M. R. (1993a). Effect of pH and nitrogen source on pigment production by *Monascus purpureus*. *Applied Microbiology and Biotechnology* **40**, 132-138.

Chen, M. H. & Johns, M. R. (1993b). Effect of pH and nitrogen source on pigment production by *Monascus purpureus*. *Applied Microbiology and Biotechnology* **40**, 132-138.

Chiou, R. Y. Y. & Cheng, S. L. (2001). Isoflavone transformation during soybean koji preparation and subsequent miso fermentation supplemented with ethanol and NaCl. *Journal of Agricultural and Food Chemistry* **49**, 3656-3660.

Chiu, C. H., Ni, K. H., Guu, Y. K. & Pan, T. M. (2006). Production of red mold rice using a modified Nagata type koji maker. *Applied Microbiology and Biotechnology* **73**, 297-230.

Da Lozzo, E. J., Oliveira, M. B. M. & Carnieri, E. G. S. (1998). Citrinin-induced mitochondrial permeability transition. *Journal of Biochemistry and Molecular Toxicology* **12**, 291-297.

Davignon, J. (2004). Beneficial cardiovascular pleiotropic effects of statins. *md circulation* **109**, 39-43.

De Doever, P., Wouters, R. & Verstraete, W. (2001). Combined use of *Lactobacillus reuteri* and soygerm powder as food supplement. *Letters in Applied Microbiology* **33**, 420-424.

Dhale, M. A., Divakar, S., Umesh-Kumar, S. & Vijayalakshmi, G. (2007). Isolation and charecterization of dihydromonacolin-MV from *Monascus purpureus* for antioxidant properties. *Applied Microbiology and Biotechnology* **73**, 1197-1202.

Dhale, M. A., Divakar, S., Umesh-Kumar, S. & Vijayalakshmi, G. (2007a). Isolation and charecterization of dihydromonacolin-MV from *Monascus purpureus* for antioxidant properties. *Applied Microbiology and Biotechnology* **73**, 1197-1202.

Dhale, M. A., Divakar, S., Umesh-Kumar, S. & Vijayalakshmi, G. (2007b). Characterization of dehydromonacolin-MV2 from *Monascus purpureus* mutant. *Journal of Applied Microbiology* **103**, 2168-2173.

Dhale, M. A. (2007c). Physiology of *Monascus purpureus* in relation to metabolite production and application as functional food. In *Food Microbiology*, pp. 110. Mysore: Thesis, University of Mysore.

Dhale, M. A., Mohan Kumari, H. P., Umesh Kumar, S. & Vijayalakshmi, G. (2009). Production of *Monascus purpureus* pigments; influenced by amidase and acid protease activity. *Journal of Food Biochemistry*.

Eckert, G. P., Wood, W. G. & Muller, W. E. (2005). Statins: Drugs for Alzheimer's disease? *Journal of Neural Transmmission* **112**, 1057-1071.

Eisenbrand, G. (2006). Toxicological evaluation of red mould rice. *Molecular Nutrition and Food Research* **50**, 322 - 327.

Ellman, G. L. (1959). Tissue sulfhydryl groups. *Archives of Biochemistry and Biophysics* **82**, 70-77.

Endo, A. (1979). Monacolin K, a new hypocholesterolemic agent produced by *Monascus* species. *Journal of Antibiotic* **32**, 852-854.

Endo, A. (1980). Monacolin K a new hypocholesterolemic agent that specifically inhibits 3-hydroxy-3-methyl-glutaryl coenzyme A reductase. *Journal of Antibiotics* **33**, 334-336.

Endo, A. (1985). Compactin (ML236B) and related compounds as potential cholesterol-lowering agents that inhibit HMG-CoA reductase. *Journal of Medcinal Chemicstry* **28**, 401-405.

Endo, A., Hasum, I. K. & Negishi, S. (1985a). Monacolin-J and monacolinl new inhibitors of cholesterol-biosynthesis produced by *Monascus ruber*. *Journal of Antibiotics* **38**, 420-422.

Endo, A., Hasumi, K., Nakamura, T., Kunishima, M. & Masuda, M. (1985b). Dihydromonacolin-L and monacolin-x, new metabolites those inhibit cholesterol-biosynthesis. *Journal of Antibiotics* **38**, 321-327.

Endo, A., Hasumi, K., Yamada, A., Shimoda, R. & Takeshima, H. (1986). The synthesis of compactin (ML-236B) and monacolin K in fungi. *Journal of Antibiotics* **39**, 1609-1610.

Endo, A., Komagata, D. & Shimada, H. (1986a). Monacolin M: A new inhibitor of cholesterol biosynthesis. *Journal of Antibiotics (Tokyo)* **39**, 1670-1673.

Endo, A., Hasumi, K., Yamada, A., Shimoda, R. & Takeshima, H. (1986b). The synthesis of compactin (ML-236B) and monacolin K in fungi. *Journal of Antibiotics* **39**, 1609-1610.

Endo, A. (2004). The origin of statins. *International Congress Series* **1262**, 3-8.

FAO (2001). Evaluation of health and nutritional properties of powdered milk and live lactic acid bacteria. Geneva, Switz., and Agriculture Organization of the United Nations andWorld Health Organization Expert Consultation Report. Edited by F. a. A. O. o. t. U. N. a. W. H. O. FAO.

Farnworth, E. R. (2005). The beneficial health effects of fermented foods -potential probiotics around the world. *Journal of Nutraceuticals, Functional & Medical Foods* **4**, 93-117.

Fletcher, M. J. (1968). Colorimetric method for estimating serum triglycerides. *Clinical Chemical Acta* **22**, 303-307.

Flohe, L. & Gunzler, W. A. (1984 a). Assays of glutathione peroxidase. *Methods Enzymology* **105**, 114-121.

Flohe, L. & Otting, F. (1984 b). Assays of glutathione peroxidase. *Methods Enzymology* **105**, 101-103.

Folch, J., Lees, M. & Sloane, S. G. (1957). A simple method for the isolation and purification of total lipids from animal tissues. *Journal of Biological Chemistry* **226**, 497-509.

Fowell, A. D. G., Robertson, A. & Whelly, W. B. (1956). Monascorubramine. *Journal of Chemical Society Special Publication* **5**, 27-34.

Gbewonyo, K., Hunt, G. & Buckland, B. (1992). Interactions of cell morphology and transport processes in the lovastatin fermentation. *Bioprocess Biosystem Engineering* **8**, 1-7.

George, A. M. (1997). Genetic regulation of nitrogen metabolism in the fungi. *Microbiology and Molecular biology reviews* **21**, 17-32.

Gheith, O., Sheashaa, H., Abdelsalam, M., Shoeir, Z. & Sobh, M. (2009). Efficacy and safety of *Monascus purpureus* Went rice in children and young adults with secondary hyperlipidemia: a preliminary report. **20**, 57-61.

Gilliland, S. E., Nelson, C. R. & Maxwell, C. (1985). Assimilation of cholesterol by *Lactobacillus acidophilus*. *Applied Environmental Microbiology* **49**, 377- 381.

Gilliland, S. E. & Walker, D. K. (1990). Factors to consider when selecting a culture of *Lactobacillus acidophilus* as a dietary adjunct to produce a hypocholesterolemic effect in humans. *Journal of Dairy Science* **73**, 905-911.

Hadfield, J. R., Holker, J. S. E. & Stanway, D. N. (1967). The biosynthesis of fungal metabolites Part II. The L-oxo-lactone equivalences in rubropunctatin and monascorubrin. *Journal of Chemical Society* **19**, 751-755.

Hajjaj, H., Blanc, P., Groussac, E., Uribelarrea, J., Goma, G. & Loubiere, P. (2000). Kinetic analysis of red pigment and citrinin production by *Monascus ruber* as a function of organic acid accumilation. *Enzyme and Microbial Technology* **27**, 619-625.

Hajjaj, H., Niederberger, P. & Duboc, P. (2001). Lovastatin biosynthesis by *Aspergillus terreus* in a chemically defined medium. *Applied and Environmental Microbiology* **67**, 2596-2602.

Hamdi, M., Blanc, P. J. & Goma, G. (1997). A new process for red pigment production by submerged culture of *Monascus purpureus*. *Bioprocess Engineering* **17**, 75-79.

Hartmann, M. A. (1998). Plant sterols and the membrane environment. *Trends in Plant Science* **3**, 170-175.

Harwood, H. J. J., Chandler, C. E., Pellarin, L. D. & other authors (1993). Pharmacologic consequences of cholesterol absorption inhibition: alteration in cholesterol metabolism and reduction in plasma cholesterol concentration induced by the synthetic saponin b-tigogenin cellobioside (CP-88818; tiqueside). *Journal of Lipid Research* **34**, 377-395

Haws, E. J., Holker, J. S. E., Kelly, A., Powell, A. D. G. & Robertson, A. (1959). *The chemistry of Fungi. Part XXXVII the structure of rubropuntatin*.

Heath, R. J. & Rock, C. O. (2002). The Claisen condensation in biology. *Natural Proceedings of Reproduction* **19**, 581-596.

Heber, D., Yip, I., Ashley, J. M., Elashoff, D. A., Elashoff, R. M. & Go, V. L. W. (1999). Cholestero-lowering effects of proprietary chinese red yeast rice dietary suppliment. *American Journal of Clinical Nutrition* **69**, 231-236.

Hendrickson, L., Davis, C. R., Roach, C., Nguyen, D. K., Aldrich, T., McAda, P. C. & Reeves, C. D. (1999). Lovastatin biosynthesis in *Aspergillus terreus:* charecterization of blocked mutants, enzyme activities and a multifunctional polyketide synthase gene. *Chemistry and Biology* **6**, 429-439.

Henry, J. B. (1979). Clinical Diagnosis and Management by Laboratory Methods. W.B. Saunders Company, Philadelphia, PA. 365.

Hesseltine, C. W. (1965). A millenium of fungi, food and fermentation. *Mycologia* **57**, 149-197.

Hiroi, T., Shima, T., Isobe, A. & Kimura, S. (1975). Studies on the structure of two pigment obtained from *Monascus* sp. *Journal of Japanies Society Food and Nutrition* **28**, 497-502.

Ho, B. Y. & Pan, T. M. (2009). The *Monascus* metabolite monacolin K reduces tumor progression and metastasis of Lewis lung carcinoma cells. *Journal of Agricultural and Food Chemistry* **57**, 8258-8265.

Ho, B. Y. & Pan, T. M. (2009). The Monascus metabolite monacolin K reduces tumor progression and metastasis of Lewis lung carcinoma cells. *Journal of Agricultural and Food Chemistry* **57**, 8258-8265.

Hoopwood, D. A. (1997). Genetic contribution to understanding polyketides synthases. *Chemcal review* **97**, 2465-2497.

Huang, H. C. (1985). Review and perspective of *Monascus* studies. In *Seminars Proceedings of Biological Research Center, National Science Council*, pp. 109-124. Taipei, Taiwan.

Huang, X., Chen, H. & Miller, W. C. e. a. (2007). Lower low-density lipoprotein cholesterol levels are associated with Parkinson´s disease. *Mov Disord* **22**, 377-381.

Huang, Z., Xu, Y., Li, L. & Li, Y. (2008). Two New *Monascus* Metabolites with Strong Blue Fluorescence Isolated from Red Yeast Rice. *Journal of Agriculture and Food Chemistry* **56**, 112-118.

Hulcher, F. H. & Wayne, H. O. (1973). Simplified spectrophotometric assay for microsomal 3-hydroxy-3-methylglutaryl CoA reductase by measurement of coenzyme. *Journal of Lipid Research* **14**, 625-631.

Hutchinson, C. R. (1999). Microbial polyketide synthase: more and more prolific. *Proceedings of the National Academy of Sciences of the United States of America* **96**, 3336-3339.

Hutchinson, C. R., Kennedy, J., Park, C., Kendrew, S., Auclair, K. & Vederas, J. (2000). Aspects of the biosynthesis of non-aromatic fungal polyketides by iterative polyketide synthases. *Antonie Van Leeuwenhoek* **78**, 287-295.

Hutter, R. & Niederberger, P. (1983). Biochemical pathways and mechanisms nitrogen, amino acid and carbon metabolism. *Biotechnology advances* **1**, 179-191.

Ikeda, I. & Sugano, M. (1998). Inhibition of cholesterol absorption by plant sterols for mass intervention. *Current Opinion Lipidology* **9**, 527-531.

Imada, A., Igarasi, S., Nakahama, K. & Isono, M. (1973). Asparaginase and glutaminase activies of micro-organisms. *Journal of General Microbiology* **76**, 85-99.

Istvan, E. S. & Deisenhofer, J. (2001). Structural mechanism for statin inhibition of HMG-CoA reductase. *Science* **292**, 1160-1164.

Izawa, S., Harada, N., Watanabe, T., Kotokawa, N., Yamamoto, A., Hayatsu, H. & Arimoto-Kobayashi, S. (1997). Inhibitory effects of food-coloring agents derived from *Monascus* on the mutagenicity of heterocyclic amines. *Journal of Agricultural and Food Chemistry* **45**, 3980-3984.

Jean, Y. C. & Eric, C. (2001). Effect of cultural conditions on ergosterol as an indicator of biomass in the aquatic hyphomycetes. *Applied and Environmental Microbiology* **67**, 2051-2055.

Jeon, T., Hwang, S. G., Hirai, S., Matsui, T., Yano, H., Kawada, T., Lim, B. O. & Park, D. K. (2004). Red yeast rice extracts suppress adipogenesis by down-regulating adipogenic transcription factors and gene expression in 3T3-L1 cells. *Life Sciences* **75**, 3195-3203.

Jia, X. Q., Xu, Z. N., Zhou, L. P. & Sung, C. K. (2009). Elimination of the mycotoxin citrinin production in the industrial important strain *Monascus purpureus* SM001. *Metabolite Engineier*.

Johns, M. R. & Stuart, D. M. (1991). Production of pigments by *Monascus purpureus* in soild culture. *Journal of Industrial Microbiology* **8**, 23-28.

Jongrungruangchok, S., Kittakoop, P., Yongsmith, B., Bavovada, R., Tanasupawata, S., Lartpornmatulee, N. & Thaebtaranonth, Y. (2004). Azaphilone pigments from a yellow mutant of the fungus *Monascus kaoliang*. *Phytochemistry* **65**.

Journoud, M. L. & Jones, P. J. (2004). Red yeast rice: a new hypolipidemic drug. *Life Sciences* **74**, 2675-2683.

Juzlova, P., Martinkova, L. & Kien, V. (1996a). Secondary metabolites of the fungus *Monascus*: a review. *Journal of Industrial Microbiology* **16**, 163-170.

Kasiske, B. L., O'Donnell, M. P., Schmitz, P. G., Kim, Y. & F, K. W. (1990). Renal injury of diet-induced hypercholesterolemia in rats. *Kidney Int* **37**, 880-891.

Kate, M. (1964). Bacterial lipids. *Advance in Lipid Research*, 217-290.

Keithley, J. K., Swanson, B., Sha, B. E., Zeller, J. M., Kessler, H. A. & Smith, K. Y. (2002). A pilot study of the safety and efficacy of cholestin in treating HIV-related dyslipidemia. *Nutrition* **18**, 201-204.

Kerr, D. I. B. & Ong, J. (1995). $GABA_B$ receptors. *Pharmacology and Therapeutics* **67**, 187-246.

Kim, J. H., Kim, H. J., Kim, C., Jung, H., Kim, Y. O., Ju, J. Y. & Shin, C. S. (2006). Development of lipase inhibitors from various derivatives of *Monascus* pigment produced by *Monascus* fermentation. *Food Chemistry*.

Kimura, K., Komagata, D., Murakawa, S. & Endo, A. (1990). Biosynthesis of monacolins: conversion of monacolin J to monacolin K (mevinolin). *Journal of Antibiotics (Tokyo)* **43**, 1621-1622.

Klimek, M., Wang, S. & Ogunkanmi, A. (2009). Safety and Efficacy of Red Yeast Rice (*Monascus purpureus*) as an Alternative Therapy for Hyperlipidemia. *Pharmocology and Toxicology* **34**, 313-327.

Kogika, M. M., Hagiwara, M. K. & Mirandola, R. M. (1993). Experimental citrinin nephrotoxicosis in dogs: renal function evaluation. *Veterinary and Human Toxicology* **35**, 136-140.

Kohama, Y., Matsumoto, S., Mimura, T., Tanabe, N., Inada, A. & Nakanishi, T. (1987). Isolation and identification of hypotensive principles in red-mold rice. *Chemistry and Pharmacetutical Bulletin* **35**, 2484-2489.

Komagata, D., Shimada, H., Murakawa, S. & Endo, A. (1989). Biosynthesis of monacolins: conversion of monacolin L to monacolin J by a monooxygenase of *Monascus ruber*. *Journal of Antibiotics (Tokyo)* **42,** 407-412.

Komatsuzaki, N., Shima, J. & Kawamoto, S. e. a. (2005). Production of c-aminobutyric acid (GABA) by *Lactobacillus paracasei* isolated from traditional fermented foods. *Food Microbiology* **22**, 497-504.

Komatsuzaki, N., Tsukahara, K. & Toyoshima, H. e. a. (2007). Effect of soaking and gaseous treatment on GABA content in germinated brown rice. *Journal of Food Microbiology* **78**, 556-560.

Kono, I. & Himeno, K. (2000). Changes in gama-amibnobutyric acid content during *bini-koji* making. *Bioscience Biotechnology and Biochemistry* **64**, 617-619.

Krairak, S., Yamamura, K., Irie, R., Nakajima, M., Shimizu, H., Chim-Anage, P., Yongsmith, B. & Shioya, S. (2000). Maximizing yellow pigment production in fed-batch culture of *Monascus* sp. *Journal of Bioscience and Bioengineering* **90**, 363-367.

Krejci, M. E., Bretz, N. S. & Koechel, D. A. (1996). Citrinin produces acute adverse changes in renal function and ultrastructure in pentobarbital-anesthetized dogs without concomitant reductions in [potassium](plasma). *Toxicology* **106**, 167-177.

Kroon, P. A., Hand, K. M., Huff, J. W. & Alberts, J. W. (1982). Effects of mevinolin on serum cholesterol levels of rabits with endogenous hypercholesterolemia. *Atherosclerosis* **44**, 41-47.

Krysiak, R., Okopien, B. & Herman, Z. (2003). Effects of hmg-coa reductase inhibitors on coagulation and fibrinolysis processes. *Drugs* **63**, 1821-1854

Kuba, M., Tanaka, K., Sesoko, M., Inoue, F. & Yasuda, M. (2009). Angiotensin I-converting enzyme inhibitory peptides in red-mold rice made by *Monascus purpureus Process Biochemistry* **44**, 1139-1143.

Kuehbacher, T., Ott, S. J., Helwig, U. & other authors (2006). Bacterial and fungal microbiota in relation to probiotic therapy (VSL#3) in pouchitis. *Gut*.

Kulkarni, A. P., Aradhya, M. & Divakar, S. (2004). Isolation and identification of a radical scavenging antioxidant- punicalgin from pith and carpellary membrane of pomegranate fruit. *Food chemistry* **87**, 551-557.

Kumar, D., Jain, V., Shanker, G. & Srivastava, A. (2003). Citric acid production by solid state fermentation using sugarcane bagasse. *Process Biochemistry* **38**, 1731-1738.

Kumar, M. S., Jana, S. K., Senthil, V., Shashanka, V., Kumar, S. V. & Sadhukhan, A. K. (2000). Repeated fed-batch process for improving lovastatin production. *Process Biochemistry* **36**, 363-368.

Kuo, C.-F., Hou, M.-H., Wang, T.-S., Chyau, C.-C. & Chen, Y.-T. (2009a). Enhanced antioxidant activity of *Monascus pilosus* fermented products by addition of ginger to the medium. *Food Chemistry* **116**, 915-922.

Kuo, C. F., Chyau, C. C., Wang, T. S., C.R., L. & Hu, T. J. (2009b). Enhanced Antioxidant and Anti-inflammatory Activities of *Monascus pilosus* Fermented Products by Addition of Turmeric to the Medium. *Journal of Agriculture and Food Chemistry*.

Kurono, M., Nakanishi, K., Shindo, K. & Tada, M. (1963). Biosynthesis of monascorubrine and monascoflavin. *Chemical Pharmacological Bulltein* **11**, 358-362.

Kuyper, H. P. (1905). Die Perithecien Entwicklung von *Monascus purpureus* Went und *Monascus* barkeri Dang. und die systematische Stellung dieser Pilze. *Annals of Mycology* **3**, 32-81.

Kycko, S., Shiho, S. S., Yoko, K. & Tamio, M. (1998). Analytical method for citrinin in *Monascus* colour. *Japanese Journal of Food Chemistry* **5**, 64-68.

Laurent Dufosse´, L., Galaupa, P., Yaronb, A., Aradb, S. M., Philippe Blancc, P., N., M. K. C. & Ravishankar, G. A. (2005). Microorganisms and microalgae as sources of pigments for food use: a scientific oddity or an industrial reality? *Trends in Food Science and Technology* **16**, 389-406.

Law, M. (2000). Plant sterol and stanol margarines and health. *British Medical Journal* **320**, 861-864.

Lee, C.-L., Wang, J. J., Kuo, S.-L. & Pan, T.-M. (2006). *Monascus* fermentation of dioscorea for increasing the production of cholesterol-lowering agent-monacolin K and antiinflammation agent—monascin. *Applied Microbiology and Biotechnology*.

Lee, C.-L., Chen, W.-P., Wang, J.-J. & Pan, T.-M. (2007). A Simple and Rapid Approach for Removing Citrinin while Retaining Monacolin K in Red Mold Rice. *Journal of Agricultural Food Chemistry* **55**, 11101-11108.

Lee, Y.-L., Yang, J.-H. & Maua, J.-L. (2008). Antioxidant properties of water extracts from *Monascus* fermented soybeans. *Food Chemistry* **106**, 1128-1137.

Levy, R. I., Troendle, A. J. & Fattu, J. M. (1993). From phenotypes to genotypes: a remarkable quarter century. A quarter century of drug treatment of dyslipoproteinemia, with a focus on the new HMG-CoA reductase inhibitor fluvastatin. *Circulation* **87**, III45-III53.

Li, C., Zhu, Y., Wang, Y., Zhu, J., Chang, J. & Kritchevsky, D. (1998). *Monascus purpureus* fermented rice (red yeast rice): a natural food product that lowers blood cholesterol in animal models of hypercholesterolemia. *Nutrition Research* **18**, 71-78.

Li, Y.-g., Zhang, F., Wang, Z.-t. & Hu, Z.-b. (2004). Identification and chemical profiling of monacolins in red yeast rice using high-performance liquid chromatography with photodiode array detector and mass spectrometry. *Journal of Pharmaceutical and Biomedical Analysis* **35**, 1101-1112.

Liang, T.-W., Lin, J.-J., Yen, Y.-H., Wang, C.-L. & Wang, S.-L. (2006). Purification and characterization of a protease extracellularly produced by *Monascus purpureus* CCRC31499 in a shrimp and crab shell powder medium. *Enzyme and Microbial Technology* **38**, 74-80.

Liao, J. K. & Ulrich, L. (2005). Pleiotropic effects of statins. *Annual review of Pharmacology and Toxicology* **45**, 89-118.

Libby, P. (2002). Inflammation in atherosclerosis. *Nature* **420**, 868-874.

Lin, C.-C., Li, T.-C. & Lai, M.-M. (2005). Efficacy and safety of *Monascus purpureus* Went rice in subjects with hyperlipidemia. *Europian Journal of Endocrinology* **153**, 679-686.

Lin, C. Y. (1973). Isolation and culture condition of *Monascus* sp for the production of pigment in a submerged culture. *Journal of Fermentation Technology* **51**, 407-414.

Lin, D. C. (2003). Probiotics as functional foods. *Nutrition and Clinical Practice* **18**, 497-506.

Lin, T. F. & Demain, A. L. (1991a). Effect of nutrition of *Monascus* sp. on formation of red pigments. *Applied Microbiology and Biotechnology* **36**, 70-75.

Lin, T. F. & Demain, A. L. (1991b). Effect of nutrition on formation of *Monascus* red pigments. *Applied Microbiology and Biotechnology* **36**, 70-75.

Lin, W.-Y., Song, C.-Y. & Pan, T.-M. (2006). Proteomic analysis of Caco-2 cells treated with monacolin-K. *Journal of Agriculture and Food Chemistry* **54**, 6192-6200.

Lindsey, K., Pullen, M. L. & Topping, J. F. (2003). Importance of plant sterols in pattern formation and hormone signalling. *Trends in Plant Science* **8**, 1360-1385.

Liu, F., Tachibana, S., Taira, T., Ishihara, M. & Yasuda, M. (2004). Purification and characterization of a new type of serine carboxypeptidase from *Monascus purpureus*. *Journal of Indudstrial Microbiology and Biotechnology* **31**, 23-28.

Liu, Y. L. & Ng, T. K. (2000). Electronic transport through a quantum wire coupled to a quantum dot. *Physical review B* **61**, 2911-2920.

Lockard, V. G., Phillips, R. D., Hayes, A. W., Berndt, W. O. & O'Neal, R. M. (1980). Citrinin nephrotoxicity in rats: a light and electron microscopic study. *Experiment and molecular pathalogy* **32**, 226-240.

Long-Shan, T. L., Tai-Her, T., Te Chi, W. & Tsung-Yao, C. (2005). The influence of culturing environments on lovastatin production by Aspergillus terreus in submerged cultures. *Enzyme and Microbial Technology* **36**, 737-748.

Lotong, N. & Suwanarit, P. (1990). Fermentation of Ang-Kak in plastic bags and regulation of pigmentation by initial moisture-content. *Journal of Applied Bacteriology* **68**, 565-570.

Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951). Protein measurement with folin phenol reagent. *Journal of Biological Chemistry* **193**, 265-271.

Ma, J., Li, Y., Ye, Q., Li, J., Hua, Y., Ju, D., Zhang, D., Cooper, R. & Chang, M. (2000). Constituents of red yeast rice, a traditional Chinese food and medicine. *Journal of Agricultural and Food Chemistry* **48**, 5220-5225.

Maatat, K., Lampi, A. M., Petterson, J., Fogelfors, B. M., Piironen, V. & Kamal-Eldin, A. (1999). Phytosterol content in seven oat cultivars grown at three locations in Sweden. *Journal of the Science of Food and Agriculture* **79**, 1021-1027.

Manchand, P. S., Whalley, W. B. & Chen, F. C. (1973). Isolation and structure of ankaflavin. *Phytochemistry* **12**, 2531-2538.

Manzoni, M., Bergomi, S., Rollini, M. & Cavazzoni, V. (1999). Production of statins by filamentous fungi. *Biotechnology Letters* **21**, 253-257.

Manzoni, M. & Rollini, M. (2002). Biosynthesis and biotechnological production of statins by filamentous fungus and application of these cholesterol-lowering drugs. *Applied Microbiology and Biotechnology* **58**, 555-564.

Martinkova, L., Juzlova, P., Kren, V. & other authors (1999). Biological activities of oligoketide pigments of *Monascus purpureus. Food Additives and Contaminants* **16**, 15-24.

Masahiko, H., Ogawa, K. & Yoshikawa, H. (1993). Application of computer to monitoring and control of fermentation process: microbiological conversion of ML-236B Na to pravastatin. . *Biotechnology and Bioengineering* **42**, 815-820.

Matsubara, N., Fuchimoto, S., Iwagaki, H., Nomaka, Y., Kimura, T., Kashimo, H., Edamatsu, R., Hiramatsu, M. & Orita, K. (1991). The possible involvement of free radicals scavenging properties in the action of cytokine. *Research Communication Chemical pathalogy and Pharmacology* **71**, 239-242.

McHan, F. & Johnson, G. T. (1970). Zinc and amino acids: important components of a medium promoting growth of *Monascus purpureus*. *Mycologia* **62**, 1018-1031.

Mensink, R. P., Ebbing, S., Lindhout, M., Plat, J. & van Heutgen, M. M. (2002). Effects of plant stanol esters supplied in low-fat yoghurt on serum lipids and lipoproteins, non-cholesterol sterols and fat soluble antioxidant concentrations. *Atherosclerosis* **160**, 205-213.

Miettinen, T. A. & Gylling, H. (2004). Plant stanol and sterol esters in prevention of cardiovascular diseases. *Annals of Medicine* **36**, 126- 134.

Mishra, O. P. & Kovachich, G. B. (1984). Inhibition of the auto-oxidation of ascorbic acid and norepinephrine by extracts of Clostridium butyrium, Megasphaera elsdelii and E. coli. *Life Sciences*, 849-854.

Miura, D., Ito, Y. & Mizukuchi, A. e. a. (2006). Hypercholesterolemic action of pre-germinated brown rice in hepatoma-bearing. *Life Science* **79**, 259-264.

Miyake, T., Mori, A., Kii, T., Okuno, T., Usui, Y., Sato, F., Sammoto, H., Watanabe, A. & Kariyama, M. (2005). Light effects on cell development and secondary metabolism in *Monascus*. *Journal of Industrial Microbiology* **32**, 103-108.

Miyake, T., Uchitomi, K., Zhang, M.-Y., Kono, I., Nozaki, N., Sammoto, H. & Inagaki, K. (2006a). Effect of principal nutrients on lovastatin production by *Monascus pilosus*. *Bioscience Biotechnology and Biochemistry* **70**, 1154-1159.

Miyake, T., Zhang, M.-Y., Kono, I., Nozaki, N. & Sammoto, H. (2006b). Repression of secondary metabolites production of exogenous c-AMP in *Monascus. Bioscience Biotechnology and Biochemistry* **70**, 1521-1523.

Moghadasian, M. H. & Frohlich, J. J. (1999). Effects of dietary phytosterols on cholesterol metabolism and atherosclerosis: clinical and experimental evidence. *Ammerican Journal of Medicine* **107**, 588-594.

Mohan Kumari, H. P., Akhilender Naidu, K., Vishwanatha, S., Narasimhamurthy, K., Vijayalakshmi, G. & (2009). Safety evaluation of *Monascus purpureus* red mould rice in albino rats. *Food and Chemical Toxicology*, 1739-1746.

Moon, J. H. & Terao, J. (1998). Antioxidant effect of caffeeic acid and dihydrocaffeeic acid in lard and human low-density lipoprotein. *Journal of Agricultural and Food Chemistry* **46**, 5062-5065.

Moon, S. H. & Parulekar, S. J. (1991). A parametric study of protein production in batch and fed-batch culture of B. Wrmus *Biotechnology and Bioengineering* **37**, 467-483.

Moreau, R. A., Whitaker, B. D. & Hicks, K. B. (2002). Phytosterols, phytostanols, and their conjugates in foods: structural diversity, quantitative analysis, and health-promoting uses. *Progress in Lipid Research* **41**, 457-500.

Moss, D. W. & Henderson, A. S. (1999). Clinical enzymology. In: Burtis, C.A., Ashwood, E.R. (Eds.), Tietz Textbook of Clinical Chemistry, third ed. W.B. Saunders Company, Philadelphia. 617-721.

Nagasawa, T. & Yamada, H. (1995). Microbial production of commodity chemicals. *Pure and Applied Chemistry* **67**, 1241-1256.

Negishi, S., Huang, Z. C., Hasumu, K., Murakawa, S. & Endo, A. (1986). Productivity of monacolin K (mevinolin) in the genus *Monascus* (in Japanese). *Hakko Kogaku Kaishi* **64**, 584-590.

Nozaki, H., Date, S., Kondo, H., Kiyohara, H., Takaoda, D., Tada, T. & Nakayama, M. (1991). Ankalactone, a new R,â-unsaturated ç-lactone from *Monascus* anka. *Agricultural and Biological Chemisrty* **55**, 899-990.

Ohkawa, H., Ohishi, N. & Yagi, K. (1979). Assay for lipid peroxides in animal tissue by thiobarbituric acid reaction. *Analytical Biochemistry* **95**, 351-358.

Omaye, S. T., Turnbull, J. D. & Sauberlich, H. E. (1973). Selected methods for the determination of ascorbic acid in animal cells, tissues and fluids. *Methods Enzymology* **62** 3-11.

Osterud, B. & Bjorklid, E. (2003). Role of monocytes in atherogenesis. *Physiological Reviews* **83**, 1069-1112.

Oyaizu, M. (1986). Studies on products of browning reactions antioxidative activities of products of browning reaction prepared from glucosamine. *Japanese Journal of Nutrition* **44**, 307-315.

Palo, M. A., Vidal Adeva, L. & Meceda, L. M. (1960). A study on ang-kak and its production. *philippine Journal of science* **89**, 1-19.

Park, J. W., Lee, J. K., Kwon, T. J., Yi, D. H., Kim, Y. J., Moon, S. H., Suh, H. H., Kang, S. M. & Park, Y. I. (2003). Bioconversion of compactin into pravastatin by Streptomyces sp. *Biotechnology Letters* **25**, 1827-1831.

Park, K. B. & Oh, S. H. (2007). Production of yogurt with enhanced levels of gamma-aminobutyric acid and valuable nutrients using lactic acid bacteria and germinated soybean extract. *Bioresoure Technology* **98**, 1675-1679.

Pattanagul, P., Pinthong, R., Phianmongkhol, A. & Tharatha, S. (2008). Mevinolin, citrinin and pigments of adlay angkak fermented by *Monascus* sp. *International Journal of Food Microbiology* **126**, 20-23.

Peters, J. M. & Boyd, E. M. (1966). Organ weights and water levels of the rat following reduced food intake. *Journal of Nutrition* **90**, 354-360.

Pfeiffer, C. J. (1968). A mathematical evaluation of the thymic weight parameter. *Toxicology and Applications Pharmacology* **13**, 220-227.

Piironen, V., Lindsay, D. G., Miettinen, T. A., Toivo, J. & Lampi, A. M. (2000). Plant sterols : biosynthesis, biological function and their importance to human nutrition. *Journal of science and food agriculture* **80**, 939-966.

Pisareva, E., Savov, V. & Kujumdzieva, A. (2005). Pigments and citrinin biosynthesis by fungi belonging to genus *Monascus*. *Zeitschrift Fur Naturforschung C-a* **Journal of Bioscience**, 116-120.

Plat, J. & Mensink, R. P. (2001). Effects of plant sterols and stanols on lipid metabolism and cardiovascular risk. *Nutrition Metabolism and Cardiovascular Disorder* **11**, 31-34.

Pyo, Y. H. & Seong, K. S. (2009). Hypolipidemic effects of *Monascus*-fermented soybean extracts in rats fed a high-fat and -cholesterol diet. *Journal of Agricultural and Food Chemistry* **57**, 8617-8622.

Qin, S., Zhang, W., Qi, P. & other authors (1999a). Elderly patients with primary hyperlipidemia benefited from treatment with a *Monascus purpureus* rice preparation: a placebo-controlled, double-blind clinical trial. In *The 39th Annual Conference on Cardiovascular Disease Epidemiology and Prevention*, pp. 1123. Orlando, Florida, USA.

Raimbault, M. (1998). General and microbiological aspects of solid substrate fermentation. *Electronic Journal of Biotechnology* **3**.

Rasheva, T., Kujumdzieva, A. & Hallet, J.-N. (1997). Lipid production by *Monascus purpureus* albino strain. *Journal of Biotechnology* **56**, 217-224.

Rasheva, T. V., Nedeva, T. S., Hallet, J.-N. & Kujumdzieva, A. (2003). Chareterization of a non-pigment producing *Monascus purpureus* mutant strain. *Antonie Van Leeuwenhoek* **83**, 333-340.

Ratty, A. K., Sunamoto, J. & Das, N. P. (1988). Interaction of flavinoids with 1, 1-Diphenyl-1-picrylhydrazyl free radical, liposomal membrane and soyabean lipoxygenase -1. *Biochemistry and Pharmacology* **37**, 989-995.

Ravi-Kumar, K. & Umesh-Kumar, S. (2006). Bioprocessing of starch using enzyme technology. In *Food Biotechnology*. Edited by K. Shetty, G. Paliyath, A. Pometto & R. E. Levin. Florida: Taylor and Francis Group.

Raza, J. A., Babb, J. D. & Movahed, A. (2004). Optimal management of hyperlipidemia in primary prevention of cardiovascular disease. *International Journal of Cardiology* **97**, 355-366.

Reid, G., Charbonneau, D., Erb, J., Kochanowski, B., Beuerman, D., Poehner, R. & Bruce, A. W. (2003). Oral use of *Lactobacillus rhamnosus* GR-1 and L. fermentum RC-14 significantly alters vaginal flora: randomized, placebo-controlled trial in 64 healthy women. *FEMS Immunology and Medicinal Microbiology* **35**, 131-134.

Reid, G. & Hammond, J. A. (2005). Probiotics. Some evidence of their effectiveness. *Canadian Family Physician* **51**, 1487-1493.

Rhyu, M., Kim, D., Kim, H. & Kim, B. (2000). Nitric oxide-mediated endothelium-dependant relaxation of rat thoracic aorta induced by aqueous extract of red rice fermented with *Monascus ruber*. *Journal of Ethnopharmacology* **70**, 29-34.

Rifai, N., Bachorik, P. S. & Albers, J. J. (1999). Lipids, lipoproteins and apolipoproteins. In: Burtis, C.A., Ashwood, E.R. (Eds.), Tietz Textbook of Clinical Chemistry, third ed. W.B. Saunders Company, Philadelphia. 809-861.

Rippe, J., Bonovich, K., Colfer, H. & other authors (1999).A multicenter, self-controlled study of cholestin in subjects with elevated cholestero. In *The 39th Annual Conference on Cardiovascular Disease Epidemiology and Prevention*, pp. 1123. Orlando, Florida, USA.

Riscaltadi, E., Moresi, M., Federici, F. & Petruccioli, M. (2002). Effect of pH and stirring rate on itaconate production by *Aspergillus terreus*. *Journal of Biotechnology* **83**, 219-230.

Rodriguez Porcel, E. M., Casas Lopez, J. L., Sanchez Perez, J. A. & Chisti, Y. (2007). Enhanced production of lovastatin in a bubble column by Aspergillus terreus using a two-stage feeding strategy *Journal of Chemical Technology and Biotechnology* **82**, 58-64.

Rosenson, R. S. (2004a). Statins in atherosclerosis: lipid-lowering agents with antioxidant capabilities. *Atherosclerosis* **173**, 1-12.

Rosenson, R. S. (2004b). Statins in atherosclerosis: lipid lowering agents with antioxidant capabilities. *Atherosclerosis* **173**, 1-12.

Ross, R. (1993). The pathogenesis of atherosclerosis: a perspective for the 1990s. *Nature* **362**, 801-809.

Sadik Ali, S., Bibhu Prasad, P., Saleem, J. & Mohd, A. (2006). Optimization of nutrient parameters for lovastatin production by *Monascus purpureus* MTCC 369 under submerged fermentation using response surface methodology *Applied Microbilogy and Biotechnology*, DOI 10.1007/s00253-00006-00577-00251.

Salo, P., Wester, I. & Hopia, A. (2003). Phytosterols. In: Lipids for Functional Foods and Nutraceuticals. *Gunstone, FD(ed)* **7**, 183-224.

Sarker, S. A., Sultana, S., Fuchs, G. J., Alam, N. H., Azim, T., Brussow, H. & Hammarstrom, L. (2005). *Lactobacillus paracasei* strain ST11 has no effect on rotavirus but ameliorates the outcome of nonrotavirus diarrhea in children from angladesh. *Pediatrics* **116e**, 221-228.

Sato, K. & Naito, I. (1935). Acids and alcohols as nutrients for *Monascus*. *Journal of Agricultural and Chemical Society, Japan* **11**, 473-479.

Schaefer, E. J. & McNamara, J. (1997). Overview of the diagnosis and treatment of lipid disorders. In: Rifai, N., Warnick, G.R., Dominiczak, M.H. (Eds.), Handbook of Lipoprptein Testing. AACC Press, Washington. 25-48.

Schneweis, I., Meyer, K., Hoermansdorfer, S. & Bauer, J. (2001). Metabolites of *Monascus* ruber in silages. *Journal of Animal Physiology and Animal Nutrition* **85**, 38-44.

Schrezenmeir, J. & de Vrese, M. (2001). Probiotics, prebiotics, and synbiotics-approaching a definition. *American Journal of Clinical Nutrition* **73**, 361-364S.

Searcy, R. L. & Bergquist, L. M. (1960). A new color reaction for quantification of serum cholesterol. *Clinical Chemical Acta* **5**, 192-196.

Sedlock, J. & Lindsay, R. H. (1968). Estimation of total, protein bound and nonprotein sulfhydryl groups in tissues with Ellman's reagent. *Analytical Biochemistry* **25**, 192-205.

Senok, A. C., Ismaeel, A. Y. & Botta, G. A. (2005). Probiotics: facts and myths. *Clinical Microbiology and Infection* **11**, 958-966.

Serizawa, N., Nakagawa, K., Hamano, K., Tsujita, Y., Terahara, A. & Kuwano, H. (1983). Microbial hydroxylation of ML-236B (compactin) and monacolin K (MB-530 B). *Journal of Antibiotic* **36**, 604-607.

Serizawa, N. & Watanabe, I. (1997). Actinomycete promoter. *European Patent* EP0776974 (A0776972)

Sherwin, E. R. (1978). Oxidation and antioxidant in fat and oil processing. *Journal of American Oil Chemical Society* **55**, 809-814.

Sheu, F., Wang, C. L. & Shyu, Y. T. (2000). Fermentation of *Monascus purpureus* on bacterial cellulose-nata and the color stability of M*onascus*-nata complex *Food microbiology and safety* **65**, 342-345.

Shimada, K., Fujikawa, K., Yahara, K. & Nakamura, K. (1992). Antioxiadative properties of xanthan on the autoxidation of soyabean oli in cyclodextrine emulsion. *Journal of Agricultural and Food Chemistry* **40**, 945-948.

Shimizu, T., Kinoshita, H., Ishihara, S., Sakai, K., Nagai, S. & Nihira, T. (2005). Polyketide synthase gene responsible for citrinin biosynthesis in *Monascus purpureus*. *Applied and Environmental Microbiology* **71**, 3453-3457.

Shirley, C. C., Joseph, T. J., Matthew, K., Meijer, G. W., A.Clevidence, B. & David, J. B. (2009). Phytosterol Intake and Dietary Fat Reduction are Independent and Additive in their Ability to Reduce Plasma LDL Cholesterol. *Lipids* **44**, 273-281.

Singh, R. P., Murthy, K. N. C. & Jayaprakasha, G. K. (2002). Studies on the antioxidant activity of Pomogranate (*Punica granatum*) peel and seed extract using in vitro models. *Journal of Agricultural and Food Chemistry* **50**, 81-86.

Singleton, V. L. & Rossi, J. A. (1965). Colorimetry of total phenolics with phosphomolybdic acid reagents. *American Journal of Enology and Viticulture* 144-153.

Smith, J. E. & Berry, D. R. (1975). *The Filamentous fungi - Industrial Mycology*. London: Edward Arnold (Publishers) Limited.

Somashekar, D., Venkateswaran, G., Sambaiah, K. & Lokesh, B. R. (2003). Effect of culture conditions on lipid and gamma linolenic acid production in mucoraceous fungi. *Process Biochemistry* **38**, 1719-1724.

Staunton, J. & Weissman, K. J. (2001). Polyketide biosynthesis: a millennium review. *Natural Proceedings of Reproduction* **18**, 380-416.

Su, N.-W., Lin, L.-Y., Lee, M.-H. & Ho, C.-Y. (2005). Ankaflavin from *Monascus*-fermented red rice exhibits selective cytotoxic effect and induces cell death on Hep G2 cells. *Journal of Agricultural and Food Chemistry* **53**, 1949-1954.

Su, Y.-C., Wang, J.-J., Lin, T.-T. & Pan, T.-M. (2003). Production of the secondary metabolites c-aminobutyric acid and monacolin K by *Monascus*. *Journal of Industrial Microbiology and Biotechnology* **30**, 41-46.

Su, Y. C., Chen, W. L., Fan, H. Y., Weng, H. C. & Wang, W. H. (1970). Microbial study of *Monascus*. *Journal of Chinese Agricultural Chemical Society* **8**, 46-54.

Su, Y. C. & Huang, J. H. (1976). Studies on the production of Anka-pigment. *Journal of Chinese Agricultural Chemical Society* **14**, 45-58.

Su, Y. C. & Wang, W. H. (1977). Chinese red rice-anka. Symposium on Indigenous Fermented Foods. Bangkok, Thailand.

Su, Y. C. (2001). Anka (Red-Koji) products and it's research development In *Symposium on Functional Fermentation Products*, pp. 67-112. Taipei, Taiwan.

Suh, J. & Shin, C. S. (2000). Physiological analysis on novel coculture of *Monascus sp.* J101 with *Saccharomyces cerevisiae*. *FEMS Microbiology Letters* **190**, 241-245.

Sumioka, I., Hayama, M., Shimokawa, Y., Shiraishi, S. & Tokunaga, A. (2006). Lipid-lowering effect of *Monascus* garlic fermented extract (MGFE) in hyperlipidemic subjects. *Hiroshima Journal of Medical Science* **55**, 59-64.

Syed Mubbasher, S., Imran, H. & Syed Dinawaz, A. G. (2003). Estimation of sterols in Edible Fats and Oils. *Pakistan Journal of Nutrition* **2**, 178-181.

Szymanski, H., Pejcz, J., Jawien, M., Chmielarczyk, A., Strus, M. & Heczko, P. B. (2006). Treatment of acute infectious diarrhoea in infants and children with a

mixture of three *Lactobacillus rhamnosus* strains-a randomized, doubleblind, placebo-controlled trial. *Alimentary Pharmacology & Therapeutics* **23**, 247-253.

Taira, J., Miyagi, C. & Aniya, Y. (2002). Dimerumic acid as a antioxidant from the mold, *Monascus anka*; the inhibition mechanism against lipid peroxidation and hemeprotein-mediated oxygen. *Biochemical Pharmacology* **63**, 1019-1026.

Tamara, N., Katarina, J. & Aleksa, C. (1997). Sterols and fatty acids of different *Aspergillus* species. *FEMS Microbiology letters* **149**, 201-205.

Tanganelli, E., Prencipe, L., Bassi, D., Cambiaghi, S. & Murador, E. (1982). Clinical Chemistry. **28**, 1461-1464.

Taylor, G. R. J. & Williams, C. M. (1998). Effects of probiotics and prebiotics on blood lipids. *British Journal of Nitrition* **80(suppl 1)**, S225-230.

Teng, S. S. & Feldheim, W. (2000). The fermentation of rice for anka pigment production. *Journal of Industrial Microbiology and Biotechnology* **25**, 141-146.

Thomas, L. (1998). Clinical Laboratory Diagnostics, first ed. TH-Books Verlagsgesellschaft, Frankfurt. 374-377.

Toshiro, W. & Shigeru, T. (2000). Analysis of natural food pigments by capillary electrophoresis. *Journal of Chromatography* **880**, 311-322.

Trautwein, E. A., Duchateau, G., Lin, Y., Melcnikov, S. M., Molhuizen, H. & Ntanios, F. Y. (2003). Proposed mechanisms of cholesterol-lowering action of plant sterols. *European Journal of Lipid Science and Technology* **105**, 171-185.

Tsai, M. S., Hseu, T. H. & Shen, Y. S. (1978). Purification and characterization of an acid protease from *Monascus kaoliang*. *International Journal of Peptide and Protein Research* **12**, 293-302.

Tseng, Y. Y., Chen, M. T. & Lin, C. F. (2000). Growth, pigment production and protease activity of *Moanscus purpureus* as affected by salt, sodium nitrite, polyphosphate and various sugars. *Journal of Applied Microbiology* **88**, 31-37.

Tsukahara, M., Shinzato, N., Tamaki, Y., Namihira, T. & Matsui, T. (2009). Red yeast rice fermentation by selected *Monascus* sp. with deep-red color, lovastatin production but no citrinin, and effect of temperature-shift cultivation on lovastatin production. *Applied Biochemistry and Biotechnology* **158**, 476-482.

Ueno, H. (2000). Enzymatic and structural aspects on glutamate decarboxylase. *Journal of Molecular Catalysis B: and enzymatic* **10**, 67-79.

Valera, H. R., Gomesa, J., Lakshmi, S., Gururaja, R., Suryanarayan, S. & Kumar, D. (2005). Lovastatin production by solid state fermentation using *Aspergillus flavipes*. *Enzyme and Microbial Technology* **37**, 521-526.

Van Tieghem, M. (1884). *Monascus* genre novea de l'ordre des Ascomycetes. *Bulleten Socitiety Botany France* **31**, 226-231.

Vaya, J. & Aviram, M. (2001). Nutritional antioxiants:mechanisms of action, analysis of activities and medical applications. *Current Medcinal Chemistry Immunology Endocrinology and Metabolic Agents* **18**, 99-117.

Volkovova, K., Dusinska, M. & Collins, A. R. (2006). From oxidative DNA damage to molecular epidemiology. *Journal of Applied Biomedicine* **4**, 39-43.

Walter, G., Carlos, E. & Domenech (1999). A laboratory class experiment to detect and quantify lovastatin in commercial medicaments. *Biochemical Education* **27**, 229-231.

Wang, J.-J., Lee, C.-L. & Pan, T.-M. (2004). Modified mutation method for screening low citrinin-producing strains of *Monascus purpureus* on rice culture. *Journal of Agricultural and Food Chemistry* **52**, 6977-6982.

Wang, J.-J., Shieh, M.-J., Kuo, S.-L., Lee, C.-L. & Pan, T.-M. (2006a). Effect of red mold rice on antifatigue and exercise-related changes in lipid peroxidation in endurance exercise. *Applied Microbiology and Biotechnology* **70**, 247-253.

Wang, J.-J., Shieh, M.-J., Kuo, S.-L., Lee, C.-L. & Pan, T.-M. (2006b). Effect of red mold rice supplements on serum and meat cholesterol levels of broilers chicken. *Applied Microbiology and Biotechnology* **71**, 812-818.

Wang, J., Lu, Z. & Chi, J. (1997). Multicenter clinical trial of the serum lipid-lowering effects of a *Monascus purpureus* (Red Yeast) rice preparation from traditional Chinese medicine. *Current Therapeutic Research, Clinical and Experimental* **58,** 964-978.

Wang, J. J., Lee, C. L. & Pan, T. M. (2003). Improvement of monacolin K, gamma-aminobutyric acid and citrinin production ratio as a function of environmental conditions of *Monascus purpureus* NTU 601. *Journal of Industrial Microbiology and Biotechnology* **30**, 669-676.

Wang, Y.-Z., Ju, X.-L. & Zhou, Y.-G. (2005). The variability of citrinin production in *Monascus* type cultures. *Food Microbiology* **22**, 145-148.

Warnick, G. R. & Albers, J. J. (1978). A comprehensive evaluation of heparin-manganese chloride precipitation procedure for estimating high density lipoprotein cholesterol. *Journal of Lipid Research* **19**, 65-76.

Watanabe, M., Maemura, K., Oki, K., Shiraishi, N., Shibayama, Y. & Katsu, K. (2006). Gamma-aminobutyric acid (GABA) and cell proliferation: focus on cancer cells. *Histology and Histopathology* **21**, 1135-1141.

Went, F. A. F. C. (1895). *Monascus purpureus*, Le champignon de l 'angquac, une nouvelle thelebolee. *Annals of Science Natural Botany Series* **81**, 1-18.

Wong, C. & Koehler, E. P. (1981). Production and isolation of an antibiotic from *Monascus purpureus* and its relationship to pigment production. *Journal of Food Science* **46**, 589-592.

Wong, H. C. & Bau, Y. S. (1977). Pigmentation and antibacterial activity of fast neutron- and X-ray-induced strains of *Monascus purpureus* Went. *Plant Physiology* **60**, 578-581.

Wu, C. L., Lee, C. L. & Pan, T. M. (2009). Red mold dioscorea has a greater antihypertensive effect than traditional red mold rice in spontaneously hypertensive rats. *Journal of Agricultural Food Chemistry* **57**, 5035-5041.

Wu, W.-T., Wang, P.-M., Chang, Y.-Y., Huang, T.-K. & Chien, Y.-H. (2000). Suspended rice particles for cultivation of *Monascus purpureus* in a tower-type bioreactor. *Applied Microbiology and Biotechnology* **53**, 542-544.

Yagi, K. (1984). Assay of lipid peroxides in blood plasma or serum. *Methods Enzymology* **105**, 328-331.

Yasukawa, K., Takahashi, M., Natori, S., Yamazaki, M., Takeuchi, M. & Takido, M. (1994). Azaphilones inhibit tumor promotion by 12-o-tetradecanoyl-phorbol-13-acetate. *Oncology* **45**, 108-112.

Ykema, A., Streekstra, H. & Luiten, R. G. M. (1999). Statin production by fermentation. In *International Patent WO/1999/010499*.

Young, E. M. (1931). The morphology and cytology of *Monascus ruber*. *American Journal of Botany* **7**, 499-517.