# CHARACTERIZATION OF BIOACTIVE MOLECULES FROM *MONASCUS PURPUREUS* FERMENTED FINGER MILLET (*ELEUSINE CORACANA*)

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Biotechnology

By

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(MAY -2010)

# DECLARATION

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I hereby declare that the thesis entitled **"Characterization of bioactive molecules from *Monascus purpureus* fermented finger millet (*Eleusine coracana*)"** 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 Food Microbiology Department, Central Food Technological Research institute (CSIR), Mysore, under the guidance of **Dr. G. Vijayalakshmi**, Scientist-F and Deputy Director during the period of February 2008 to May 2010.

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

**Place:** Mysore

**Date:** **(VENKATESWARAN, V.)**

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Place: Mysore (Venkateswaran, V.)

Date:

# DEDICATED TO MY BELOVED FATHER (LATE) AND OTHER FAMILY MEMBERS

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Date: 31st May 2010

Place: Mysore

# CERTIFICATE

I hereby certify that this thesis entitled **"Characterization of bioactive molecules from *Monascus purpureus* fermented finger millet (*Eleusine coracana*)"** submitted by **Shri Venkateswaran, V.** for the award of Doctor of Philosophy in Biotechnology to the University of Mysore, is the result of research work carried out by him in the Food Microbiology Department, Central Food Technological Research Institute, Mysore, under my guidance during the period February 2008 to May 2010.

(G.Vijayalakshmi )
Guide

# INTRODUCTION

# MATERIALS AND METHODS

# CHAPTER 1

## CHARACTERIZATION OF BIOACTIVE MOLECULES (STATIN AND GABA) PRODUCED BY *MONASCUS PURPUREUS* (MTCC- 410) DURING CEREAL AND MILLET FERMENTATION

# CHAPTER -2

## OPTIMIZATION OF CULTURAL CONDITIONS FOR STATIN AND GABA PRODUCTION

# CHAPTER 3

## CHARACTERIZATION OF *MONASCUS PURPUREUS* AND MUTANT IN RELATION TO STATIN AND GABA PRODUCTION

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# THE PROBLEM

# DISCUSSION

# SUMMARY

# INTRODUCTION

Microorganisms have been exploited for the past hundred years for the production of several industrial products. Notably among them are vast array of antibiotics, therapeutic compounds and several fine chemicals like vitamins. Fungi and bacteria have been used in the industrial production of drugs, organic acids and food enzymes.

Many important industrial products (chemicals, pharmaceuticals, etc.) are now produced from fungi using fermentation technology. A wide range of enzymes are excreted by fungi and play an important role in the breakdown of organic materials; many of these enzymes are now produced commercially. Most are used in food processing, however, new applications are being found all the time.

Fungi are well known as a source of antibiotics but new therapeutic compounds with novel pharmacological activities have also been developed in recent years. One such example is the cyclosporins first isolated from *Tolypocladium inflatum* in 1976 as antifungal compounds and later shown to possess immunosuppressive activity. Cyclosporin A is currently the most widely used drug for preventing rejection of human organ transplants (note: 20 years later the sexual stage of *T. inflatum* was found to be *Cordyceps subsessilis*).

*Monascus purpureus* is traditionally known as red yeast rice in East Asia particularly in China and Japan. Red yeast rice has been used in China for centuries as both food as well as medicine. In addition to its culinary use, red yeast rice is also used in traditional Chinese herbology and traditional Chinese medicine. Its use has been documented as far back as the Tang Dynasty in China in 800 A.D. Red yeast rice has been used in China as a preservative, spice, and food colourant. Its characteristic red color was used as an ingredient in fish sauce, fish paste, and

rice wine. The other names of red yeast rice are Red fermented rice, Red kojic rice, Anka, or Ang-kak., Corn silage mold, Maize silage mold (Young, 1930). In addition, this dietary supplement has been used traditionally for treating bruised muscles, hangovers, indigestion, and colic in infants. Recently, it has been discovered that red yeast rice contains substances that are similar to prescription medications that lower cholesterol. Red yeast rice's importance is also enhanced because of its use as natural food colourants and in preservation of food. This fungus is most important because of its use, in the form of red yeast rice, in the production of certain fermented foods in China. However, discoveries of cholesterol-lowering statins produced by the mold have prompted research into its possible medical uses.

## CLASSIFICATION OF *MONASCUS PURPUREUS*

The taxonomy of *Monascus* was revised as a result of an investigation of cultural and microscopic characters of type and numerous other isolates. The red mold was first investigated by a French microbiologist, Van Tieghem during 1884. Three species are recognized: *M. pilosus* K. Sato ex D. Hawksw. & Pitt sp. nov.: *M. purpureus* Went; *M. ruber* van Tieghem (Hawksworth and Pitt 1983). *Monascus purpureus* was placed in the below furnished taxonomic position (Went, 1895). The species of *Monascus* are presented in **Table 1**.

**Kingdom : Fungi**
**Division : Ascomycota**
**Class : Eurotiomycetidae**
**Order : *Incertae sedis***
**Family : Monascaceae**
**Genus : *Monascus***
**Species : *purpureus***

**Table 1. Strains of *Monascus* spp.**

| S. No. | Species | Strains of *Monascus* |
|---|---|---|
| 1 | purpureus | *Albidus, Anka, Araneosus, Kaoling, Major, Purpureus, Rubiginosus*<br>*Albidus var. glaber, Anka var. rubellus* |
| 2 | pilosus | *Pilosus, Pubigerus, Rubropunctatus, Serorubescens* |
| 3 | ruber | *Ruber, Vitreus, Barkeri, Fuliginosus, Paxii* |

## MORPHOLOGY OF *MONASCUS PURPUREUS*

*Monascus sp.* is homothallic, and its life cycle is composed of asexual propagation accompanied by one-celled conidia and sexual propagation by ascospores in asci. The asci are found inside the fruiting body called cleistothecia which develop when antheridia mate with ascogonia. (Barker 1903; Olive 1905). Asexual sporulation is a common reproductive mode in *Monascus* species. Sexual reproduction, which produces ascospores, has also been observed in submerged cultures. Pigments seem to be produced more when conidia are inhibited. Therefore, a *Monascus* sp. propagating sexually more often than asexually, is probably effective for the hyper production of pigments. The growth of *Monascus purpureus* MTCC 410 cultivated on thin layer of potato dextrose broth is presented in **Figure 1**.

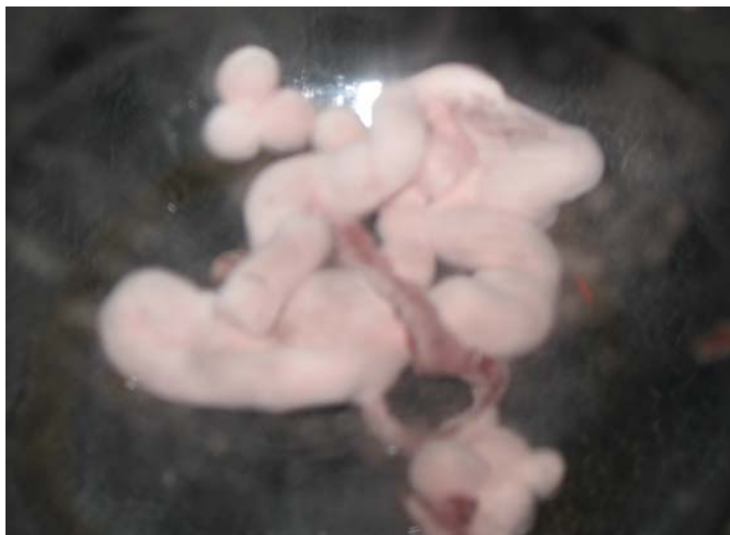


**Figure 1. Growth of *Monascus purpureus* MTCC 410 cultivated on thin layer of potato dextrose broth**

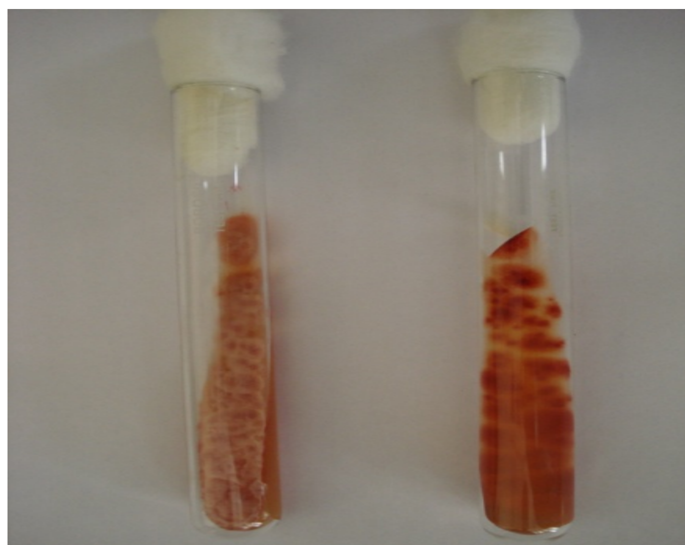


**Figure 2. *Monascus purpureus* growth on PDA slants**

## COLONY MORPHOLOGY

The colonies of *Monascus purpureus* MTCC 410 on potato-dextrose-agar (PDA) after 7 days are 20 to 30 mm in diameter, plane, eventually with small aerial development, sparse, with flocculent superficial texture, mycelium initially white (1 to 2 days), turning to orange and then to brick red as the culture develops, with formation of cleistothecia and aleurioconidia. Usually the soluble pigments formed diffuse through the agar **(Figures 2 and 3)**. The cleistothecia are spherical, from 30 to 60 μm in diameter, formed as a hyphal knot from a well defined stalk, with cell walls turning to brown with maturation; ascospores are ellipsoid, hyaline, 5-7 x 4-4,5 μm in size, with a smooth cell wall.

**Figure 3. Colony morphology of *Monascus purpureus* MTCC 410 on potato dextrose agar**

## METABOLITES AND BIOACTIVE COMPOUNDS

*Monascus purpureus*, the ascomycete fungus is highly important in relation to its metabolites production, which are used in health promotion of human beings. The pigment of the

mould Monascus spp. belongs to the polyketides and it exhibits mild bactericidal effect. The group of pigments includes the orange pigments -Monasorubin and Rubropunctatin, the yellow pigments - Monascin and Ankaflavin, the red pigments- Monascorubramin and Rubropunctamin (Meyer 1990; Margalith 1992). A *Monascus purpureus* mutant stain YLC1 produces yellow pigment alone (Slugen *et al*. 1997).

*Monascus purpureus* is normally cultivated on cooked rice to produce a range of secondary metabolite, statin *viz.* lovastatin, monacolin J, pravastatin and mevastatin (Manzoni et al 1999) known as Monacolins. These metabolites inhibit the enzymatic conversion of hydroxymethyl-glutarate to mevalonate by HMG- CoA reductase, which is the important step in the biosynthetic pathway of cholesterol (Manzoni and Rollini 2002; Heber et al 1999). Statin inhibits the action of HMG-CoA reductase by its the structural similarity with the substrate, hydroxymethyl-glutarate. Statin is commonly used as a medicine in the therapy of hypercholesterolemia (Chen and Johns 1993).

*Monascus* fermented food product contains many other synergistic nutrients with lipid-lowering properties in addition to monacolins. For example, red yeast rice 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 and Frohlich, 1999)**.** Red yeast rice also contain unsaturated fatty acids such as oleic, linoleic, and linolenic acids (Ma *et al.* 2000) 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.

Gamma amino butyric acid (GABA), antihypertensive agent is also known to be produced by *Monascus purpureus* (Rhyu *et al*. 2002). Antibacterial properties of *Monascus*

pigments were first mentioned by Wong and Bau (1977). The culture filtrate of *Monascus purpureus* is known to contain antioxidant and antibacterial principles. Red yeast rice also contains angiotensin I –converting enzyme inhibitory peptides (Kuba *et al*. 2009) and antioxidant compound (Dhale *et al*. 2007). In red fermented rice (angkak, red koji) obtained as cultures of *M. purpureus* DSM1379 and DSM1603, two compounds with identical UV absorption spectra and maxima at 306-307 nm were detected. They were isolated by HPLC, and their structures were elucidated by intensive MS and NMR studies. Monascopyridine A contains a *ç*-lactone, propenyl group, hexanoyl side chain, and a pyridine ring, whereas the more lipophilic compound, monascopyridine B, is a higher homologue of monascopyridine A with the more lipophilic octanoyl instead of the hexanoyl side chain. This is the first report of *Monascus* metabolites with a pyridine ring (Wild *et al*. 2003).

Red yeast rice obtained from cultures of *Monascus* AS3.4444 on rice is known to produce two strong blue fluorescent new *Monascus* metabolites, monasfluore A and monasfluore B, with similar fluorescence spectra (Huang *et al*. 2008). Aqueous extract of fermented rice at two dose levels showed a significant decrease in fasting blood glucose level. The total cholesterol and triglycerides were also significantly reduced where as the HDL cholesterol levels were significantly increased, which confirmed the potent anti-diabetic property of the *Monascus* fermented rice in diabetic rats, which may be due to presence of statins (Rajasekaran et al. 2009). A new compound, tetralone monaspurpurone isolated from the ethanol extract of a yellow mutant of the fungus *Monascus purpureus* BCRC 38113 grown on rice is with a strong antioxidant property (Cheng *et al*. 2009).

*Monascus spp*. have been utilised for making fermented food and preserving meat for hundreds of years. Various secondary metabolites useful as food additives and/or pharmaceuticals have reported being produced by *Monascus spp*. (Ma *et al,* 2000).

## CULTURING OF *MONASCUS PURPUREUS*

*Monascus purpureus* is capable of growing in both solid state and submerged fermentation and produces bioactive metabolites.

## SOLID STATE FERMENTATION

The contemporary method of *Monascus* fermented rice (MFR) mass production is still by the traditional solid-state fermentation on cooked whole rice kernel. Solid state fermentation gives a higher yield and productivity of pigment than liquid fermentation, but usually needs complex media, which are difficult to use for the elucidation of metabolic pathways and other factors during growth.

Successful productions of MFR are often determined by the following factors: the type of substrates (predominantly non-glutinous rice kernel), type of selected *Monascus* strains, temperature and moisture content of the fermentation mixture thorough the process and control of contamination factors (Su 2001). Until monacolins and GABA were reported in these products, good production quality of MFR was long being regarded as higher pigment accumulation in the fermentation mixture.

Humidity plays a major role in the growth of the *Monascus* in solid state fermentation. The water content during MFR production is controlled so as to maintain the optimal substrate humidity approximately 40–50% at initial and maintained by temporarily moistening the

substrates in favor of fungal growth (Hesseltine 1965; Su and Wang 1977). Recent studies suggested that lower initial moisture content (25–30%) helps to keep a low glucoamylase activity so as to enhance the pigment yields (Lotong and Suwanarit 1990). The ideal humidity for the growth in solid substrates is around 56 – 60 %, with pH 6 (Johns 1991). However cultivation of a mutant strain *M. pilosus* M12-69 is known to yield 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 and Feldheim 2000). In the Chinese ancient process, sufficient aeration is achievable by stirring the fermentation mixture on bamboo trays every 2 h to separate grains from agglomerates. The separation is substantially carried out in laboratory scale by shaking the substrates in flasks or dissipating in plastic bags. The koji maker provides a perforated bottom plate for up-flow aeration and a plowing mixer for assisting heat removal and preventing koji agglomeration was used in Red yeast rice large scale production (Chiu et al. 2006).

Wang *et al.* 2003 has reported that the culture of M. *purpureus* NTU 601, with the addition of 0.5% ethanol as the carbon source tripled the monacolin K content, elevated the GABA production to sevenfold, and reduced the citrinin content. 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). For koji (mold rice) with high lovastatin production, separation of the growth phase and lovastatin production phase by shifting the temperature from 30 to 23 °C increased lovastatin production by nearly 20 times compared to temperature-constant cultivation. In addition, citrinin was not produced even in the lovastatin production phase, although the pigment was increased.

With temperature-shift cultivation, 225 µg lovastatin/g dry koji was reported in 14 days without citrinin (Tsukahara *et al*.2009).

The fermentation of rice with *Monascus purpureus* for three weeks was known to produce higher GABA concentration but required longer production period and red yeast rice was obtained as product. On the other hand, the germination method yielded rice grains with lower GABA but in more suitable form for consumption. Both methods are considered to be economical and efficient methods to increase GABA in rice grains, providing alternative products with higher nutritional values (Jannoey *et al*. 2009). *Monascus pilosus* NBRC4520 was selected for functional fermented food inoculation for its high lovastatin and low citrinin production with a deep-red color.

Pyo (2007) reported that soybean fermented with the fungus *Monascus pilosus* 'KFRI 1140' yielded 2.94 mg mevinolins per gram of dry weight. *Monascus pilosus* 'M12-69' when grown under SSF with rice as substrate, inoculum level of 5 ml spore suspension and incubation temperature of 30°C yielded 2.52 mg monocolin K per gram of substrate (Chen and Hu 2005). *Monascus pupureus* 'NTU601' was grown on SSF with rice as substrates, the production of monocolin K at 30 °C was 530 mg/kg (Wang *et al.* 2003). Su *et al*. (2003) reported that *Monascus pupureus* 'CCRC 31615' exhibited monocolin K yield of 378 mg / kg under SSF with the long grain rice. *Monascus purpureus* 'NTU 301' with *Dioscorea* as the substrate has resulted in 2584 mg monocolin k which is 5.37 times more when rice is used as a substrate (Lee et al 2006). Fermentation using cassava and derivatives in solid substrate fermentation is an alternative to the use of rice (Carvalho, 2001). Solid state fermentation was conducted in a 250ml Erlenmeyer flask at 30°C for 14 days with initial moisture content of 40% and inoculum size of 10% active culture. Barley, long grain rice and sago starch were found to be the suitable

substrates producing maximum lovastatin of 193.7 mg, 190.2 mg and 180.9 mg/g of dry solids (Subhagar *et al.* 2009).

## SUBMERGED FERMENTATION

The production of *Monascus* secondary metabolites by liquid/submerged fermentation has been extensively studied. Various culturing parameters including water supplement, temperature, nitrogen source, medium components, and pH value have been investigated (McHan & Johnson 1970). Lin (1973) was the first to study liquid culture conditions on the pigment production by *Monascus*. The cultural conditions for maximum pigmentation were found to be 5% rice powder (with 3.5% starch content) as the carbon source, 0.5% of sodium nitrate or potassium nitrate as the nitrogen source, initial pH of 6.0, and a temperature of 32 °C. Su and Huang (1976) reported that polished rice powder gave higher pigment productions and than rice starch, which indicates that the minor compositions in rice kernel could benefit fungal growth and pigmentation. Corn powder yielded higher fungal dry weight than rice powder but with poor pigment production. They also suggested that the addition of 1–2% alcohol during incubation has a favorable effect on the pigment production**.** Carels and Shepherd (1977) investigated the effect of different nitrogen sources on the end culture acidity and the pigment production.

During cultivation, the initial pH was around 6.5 when yeast extract or nitrate was used as the nitrogen source, and red pigments were formed, whereas when using ammonium or ammonium nitrate, the pH was around 2.5, and the pigments produced were orange. A lower pH

value (pH 4.0) promoted the fungal growth and favored the synthesis of ankaflavin however; the production of other pigments is relatively non susceptible to pH value.

Oxygen concentration in the submerged cultivation also affects the biosynthesis of *Monascus* metabolites (Hajjaj *et al.* 2000). In oxygen-limiting incubation, production of pigments and citrinin is growth-related and are both biosynthesized as primary metabolites. Under oxygen-excess condition, however, citrinin is produced as a secondary metabolite, which is mostly produced during the stationary phase. In contrast, the pigments decrease dramatically during the incubation. The formation of the pigments is partially inhibited by metabolites produced in aerobic environments such as L-maltose, succinate, and dicarboxylic acid; however, the formation of citrinin is not affected. Mohamed *et al*. (2009) reported the possibility of using a novel proximity type half-pitched, double-flight helical ribbon impeller (HRI) for the improvement of red pigment production by *Monascus purpureus* FTC5391 in a 2-L stirred-tank fermenter (STF).

Submerged fermentation using a gelatinized cassava starch block, in an attempt to increase the concentration of carbohydrates using this substrate, which is cheap and colorless. Using the starch as a block, there is plenty of carbon source for the microorganism, but it does not increase the medium viscosity with free starch, thus not compromising oxygen transfer known to be necessary (Lee, 1995). *Monascus pupureus* ‘MTCC 369’ when grown on synthetic medium under shake flask culures yielded lovastatin of 351 g/l (Sayyad *et al.* 2007).

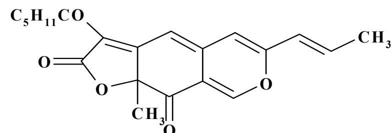
## BIOACTIVE MOLECULES FROM *MONASCUS PURPUREUS*

## PIGMENTS

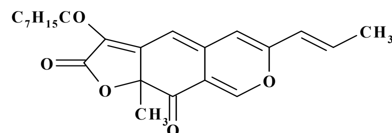
The main primary metabolites produced by *Monascus* are polyketides (Chen *et al.* 1973) are formed by the condensation of one acetyl CoA with one or more malonylcoA with a simultaneous decarboxylation as in the case of lipidic synthesis.

*Monascus* pigments are a group of fungal metabolites called azaphilones, which have similar molecular structures as well as similar chemical properties. Ankaflavine and monascine are yellow pigments, rubropunctatine and monascorubrine are orange and rubropunctamine and monascorubramine are purple. The same colour exists in two molecular structures differing in the length of the aliphatic chain. These pigments are produced mainly in the cell-bound state. They have low water solubility, are sensitive to heat, unstable in the pH range of 2–10 and fade with light.

**ORANGE PIGMENTS**

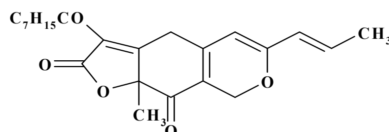



Rubropunctatin M = 354

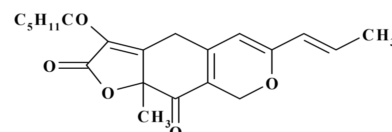



Monascorubin M = 382

**YELLOW PIGMENTS**

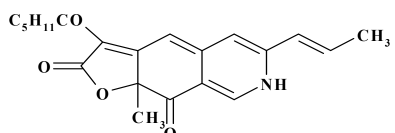



Ankaflavine M = 386

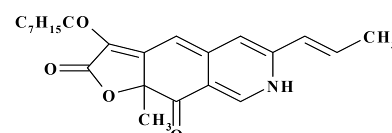



Monascine M = 358

**RED PIGMENTS**




Rubropunctamine M = 353




Monascorubramine M = 381

**Figure 4. Biochemical structure of *Monascus* pigments**

A number of methods have been developed in order to make water-soluble pigments. The principle is the substitution of the replaceable oxygen in monascorubrine or rubropunctatine by nitrogen of the amino group of various compounds such as aminoacids, peptides and proteins, changing the colour from orange to purple **(Figure 4)**. *Monascus* pigments can be reduced, oxidized and react with other products, especially amino acids, to form various derivative roducts sometimes called the complexed pigments. Glutamyl-monascorubrine and glutamyl-rubropunctatine **(Figure 6)** were isolated from the broth of a submerged culture (Blanc *et al.* 1995a). A yellowish colour pigment viz. Xanthomonasin A was identified in *Monascus anka* mutant (Martinkova *et al.* 1999). The biosynthesis of polyketide is presented in **Figure 5.**

$HO\text{-}C(=O)\text{-}CH_2\text{-}C(=O)\text{-}CoA$ + $CH_3\text{-}C(=O)\text{-}CoA$

(Malonyl-CoA) (Acetyl-CoA)

$CO2$

$CH_3\text{-}C(=O)\text{-}CH_2\text{-}C(=O)\text{-}CoA$

(Acetoacetyl-CoA)

Malonyl-CoA

repeat

$CO_2$+CoA

$CH_3\text{-}C(=O)\text{-}(CH_2\text{-}C(=O))_n\text{-}CoA$ (Polyketide)

Further modification (unknown)

Fatty acid biosynthesis

(4 $CH_3Co\text{-}SCoA$)

Hexanonate($C_6$) or

Octanoate ($C_8$)

**Figure 5. Biosynthesis of polyketide pigments in *M. purpureus* (Juzlova *et al.* 1996)**

**Glutamyl-monascorubrine M = 483** **Glutamylmonascorubrine M = 511**

**Figure 6. Structures of the complex pigments of *Monascus***

Stability of the pigments is affected by acidity, temperature, light, oxygen, water activity and time. It was shown that these pigments added to sausages or canned pates remained stable for 3 months' storage at 48 °C, while their stability ranged from 92 to 98% (Fabre *et al.* 1993). Thus, the main objective is focused on the solubilization, the stability and the extraction in solution of pigments. The pigments can easily react with amino group-containing compounds in the medium such as proteins, amino acids, and nucleic acids, to form water-soluble pigments. The synthesis of red pigment by Schiff's base reaction is presented in **Figure 7.**

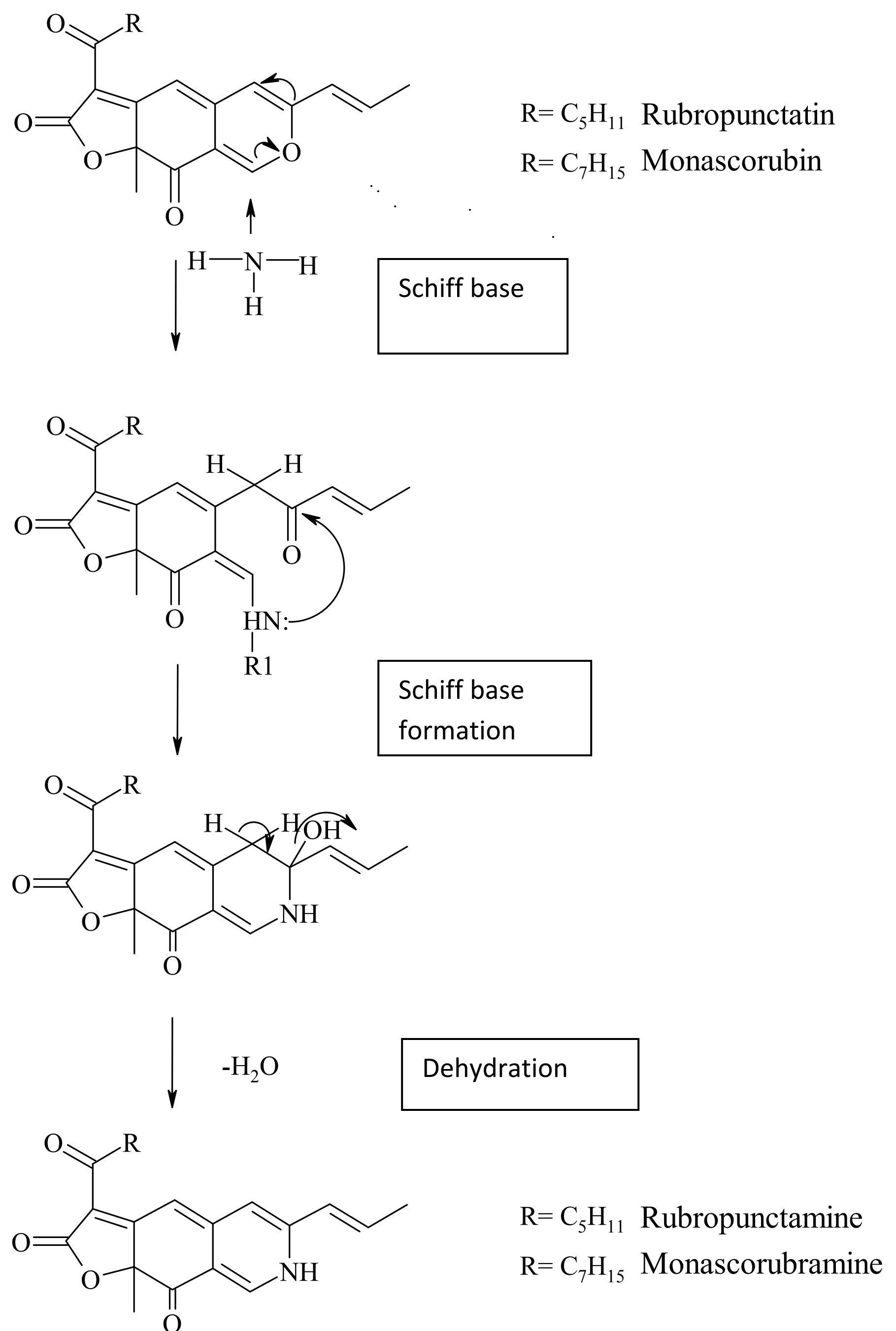



**Figure 7. Synthesis of red pigments by Schiff base reaction (Juzlova *et al.* 1996)**

The orange pigments, monascorubrin and rubropunctatin, are synthesized in the cytosol from acetyl coenzyme A, by the multienzyme complex of polyketide synthase I (Hopwood & Sherman, 1990). These compounds possess a unique structure responsible for their high affinity to compounds with primary amino groups (so called aminophiles). Reactions with amino acids yield the water-soluble red pigments, monascorubramine and rubropunctamine (Blanc *et al.* 1994*;* Lin *et al.* 1992).

The mechanism of formation of the yellow pigments, ankaflavin and monascin, has not yet been elucidated. Carels and Shepherd (1997) supposed that these compounds originated from chemical oxidation of monascorubrin and rubropunctatin. However, their structures strongly suggest that the yellow pigments are reduced derivatives of the orange ones. Thus, the suggestion of Yongsmith *et al.* (1993), that ankaflavin and monascin have their own biosynthetic pathway seems to be more probable.

Huang *et al.*(2008) has reported the production of the two new pigment compounds, namely monasfluore A and monasfluore B, respectively from Red Yeast Rice which are fluorescent in nature.

**STATINS (MONOCOLINS)**

Different types of statins are currently available: the natural statins (lovastatin and pravastatin), obtained directly by fermentation, and the semi-synthetic (simvastatin) and synthetic statins (atorvastatin and fluvastatin). 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).

Statins are fungal secondary metabolites which inhibit the enzymatic conversion of hydroxymethyl-glutarate to mevalonate by HMG- CoA reductase, which is the important step in the biosynthetic pathway of cholesterol (Alberts 1988**;** Heber *et al.* 1999; Manzoni & Rollini 2002). Statin inhibits the action of HMG-CoA reductase by its the structural similarity with the substrate, hydroxymethyl-glutarate. Statin is commonly used as a medicine in the therapy of hypercholesterolemia (Chen and Johns 1993).

Natural statins are of very similar chemical structure. They possess a common main polyketide portion, a hydroxy- hexahydro naphthalene ring system **(Figure 8)**, to which different side chains are linked at C8 (R1) and C6 (R2) **(Figure 9)**. 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 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. The structures of the synthetic statins atorvastatin (Lipitor, Parke-Davis), fluvastatin (Lescol, Novartis), and cerivastatin (Baycol and Lipobay, Bayer) are dissimilar, and quite different from the natural statins. Only the HMG CoA-like moiety, responsible for HMG-CoA reductase inhibition, is common to both natural and synthetic statins. Unlike lovastatin and simvastatin, synthetic statins are obtained in hydroxy acid form. Fluvastatin (Levy *et al.* 1993) derived from mevalolactone, was the first entirely synthetic statin available, while atorvastatin (Bakker-Arkema *et al.* 1996) and cerivastatin, pyridine derivatives, are a new generation of highly purified statins **(Figure 10)**. As on date, 18 monacolin metabolites have been identified from *Monascus sp.* Fourteen monacolins which includes monacolin K (mevinolin, lovastatin), J, L, M, X, and their corresponding hydroxyl acid forms.

Dehydromonacolin K, dihydromonacolin L, compactin and 3-hydroxy-3,5-dihydromonacolin L were also identified in red rice (Ma *et al.* 2000; Li *et al.* 2004). Dihydromonacolin-MV and dehydromonacolin-MV2 were isolated from Monascus purpureus and its mutant from this laboratory. They are characteristic for their strong antioxidant and bactericidal properties (Dhale *et al.* 2007a: Dhale *et al.* 2007b).

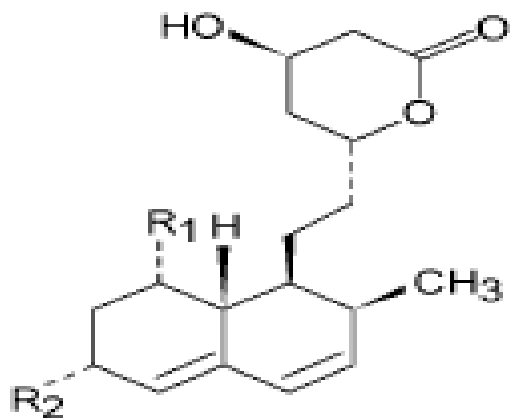



**Figure 8. Basic Hydroxy- hexahydro naphthalene ring structure**

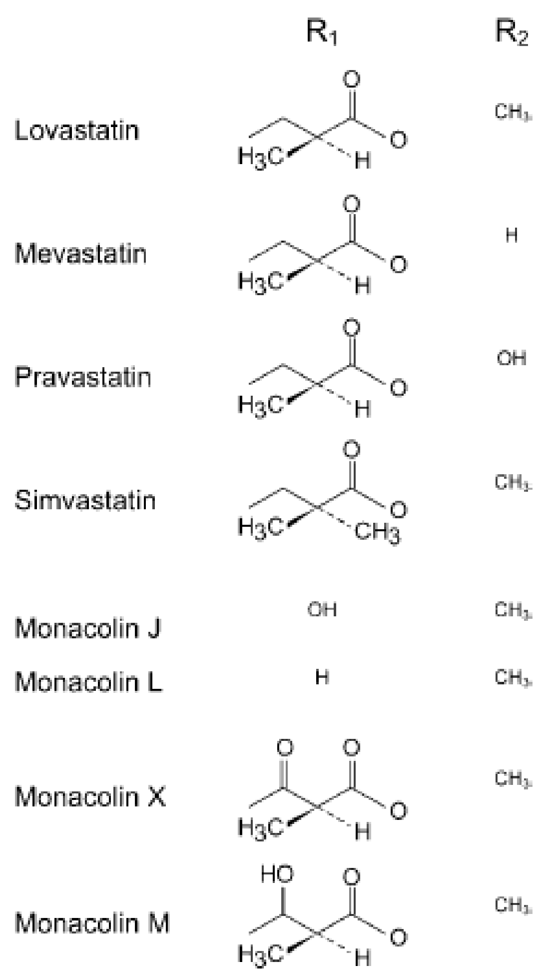



**Figure 9. Statin side chains linked at C8 (R1) and C6 (R2)**

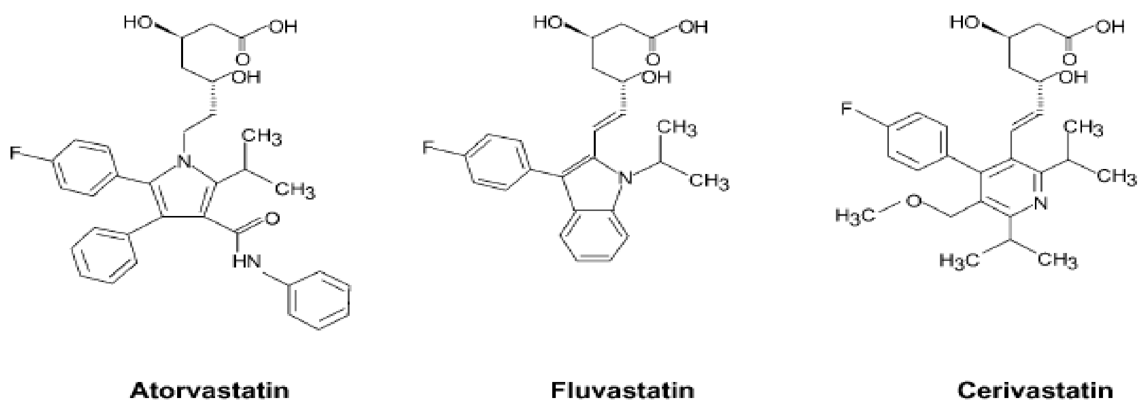



**Figure 10. Structures of synthetic statins**

Endo *et al*. (1976) described a process for the production and purification of mevastatin from *Penicillium citrinum.* After this, lovastatin was obtained from the culture of *Monascus ruber* (Negishi *et al.* 1986) and in 1980 an industrial process for its production was set up using *Aspergillus terreus* which yielded nearly 180 mg lovastatin/l, glycerol being the carbon source in a fed batch culture (Buckland *et al.* 1989).

## BIOSYNTHETIC PATHWAY OF CHOLESTEROL

Cholesterol, a steroid molecule, is an essential component of cell membranes and represents the substrate for the biosynthesis of bile acids. It is essential for adsorption of fats from the intestine and fat soluble vitamins (Alberts, 1988). Cholesterol is also involved in steroid hormone biosynthesis and is required for the production, in hepatocytes of very low-density lipoproteins (VLDL), which are responsible for the transport of fats to peripheral tissues, for subsequent metabolism or storage (Alberts 1988; Alberts *et al.* 1980).

The cholesterol biosynthetic pathway, starting from acetyl-CoA units, involves more than 25 enzymes, but the rate-limiting step is the conversion of 3-hydroxy-3- methylglutaryl-coenzyme A (HMG-CoA) to mevalonate by HMG-CoA reductase **(Figure 11)**.

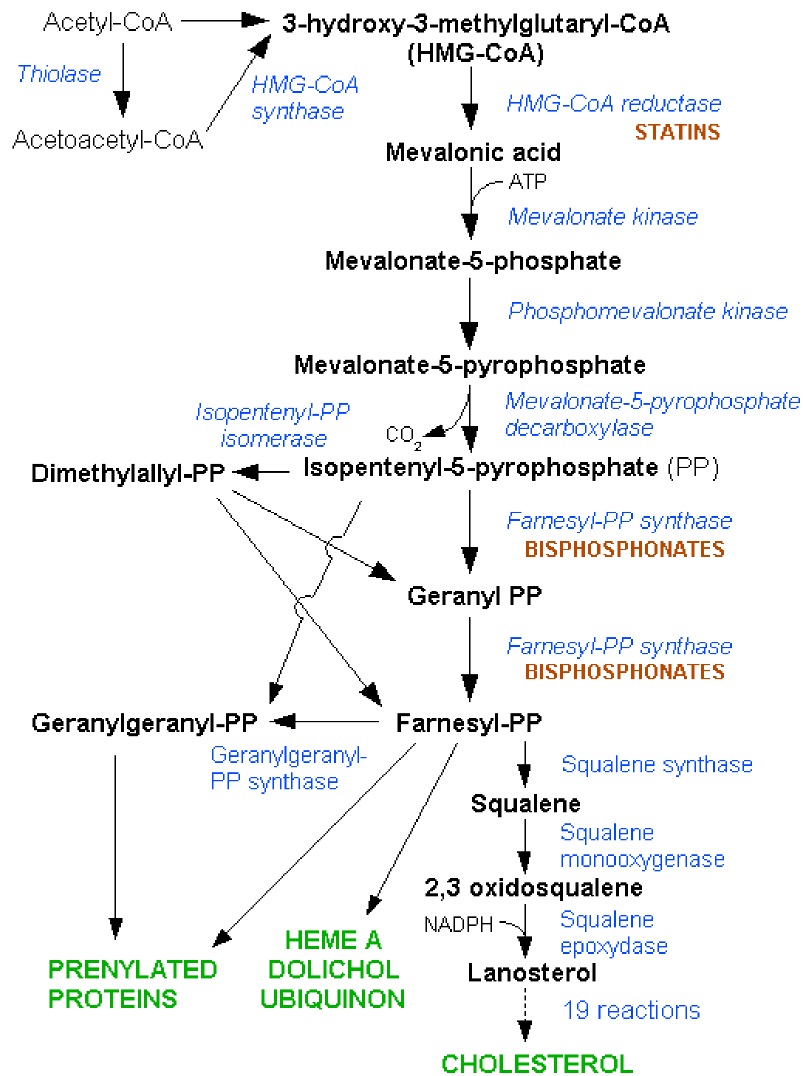



**Figure 11. Cholesterol biosynthetic pathway**

Squalene synthase is the first pathway-specific enzyme and catalyzes head-to-head condensation of two molecules of farnesyl pyrophosphate (FPP) to form squalene. Squalene is converted to cholesterol after a series of steps.

## MODE OF ACTION OF STATINS

The acid forms of statins are structurally similar to the substrate HMG-CoA up on which HMG-CoA reductase enzyme acts in the biosynthetic pathway of cholesterol. These statins remove HMG-CoA from the micelle, where HMG-CoA reductase enzyme acts, there by inhibits the enzyme action which subsequently affects cholesterol formation.

Mevinolin, compactin and their derivatives obtained by chemical modifications (pravastatin, simvastatin) have provided a new mode of therapy for hypercholesterolemia a disease characterized by an elevated plasma concentration of the low density lipoprotein (LDL)/cholesterol complex. The microsome enzyme 3-hydroxy-3-methylglutarylcoenzyme A (HMG-CoA) reductase catalyzes an early step in cholesterogenesis, ie the reduction of HMG-CoA to mevalonic acid. The specific inhibitory effect of mevinolin on this enzyme is caused by a structural relation between the 5-carbon hydroxy acid fragment of mevinolin and HMG-CoA (Nakamura and Abeles 1985).

Mevinolin can suppress tumor growth *in vivo* owing to its capability to inhibit the synthesis of nonsterol isoprenoid compounds such as dolichol, ubiquinone and isopentenyl-tRNA. Mevinolin and related compounds also shows as potential therapeutic agents for the treatment of various types of tumors (Maltese *et al.* 1985). Lovastatin and related compounds, with the exception of pravastatin, are produced as predrugs, being a mixture of the lactone and the β-hydroxyacid form. The lactone ring is converted into the corresponding β-hydroxyacid form in vivo (Alberts *et al.* 1980). The mechanism involved in the hypocholesterolemic activity of statins is based on the competitive inhibition of HMG-CoA reductase, due to the structural homology between the β-hydroxyacid form of the statins and the HMG-CoA intermediate

formed **(Figure 12)**. The affinity of the inhibitor (statins) is several times higher with respect to the intermediate (Alberts *et al.* 1980; Alberts, 1988).

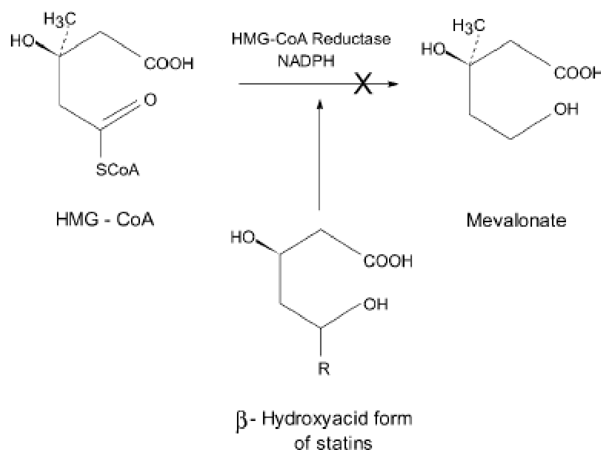



**Figure 12. Structural analogy between HMG-CoA and the Hydroxyacid form of statins and mechanism of inhibition**

Comparative kinetic analysis of HMG-CoA reductase has shown that the methyl group in lovastatin in the 6α- position confers a two- to threefold enhancement of the intrinsic inhibitory activity with respect to mevastatin (Alberts et al. 1980). The crystal structures of the catalytic portion of human HMG-CoA reductase in complex with some substrates and products (HMG-CoA, HMG, CoA, and NADPH) provide a detailed view of the enzyme active site (Istvan and Deisenhofer 2000; Istvan *et al.* 2000). The structures of the catalytic portion of HMG-CoA reductase complexed with six different statins [mevastatin, simvastatin, fluvastatin, atorvastatin, cerivastatin, (withdrawn in August 2001), and rosuvastatin (in the late stage of clinical development) were recently described. The statins occupy a portion of the HMG-CoA binding site, thus blocking substrate access to the active site of the enzyme. The tight binding of statins is

probably due to the large number of van der Waals interactions between inhibitors and HMG-CoA reductase (Istvan and Deisenhofer 2001).

The hypocholesterolemic effects of statins are evident after only a few days of therapy. Lovastatin, simvastatin, and pravastatin are well tolerated drugs; at 40 mg lovastatin a mean reduction of 30% in total plasma cholesterol, 40% in LDL- (low-density lipoprotein), 35% in VLDL- (very low-density lipoprotein) cholesterol, and 25% in triglycerides, and an increase of 10% high density lipoprotein (HDL)-cholesterol was observed (Tobert, 1987). The reduction of cholesterol, especially VLDL and LDL, involved in the translocation of cholesterol, and the increase in the number of LDL-specific membrane receptors in extrahepatic tissues, were also observed for patients treated with simvastatin and pravastatin.

In addition to reducing LDL-cholesterol, the clinical data showed that lovastatin, simvastatin, and pravastatin increase HDL, with a subsequent decrease, by almost 50%, of the LDL- to HDL-cholesterol ratio, considered the best predictor of atherogenic risk (Alberts 1988). Synthetic statins appear to be as efficacious as natural statins. In vivo statins have different effects on LDLcholesterol reduction, with dose-dependent reduction of plasma levels of up to 60%, although reductions of 20-25% are more typical with the dose commonly used in clinical practice. The greatest LDL-cholesterol reduction is obtained with atorvastatin and simvastatin (Chong *et al.* 2001), It was also observed that statins may contribute to decrease triglyceride levels, especially in hypertriglyceridemic subjects (Stein *et al.* 1998).

Moreover statins should not be used with nicotinic acid, as this leads to increased risks not only of myopathy, but also of hepatotoxicity (Farnier & Davignon, 1998). All statins are metabolized in the liver, with different pharmacokinetics. Lovastatin, simvastatin, atorvastatin,

and cerivastatin have a common metabolic pathway through the cytochrome P-450 3A4 enzyme system; fluvastatin has a metabolic pathway through the P-450 2C9 system, while pravastatin has multiple metabolic pathways (Chong *et al.* 2001). These differences are closely related to potential interactions with other drugs, which are metabolized through the same pathway (Chong et al. 2001; Horsmans 1999). Drug interactions may also depend on the plasma half-life of the statin, which, for all statins, is about 2–3 h, the exception being atorvastatin, which has a half-life of 14–20 h (Furberg, 1999).

**BIOGENESIS AND BIOSYNTHESIS OF STATINS**

Early biogenetic investigations of statins carried out on 14C-labelled monacolin J and L, employing a strain of *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.* 1985)**.** Studies on the pathway involved in monacolin synthesis have demonstrated that monacolin L is the precursor of monacolin J (Komagata *et al.* 1989).

From an overview of the early biogenetic studies carried out on the monacolins, it has been possible to demonstrate that monacolin L is the first to be synthesized from nine molecules of acetate and is, in turn, converted to monacolin J by hydroxylation; monacolin K is then derived from monacolin J. The monacolin X, i.e., the α- methyl-β-ketobutyryl ester of monacolin J, is converted to lovastatin, while it is accumulated in cultures of mutant strains producing no detectable amounts of lovastatin (Endo *et al.* 1986).

Earlier investigation of the biogenesis of lovastatin, carried out mainly in *Aspergillus terreus* strains employing labelled precursors (Greenspan & Yudrovitz, 1985), indicated that the lovastatin biosynthetic pathway **(Figure 13)** starts from acetate units (4- and 8-carbons long)

linked to each other in head-to-tail fashion to form two polyketide chains. The methyl group present in some statins in the side chain or at C6 derivatives from methionine, as frequently occurs in fungal metabolism, and is inserted in the structure before the closure of the rings (Shiao & Don, 1987). This mechanism demonstrates that mevastatin, which lacks the 6α-methyl group at C6, is not an intermediate in lovastatin biosynthesis.

The main chain is then cyclized and in some statins esterified by a side chain at C8. The oxygen atoms present in the main chain are inserted later by aerobic oxidation using a deoxygenated precursor (Greenspan and Yudrovitz 1985). Studies on the 13C incorporation in lovastatin and mevastatin carried out with *Penicillium citrinum* and *M. ruber* strains indicated a similar pathway; enzymatic hydroxylation and subsequent esterification at C8 was also observed (Endo 1985).

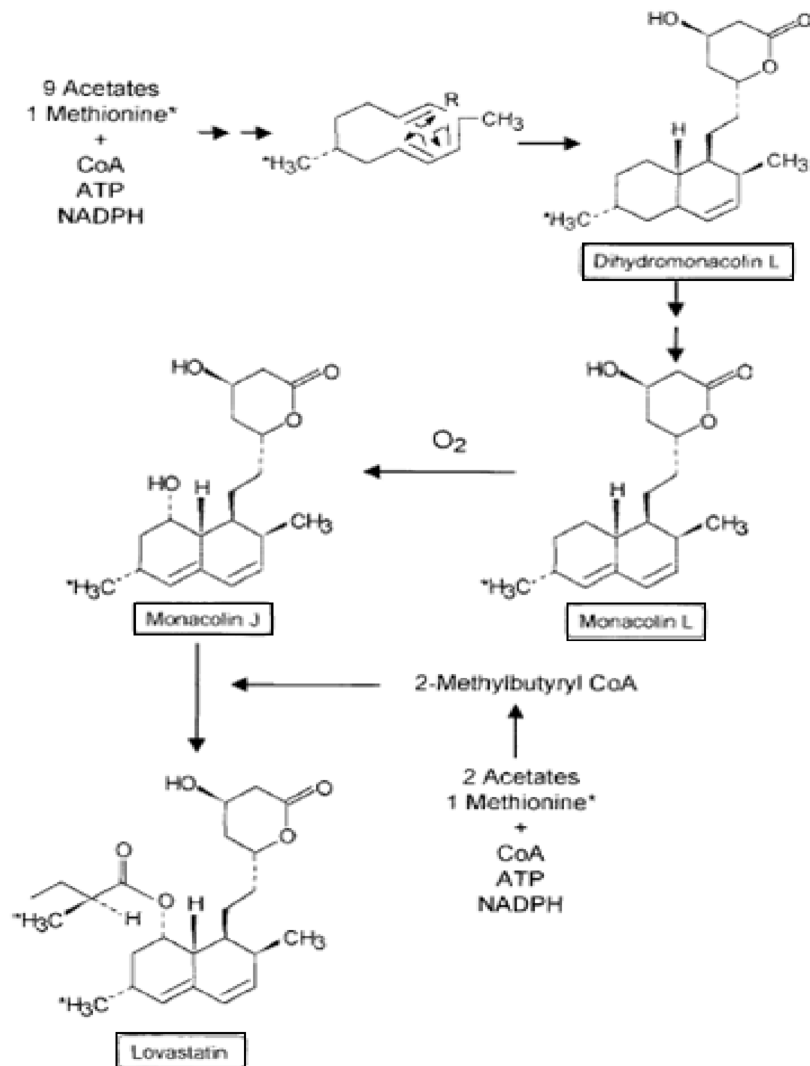



**Figure 13. Biosynthetic pathway of lovastatin**

More-recent investigations have studied enzymatic kinetics together with gene regulation and expression involved in *A. terreus* statin biosynthesis. Earlier genetic research investigated the mechanisms involved in lovastatin biosynthesis, particularly with regard to the two polyketide chains. The results, including the characterization of *A. terreus* lovastatin-blocked mutants, showed that the multifunctional polyketide synthase system (PKSs) comprises a

lovastatin nonaketide synthase (LNKS) involved in the cyclization of the main polyketide chain, to form the hexahydro naphthalene ring system, and a diketide synthase (LDKS) involved in the transfer of the methylbutyryl side chain to monacolin J. Study of the primary structure of the PKS that forms the lovastatin nonaketide provided new details of lovastatin biosynthesis (Hendrickson *et al.* 1999).

The characterization of the LNKS gene was of fundamental importance for understanding how the carbon skeletons of dihydromonacolin L and lovastatin are assembled. Other aspects of the biosynthesis of lovastatin related to PKSs have been investigated. The LNKS, product of *lovB* gene, interacts with *lovC* (a putative enoyl reductase), to catalyze the reactions in the first part of the biosynthetic pathway, leading to dihydromonacolin L (Fig. 6). In the final step of the lovastatin pathway, the LDKS, made by *lovF*, interacts with *lovD* (transesterase enzyme) that catalyzes the attachment of the 2-methylbutyric acid to monacolin J, derived from monacolin L.

The results demonstrated that the role of the *lovC* protein is to ensure correct assembly of the nonaketide chain in lovastatin by the *lovB* protein. In contrast, the construction of the methylbutyrate side chain by the LDKS (*lovF* protein) does not require *lovC* protein. The study also demonstrated that the *lovC* protein has no detectable function in post-PKS processing of dihydromonacolin L (Auclair *et al.* 2001). In conclusion, the recent advances in gene cloning have allowed the identification of most of the enzymes involved in lovastatin biosynthesis and have confirmed the biosynthetic pathways hypothesized in earlier investigations (Sutherland *et al.* 2001).

## GAMMA AMINO BUTYRIC ACID

Gamma-amino butyric acid (GABA) was first synthesized in 1883, and was first known as a plant and microbe metabolic product. In 1950, however, GABA was discovered to be an integral part of the mammalian central nervous sustem (Robert *et al.* 2003).

It is the chief neurotransmitter in the mammalian central nervous system. GABA plays an important role in regulating neuronal excitability throughout the nervous system. and disrupted GABAergic signaling, which has been implicated in numerous and varied neurological and psychiatric pathologies including movement and anxiety disorders, epilepsy, schizophrenia and addiction. GABA in insect species behaves primarily as an excitatory neurotransmitter. GABA is present in significant amount in the tissues of the nervous system. However, pancreatic islet cells and kidney contains some GABA.

GABA is a free moving amino acid having molecular mass of 103.12 g/mol and molecular formula $C_4H_9NO_2$. GABA is found mostly as a zwitterion with the carboxyl group deprotonated and the amino group protonated **(Figure 14).**

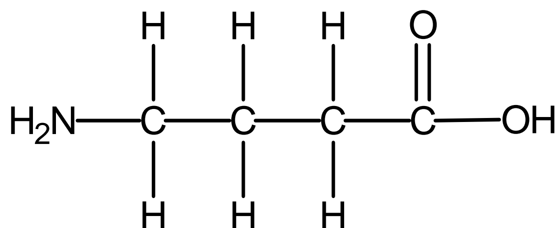



**Figure 14. Structure of GABA**

**MODE OF ACTION OF GABA**

In vertebrates, GABA acts at inhibitory synapses in the brain by binding to specific transmembrane receptors in the plasma membrane of both pre-and postsynaptic neuronal processes. This binding causes the opening of ion channels to allow the flow of either negatively charged chloride ions into the cell or positively charged potassium ions out of the cell. This action results in a negative change in the transmembrane potential,usually causing hyperpolarization. Three general classes of GABA receptors namely 1) $GABA_A$ and 2) $GABA_C$ ionotropic receptors, which are ion channels themselves and 3) $GABA_B$ metabotropic receptors, which are G protein-coupled receptors that open ion channels via intermediaries (G proteins). When the net positive ionic current is directed into the cell, GABA is excitatory, when the net positive ionic current is directed out of the cell, GABA is inhibitory.

**BIOSYNTHESIS OF GABA**

Gamma-amino butyric acid (GABA) produced by *Monascus purpureus* is an effective antihypertensive metabolite which has several physiological functions such as neurotransmitting, diuretic and hypotensive effect (Kiesuke *et.al*., 1992). GABA is formed by the decarboxylation of glutamic acid by a key enzyme, glutamate decarboxylase produced by the fungus. Glutamic acid is produced by an acid protease and an acid carboxypeptidase that are secreted upon growth of *Monascus purpureus* (Narahara, 1994).

## FUNCTIONS OF GABA

*Monascus* fermented rice contains good amount of GABA which exhibits an anti-hypertensive effects for humans (Kohama *et al.* 1987). It has several physiological functions, such as neurotransmitting, hypotensive and diuretic effects (Kiesuke *et.al*., 1992; Ueno *et al.* 1997). Consumption of GABA-enriched foods can inhibit cancer cell proliferation (Park & Oh, 2007) in mammals. GABA has been applied to clinical medicine for treatment of hypertension, Parkinson's disease, hypochondria and epilepsy.

## PRODUCTION OF GABA BY *MONASCUS PURPUREUS*

Kohama *et al*. (1987) repoted that the aqueous extract of the *Monascus koji* decreased blood pressure *in vivo. Monascus koji* grown on soybean paste exhibited antihypertensive effect in 4 weeks old spontaneously hypertensive rats.(Rhyu *et al.* 2002). *Monascus purpureus* CCRC 31615 produced GABA to 1276.6 mg/kg by the addition of sodium nitrate during solid state fermentation with long grain rice. GABA productivity increased further to 1493.6 mg/kg when dipotassium hydrophosphate was added to the medium (Su *et al.* 2003). *Monascus purpureus* strain CMU001 produced GABA of 28.37 mg/g at 3 weeks of fermentation with glutinous rice (Jannoey *et al.* 2010).

## STEROLS

Sterols are also known as steroid alcohols. They are a subgroup of steroids with a hydroxyl group at the 3-position of the A-ring (Alberts 2001). They are amphipathic lipids synthesized from acetyl-coenzyme A *via* the HMG-CoA reductase pathway. They are cell membrane protective compounds.

Plants have more than 40 sterols (Law, 2000) predominantly present in oil seeds and are commonly termed as phytosterols. The monst abundantly occurring phytosterols are β-sitosterol, campesterol and stigmasterol. In fungi and yeast the natural sterol is ergosterol. Ergosterol (ergosta-5, 7, 22-trien-3β-ol), a sterol, is a biological precursor (a provitamin) to Vitamin $D_2$. It is turned into viosterol by ultraviolet light, and is then converted into ergocalciferol, which is a form of Vitamin D (Rajakumar *et al.* 2005). Ergosterol is a component of fungal cell membranes, serving the same function that cholesterol serves in animal cells. Ergosterol is also used as an indicator of fungal biomass in soil. Though ergosterol does degrade over time, if kept below freezing in a dark environment, this degradation can be slowed or even stopped completely. Plant sterols have the ability to reduce blood cholesterol and the risks of cancer and improve immune system (Moreau *et al.* 2002).

Red yeast rice also contains sterols such as β-sitosterol, campesterol, stigmasterol, saponin and sapogenin; isoflavones and isoflavone glycosides; selenium, and zinc (Wang *et al.* 1997; Heber *et al.* 1999).

## MODE OF ACTION OF STEROLS

The structural similarity of fungal sterols and stanols to cholesterol enables them to compete with cholesterol for incorporation into micelles, the particles which transport lipids and cholesterol into the intestinal mucosa. This competition reduces dietary and biliary cholesterol absorption in the gastrointestinal tract (Lichtenstein, 2002). Decreased cholesterol absorption up-regulates LDL-receptor concentration and therefore decreases LDL serum levels. In response to decreased absorption, the liver and other tissues increase cholesterol synthesis, but this effect is

less significant than that of inhibited absorption (Clifton, 2002). Dietary sterol helps in decreasing LDL serum level there by reducing total cholesterol without affecting HDL and triglycerides level (Law 2000, Ostlund 2002). Statin in combination with dietary sterols produced by the fungi has been suggested as a more effective means of lowering cholesterol level than statin alone in the therapeutic use of anticholesterolemia (Plat and Mensink 2001).

**FATTY ACIDS**

Fatty acid is a carboxylic acid often with a long unbranched aliphatic tail (chain), which is either saturated or unsaturated. Carboxylic acids as short as butyric acid (4 carbon atoms) are considered to be fatty acids, whereas fatty acids derived from natural fats and oils may be assumed to have at least eight carbon atoms, caprylic acid (octanoic acid), for example, most of the natural fatty acids have an even number of carbon atoms, because their biosynthesis involves acetyl-CoA, a coenzyme carrying a two-carbon-atom group. Fatty acids are produced by the hydrolysis of the ester linkages in a fat or biological oil (both of which are triglycerides), with the removal of glycerol (Wild *et al*, 2003).

*Monascus* on solid state fermentation produces mono unsaturated long-chain fatty acids. The red and albino mutants of the *M. purpureus,* shows that fatty acids of wide range from $C_{14}$ to $C_{24}$. Thirty-nine fatty acids were identified so far form *Monascus* fermented samples, 22 saturated (including iso and anteiso), 14 monoenoic, two dienoic and one α-linolenic acid. The fatty acid composition differs between various mutant stains of *Monascus purpureus* (Kennedy *et al.* 1999).

The composition of FAs in the red and albino mutant of *M. purpureus* are are known to contain similar proportions of palmitic, oleic, linoleic and linolenic acid as in other ascomycetes.

The major components of fatty acids are C18:1, C18:2, C16:0, C18:0 and C16:1 (Nishikaw *et al.* 1989). The mutant strains produce less linoleic acid (11.4-15.9% compared to 26.8-53.1%) and oleic acid (9.6-19.3% compared to 21.2-44.6%) to that of parent stain. The concentration of palmitic acid was nearly the same (9.6-18.2% compared to 12.9-21.6%) (Juzlova *et al.* 1996). Red mold rice contains unsaturated fatty acids such as oleic, linoleic and linolenic acids (Ma *et al.* 2000). These fatty acids also help in reducing serum cholesterol (Wang *et al.* 1997).

## ANTIMICROBIAL PRINCIPLES

Antibacterial properties of *Monascus* pigments were first mentioned by Wong & Bau (1977). The monascidin A was effective against *Bacillus, Streptococcus* and *Pseudomonas*. It was shown that this molecule was citrinin (Blanc *et al.* 1995) and its production by various *Monascus* species was studied using different culture media and conditions (Blanc *et al.* 1995). Statins have been shown to exert antifungal activity against the pathogenic yeasts *Candida spp*. and *Cryptococcus spp* and non-pathogenic *Saccharomyces cerevisiae* (Lorenz & Parks, 1990; Chin *et al.* 1997).

The new *Monascal* pigment dehydromonacolin- MV2 produced by a mutant strain shows similar antibacterial activity. Chloroform extract of rice fermented with a hyper pigment-producing mutant of *M. purpureus* (CFR 410-11) was found to contain metabolites that inhibited the growth of *Bacillus, Pseudomonas* and *Streptococcus* in agar gel diffusion assays (Dhale *et al.* 2007b). Dehydromonacolin-MV2 apparently originated in the mutant by hydroxylation and oxidation of monacolin-J, an intermediate of monacolin biosynthetic pathway.

## ANTIOXIDANT PRINCIPLES

Dhale et al. (2007a) reported that the extract obtained from the *Monascus purpureus* exhibited strong 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity. The antioxidant activity was strengthened by repeated fractionation of red yeast rice extract. The antioxidant isolated was identified as Dihydromonacolin-MV. The compound has significant radical scavenging activity and strongly inhibited lipid peroxidation in a liposome model.

## ANTICARCINOGENIC PRINCIPLES

Ankaflavin and monascin isolated from the extracts of *Monascus* spp. are known to exhibit anti-tumor initiating activity through oral administration on the two-stage carcinogenesis of mouse skin tumor induced by peroxynitrite or UV treatment. Monascin showed inhibitory activity on both chemical or UV induced skin carcinogenesis. Akihisa *et al*. (2005) reported that monascin compound can be used in the prevention of chemical and environment oriented cancer.

## MONASCIDIN A (CITRININ)

Monascidin A, produced by *Monascus spp*. was used as food preservative which was later identified as a mycotoxin (Hajjaj, 1999). The production of citrinin limited the usage of red yeast rice as functional food. Various metabolic engineering and traditional mutagenesis technologies were followed to eliminate the production of citrinin (Jia *et al.* 2009). Wang et al. (2004) reported that a simple and quick selection method for mutant strains of *Monascus purpureus* NTU 601 was designed based on the fact that citrinin possesses antibacterial activity for *Bacillus subtilis* and will form an inhibition zone around the colony of the *Monascus* strain. The mutant strain *M. purpureus* N301 only produced 0.23 (0.01 ppm citrinin, which was 50% less than that of the parent strain, and the monacolin K production was 481.29 (7.98 ppm and

maintained 91% productivity. *M. purpureus* N 310, the other mutant strain, produced 0.27 % (0.01 ppm) citrinin, which was 41% less than that of the parent strain, and the monacolin K production was 526.29 (5.54 ppm), which showed no significant changes when compared with the parent strain. The GABA content of the two strains was 5000 ppm, which is similar to that of the parent strain. The results showed that the method could be used to select red mold rice with low citrinin production.

**ALTERNATE SUBSTRATE FOR SOLID STATE FERMENTATION**

Millets can be cultivated in a wide range of soils and climates and because of their short growing seasons, they are of specific importance in semi-arid regions. Finger millet is consumed mainly as a pap (porridge) and mudde. These porridges are particularly used as weaning foods in developing countries. Sprouting has been reported to improve the nutritional quality of seeds by increasing the contents and availability of essential nutrients and lowering the levels of antinutrients (Chavan & Kadam, 1989). Total mineral analysis of finger millet consists of Ca-302.33±0.57, P-237±1.0, Fe-4.33±0.08, Mg-109.66±0.57, Zn-2.11±0.08, Cu - 0.52 ± 0.00 mg/100g dry matter.

Among millets, finger millet was reported to contain high amounts of tannins (Ramachandra *et al.* 1977) ranging from 0.04 to 3.47 per cent (catechin equivalent). Tannins form complex with proteins and carbohydrates, thereby decreasing nutrient bioavailability (Udayasekhara Rao *et al.* 1991). Phytate or phytic acid, naturally occurring phosphate in the grain is the other group of antinutritional factor which significantly influences the functional and nutritional properties of foods. It is the main phosphorus store in mature seeds. Phytic acid has a strong binding capacity, readily forming complexes with divalent and multivalent cations and proteins. Phytate binding renders several minerals biologically unavailable to animals and

humans. However, these antinutrients can be removed by processing techniques, germination, fermentation, dehulling etc. Sankara Rao & Deosthale (1983) reported that malting of the grain significantly reduced the phytate phosphorus in finger millet. This reduction of phytic acid was accompanied by significant increase in availability of nutrients. Considering the above benefits of germination, germinated finger millet was also included as a substrate in this present study.

Finger millet is grown in more than 25 countries in Africa and Asia. Uganda, India, Nepal, and China are the major *ragi* producers of world. In India, it is extensively grown in Karnataka, Tamil Nadu, Andhra Pradesh, Orissa, Bihar, Gujarat and Maharashtra and the hilly regions of Uttar Pradesh and Himachal Pradesh. The area under finger millet has declined from 2.6 million ha in early sixties to around 1.66 million ha in 2003-04. However, the annual production is maintained around 2.6 million tonnes with a productivity of around 1400 kg/ha **(Table 2)**.

**Table 2. Area, production and productivity of finger millet in important states of India in 2004-05**

| State | Area (000 ha) | Production (000 t) | Productivity (kg/ha) |
|---|---|---|---|
| Andhra Pradesh | 69.0 | 87.0 | 1261 |
| Bihar | 15.6 | 10.5 | 673 |
| Chattisgarh | 10.3 | 2.7 | 262 |
| Gujarat | 24.3 | 25.8 | 1062 |
| Himachal Pradesh | 3.0 | 3.0 | 1000 |
| Jharkhand | 18.0 | 11.0 | 811 |
| Karnataka | 893.0 | 1733.0 | 1941 |
| Kerala | 0.5 | 0.6 | 1200 |
| Madhya Pradesh | 0.5 | 0.1 | 200 |
| Maharashtra | 145.0 | 147.0 | 1014 |
| Orissa | 78.0 | 44.9 | 576 |
| Tamil Nadu | 108.9 | 154.1 | 1415 |
| Uttarakhand | 167.0 | 190.0 | 1138 |
| Uttar Pradesh | 0.7 | 0.9 | 1286 |
| West Bengal | 12.4 | 15.1 | 1218 |
| **India** | **1552.7** | **2432.4** | **1567** |

**Source:** Fertilizer association of India, 2006

Considering the importance of metabolites produced during fermentation by *Monascus purpureus*, providing due importance to finger millet for its abundant availability, high nutritive value, low cost, and as there are no report on the metabolites production by *Monascus purpureus* from finger millet in the past, the present research study was carried out to develop a food constituent with improved nutritive value, digestibility, nutraceutical and therapeutic properties.

# THE PROBLEM

Finger millet (*Eleusine coracana*), also known as ragi, is valued as staple food in Asia and Africa. In India, it is cultivated as kharif crop in southern parts and hilly areas. The area under cultivation of ragi is 15,52,700 ha with annual production of 24,32,400 tonnes. It is a rich source of calcium, dietary fibre and well balanced protein. It is used as source of food for growing children and aged people. Finger millet grain is usually made into flour for the preparation of puddings and mudde. The porridge prepared from finger millet is particularly used as weaning food in developing countries. The malted finger millet is used as a food constituent for diabetic patients.

Finger millet grain is rich in minerals. However, the nutritional availability of these is hindered by the presence phytic acid, which has a strong binding capacity, readily forming complexes with divalent and multivalent cations and proteins thereby rendering several minerals biologically not available. Germination of finger millet helps in the degradation of phytic acid, thereby releasing the minerals in the available form.

Fermentation of finger millet helps in the degradation of phytic acid and thereby enhances further release of minerals from the phytic acid-mineral complex. Fermentation degrades tannin carbohydrate and protein complexes into simple sugars and amino acids and makes them available. In addition to increase in dietary fibre and lipid content, fermentation also supports the production of various enzymes, amino acids, vitamins and metabolites by the microorganism.

The fermentation of germinated finger millet by *Monascus purpureus* thus able to include value addition by increasing the nutritional availability, converting complex carbohydrates and proteins to simple sugars and amino acids and supporting the production of health promotive

metabolites like statins, dietary sterols, unsaturated fatty acids, γ-amino butyric acid, antioxidant and antimicrobial compounds. Considering the importance of the metabolites produced during fermentation of finger millet by *Monascus purpureus*, providing due importance to finger millet for its high nutritive value, low cost, and as there are no report on the metabolites production by *Monascus purpureus* from finger millet in the past, the research study entitled "Characterization of bioactive molecules from *Monascus purpureus* fermented finger millet (*Eleusine coracana*)" was carried out wherein germinated finger millet was used as a substrate for culturing *Monascus purpureus* to develop a food constituent with improved nutritive value, digestibility, nutraceutical and therapeutic properties.

Monascus purpureus MTCC 410 produced pigments, antihypercholesterolemic metabolites (statins and sterols), unsaturated fatty acids and GABA during its growth in various starch rich cereals and millets. Germinated finger millet used as a substrate in solid state fermentation with *Monascus purpureus* exhibited higher production of statin due to value addition by germination and fermentation. The details of the study are elaborated in the **First chapter.**

Enhancement of statin production and optimization of GABA yield by Monascus purpureus MTCC 410 by supplementing finger millet solid state media with different media and nutrient constituent using response surface methodology were conducted. An animal experiment to study the effect of *Monascus* fermented finger millet on production performance, yolk cholesterol and serum lipid in layer hens was carried out. The outcome of the above experiments was explained in the **Second chapter**.

The ability of wild type and its two mutants *viz.* albino and hyper pigment producer of Monascus purpureus MTCC 410 for statin and GABA production were characterized by genetics

and physiological studies respectively. The details of the experiments are discussed in the **Third chapter** of the thesis.

# MATERIALS AND METHODS

## MICROBIOLOGICAL METHODS

All media preparation and culturing of microorganisms were carried out by routine microbiological methods. Culture media were sterilized at 121 °C for 20 min (15 lbs pressure) and glass ware were sterilized in hot air oven maintained at 180 °C for 3 h. Inoculation of cultures was carried out in laminar air flow chamber and all other aseptic methods advised for microbiological work were generally followed. All media preparations were carried out using double glass distilled water. Ultra pure water obtained from Milli pore filtration unit was used for buffer preparations for enzyme assay and extraction purposes.

## CHEMICALS

All microbiological media were prepared using laboratory grade chemicals. For enzymatic assay and extraction purposes, analytical grade or pure chemicals were used. Microbiological media were obtained from Hi-media, India and chemicals were obtained from Ranbaxy Laboratories Ltd., India; SRL Chemicals, India and Sigma Chemicals. St. Louis, USA.

Solvents used for spectrophotometric studies, high performance liquid chromatography, gas chromatography and thin layer chromatography were of analytical and HPLC grades, obtained from Merck India Ltd., India.

The standard chemicals of pravastatin, lovastatin and GABA enzyme assay were obtained from Sigma Chemicals, St. Louis, USA. Serum cholesterol assay kits for total cholesterol, low

density lipoproteins, high density lipoproteins and triglycerides were procured from the Spam diagnostics, India.

## THE ORGANISMS

The isolates of *Monascus purpureus* (MTCC 410) and *Aspergillus terreus* (MTCC 2803) were obtained from IMTECH, Chandigarh, India. The two UV mutants of *Monascus purpureus* viz. albino (CFR 410-22) and hyper (CFR 410-11) mutants from Food Microbiology Department, CFTRI.

## MAINTENANCE OF CULTURES

*Monascus purpureus* and its mutants and *Aspergillus terreus* were maintained on potato dextrose agar slants at 4 °C and they were sub-cultured in the interval of every two months.

## PREPARATION OF INOCULUM

Seed culture was prepared by inoculating a loopful of spores from Potato Dextrose Agar slant into 500 ml Erlenmeyer flask containing 100 ml of sterile basal medium. The culture was incubated at 28 °C for 48 h at 110 rpm.

**Basal Medium composition**

| | |
|---|---|
| Glucose | 10%, |
| Peptone | 1%, |
| Potassium nitrate | 2%, |
| Ammonium hydrogen phosphate | 2% |
| Magnesium sulphate heptahydrate | 0.5% |
| Calcium chloride | 0.1% |
| pH | 6.0 |

## SUBSTRATES USED IN FERMENTATION

*Oryza sativa* (Raw Rice, Parboiled Rice and Njavara Rice), *Eleusine coracana* (Finger millet and germinated Finger millet) *Triticum aestivum* (Wheat), *Sorghum vulgare* (Jowar), *Hordeum vulgare* (Barley) and *Zea mays* (Maize) were procured from the local market of Mysore.

## GERMINATION OF FINGER MILLET

Finger millet seeds were weighed and soaked for 10 h in distilled water. Wet filter papers were placed in a Petri dish. The soaked seeds were uniformly sprinkled over the wet filter paper. The lid of the dish was closed and incubated at 28 ± 2°C temperature for a period of 72 h. Germinated seeds were dried in a dryer set at 40 °C for 24 h to a moisture content of

approximately 12%. This was used as a substrate for solid state fermentation of *Monascus purpureus*.

## PREPARATION OF SUBSTRATES FOR SOLID STATE FERMENTATION

20 g of dried substrate was taken in 500 ml Erlenmeyer flask and mixed with 40 ml of distilled water (34 ml of distilled water in case of germinated finger millet and 30 ml of distilled water for ungerminated finger millet). The substrates were sterilized at 121°C for 30 min. Sterilized substrates were allowed to cool over night in room temperature.

## CULTIVATION OF *MONASCUS PURPUREUS* BY SSF

Sterilized substrates were mixed well using a sterile glass rod under aseptic condition to facilitate separation of cooked grains. Seed medium of *Monascus purpureus* at the rate of 2% was added as inoculum to the substrates and the flasks were shaken well to uniformly distribute the spores within the substrate. Then the flasks were kept in slanting position in an incubator maintained at 28 °C for 7 days with intermittent mixing of substrates by hand shaking for statin production **(Figure 15)**. In case of optimization of statin and GABA 10 days incubation was allowed and germinated finger millet was used as the substrate for fermentation.

**Figure 15. Cultivation of *Monascus purpureus* in Erlenmeyer flasks**

## pH ANALYSIS

A digital pH meter (APX-175E, CD Instrumentation Pvt. Ltd., Bangalore, India) with a glass electrode was used for measuring the pH of the substrates (before and after fermentation). 5.0 g of substrate (both fermented and unfermented) and 25 ml of de-ionized water were homogenized individually using a mortar and pestle, and then filtered through single layer cheesecloth to measure pH (No and Meyers 2004).

## MOISTURE CONTENT

Moisture content was determined by drying 5 g of Monascus fermented samples, taken in pre weighed petriplate. About 5 ml of alcohol was added, kept in hot air oven at 150° C for 3 h till a constant weight was reached , then placed in a desiccator and cooled to room temperature. The final weight was noted down and repeated till constant weight was obtained (AOAC, 1990).

## LOSS OF ORGANIC MATTER

Fermented samples were 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.

$$LOM = \frac{Wi - Wf}{Wi} \times 100\ \%$$

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. It was assumed that loss of organic matter is due to CO2 formation by fungal metabolic activities during the course of fermentation. Since there is no

direct method for biomass estimation in SSF, LOM was used to express biomass and metabolic activity in an indirect way (Valera *et al.* 2005).

## EXTRACTION AND ESTIMATION OF STATIN

Fermented substrates were dried at 45 °C for 24 h to a moisture content of approximately 12% and powdered using pestle and mortar. 1.0 g of the powdered material was extracted with 50 ml of methanol: water mixture (1:1, v/v) of pH 7.0 in 250 ml Erlenmeyer flask and incubated on a rotary shaker for 2 h at 150 rpm. The content was passed through Whatmann No.1 filter paper and the filtrate was flash evaporated using Roto evaporator (Buchi, Switzerland). The flash evaporated residue was washed with methanol and the washing was passed through 0.45µ syringe filter and the resultant supernatant was collected. The statin content (pravastatin and lovastatin) was analysed by HPLC (isocratic) at 238 nm with C-18 Agilent column and acetonitrile:water 72:28 (v/v) as mobile phase.

## EXTRACTION AND ESTIMATION OF STEROL

One gram of the dried powdered fermented substrate was taken in 50ml Erlenmeyer's flask with 10 ml of 2.5 N alkali and autoclaved at 15 lbs for 1 h (Kieber *et al.* 1955). After cooling, equal volume of diethyl ether was added to the contents and kept on incubator shaker for 1 h. The mixture was allowed to separate at cold 4°C. Ether layer containing sterol was separated using a separating funnel. Trace amount of sodium sulphate was added to the content and dried using nitrogen gas to remove any excess moisture. The dry residue was dissolved in one ml of chloroform and the content was passed through 0.45µ membrane filter. The filtrate was collected in a tube and the solvent was evaporated by keeping the tube in an incubator maintained at 40 °C. The cholesterol residue in the tube was dissolved in one ml of choloroform

and the total sterol content was measured spectrophotometrically at 640 nm using cholesterol as standard (Sabir *et al.* 2003).

**EXTRACTION AND ESTIMATION OF FATTY ACIDS**

One gram of powdered fermented sample was taken in 50ml Erlenmeyer flask. About 20 ml of chloroform: methanol (2:1) (v/v) was added and incubated overnight in reciprocating shaker set at 150 rpm and temperature 20°C. The content in the flask was filtered to remove solid debris. The filtrate was collected in separate 50ml Erlenmeyer flask to which an equal volume of 0.88% potassium chloride was added. The mixture was taken in separating funnel and chloroform layer containing lipid was collected in test tubes, a pinch of anhydrous sodium sulphate was added to lipid containing chloroform and dried under nitrogen gas flushing. After drying 1.0 ml of chloroform was added to the dried layer in the test-tube and the lipid was dissolved. The chloroform with lipid was pipette out into separate test-tubes and the solvents were allowed to dry by keeping the test tubes at 45°C in an incubator. Thus the lipid was obtained was weighed (Folch *et al.* 1957).

**PREPARATION OF FATTY ACID METHYL ESTERS (Kates, 1964)**

To the lipid residues in the test-tubes 1.0 ml of methanol acetyl chloride (95:5) (v/v) was added and kept in a water bath at 70 °C. To the residue 1ml of chloroform was added and shaken thoroughly the resultant solution was passed through 0.45μ membrane filter and the contents were collected separately and dried at 45 °C. The samples were analyzed for different fatty acid constituents using Gas chromatography (Shimadzu, Japan). Standard fatty acids were also run in the same conditions for quantifying the fatty acids present in the samples.

**GC Conditions**

| | |
|---|---|
| Column | DEGS packed column. |
| Oven temperature | 180°C |
| Injection block | 220°C |
| Detector block | 230°C |
| Flow rate | 40ml/min |
| Detector | flame ionization detector (FID) |

## ESTIMATION OF CARBOHYDRATES

Carbohydrates content was determined by anthrone reagent method (Hedge and Hofreiter 1962) and reducing sugar was estimated using Nelson-Somogyi method (Somogyi 1952).

## ESTIMATION OF PROTEINS

Total and soluble protein was estimated using Folin Ciocalteau reagent (Lowry et al. 1951).

## ESTIMATION OF FAT

Total fat was estimated by standard procedure of AACC (2000).

## ESTIMATION OF DIETARY FIBRE

The dietary fibre content of raw and processed finger millet was determined by following the standard procedure of AOAC (1990).

## ESTIMATION OF NUTRIENTS

Finger millet was pulverized to fine powder and mineral analysis was carried out by wet acid digestion of the samples using nitric/perchloric acid mixture (2:1). Nutrients like calcium, iron and phosphorous was analysed by following the standard procedure of AOAC (1990). Magnesium was estimated by Versanate titration method and the results were expressed as a percentage of the dry weight (Jakson 1973). Trace minerals iron, copper and zinc were estimated by using Perkin-Elmer 3110 atomic absorption spectrophotometer.

$$\text{Element concentration (mg/g)} = \frac{\text{AAS readings (ppm) x volume made up x dilution (if any)}}{\text{Weight of the sample}}$$

## ESTIMATION OF ANTINUTRIENT FACTORS

Tannin was estimated colorimetrically based on the measurement of blue colour formed by the reduction of phosphotungstomolybdic acid in alkali solution (AOAC 1975). The estimation of phytic acid was based on the principle that the phytate is extracted with trichloroacetic acid and precipitated as ferric salt. The iron content calculated from this value assuming a constant 4 Fe: 6 molecular ratios in the precipitate. Phytates were estimated as phytic acid and expressed as phytate phosphorus (Wheeler and Ferrel 1971). The phytate phosphorus was calculated by the following formula.

$$\text{Phytate phosphorus (mg/100g sample)} = \frac{\mu g \text{ Iron} \times 15}{\text{Wt. of sample}} \times \frac{1}{100}$$

## ESTIMATION OF HCl- EXTRACTABILITY OF MINERALS

HCl extractability of minerals was carried out by continuous shaking 1 g of sample with 50 ml of 0.03 N HCl at 150 rpm for 3 h at 37° C; the mixture was filtered through Whatman No. 42 filter paper and the clear supernatant was oven dried at 100°C. Mineral analysis was done by wet acid digestion of the samples using nitric/perchloric acid mixture (2:1) and the minerals were estimated in the acid digest as described earlier (Chompreeda and Fields 1984).

## SOLID STATE FERMENTATION (SSF) FOR STATIN OPTIMIZATION

Forty grams of solid substrate which includes dried coarsely ground finger millet (variety Indaf-5), glycerol, ammonium nitrate and soybean flour as per the experimental design was taken in 500 ml Erlenmeyer flask and mixed with 60 ml of distilled water. Then the substrates were sterilized at 121°C for 30 min. Sterilized substrates were allowed to cool over night at room temperature. The medium was inoculated with seed medium at the rate of 2%). *Monascus purpureus* was cultivated on cooked germinated finger millet in 500 ml conical flasks kept in slanting position with intermittent mixing of substrates by hand shaking in an incubator maintained at 30°C for 10 days. After completion of prescribed incubation period, the flasks were withdrawn from the incubators and the fermented finger millet was dried at 45°C for 24 h to a moisture content of approximately 12 % and stored in deep freezer set at -20°C until it was used for total statin extraction.

## RESPONSE SURFACE METHODOLOGY EXPERIMENTAL DESIGN

A response surface methodology study as described by Box and Wilson (1951) was conducted to determine the effect of three predictor variables (soybean flour, glycerol and ammonium nitrate) on the total statin production by *Monascus purpureus*. A CCRD was constructed using the software package Statistica (1999). The limits for the each independent variable were fixed based on the prior trials conducted in the laboratory. Table 1 shows levels of predictor variables and shows the combination of predictor variable levels used in CCRD. To study 3 factors at 5 levels would require $5^3$or 125 using conventional experiments, where as use of standard experimental designs such as CCRD requires only 22 runs or experiments. The center point in the design was repeated 6 times to calculate the reproducibility of the result.

For each of the response variables, model summaries and lack of fit tests were analyzed for linear or quadratic models. From this information, the most accurate model was chosen by the sequential F tests, lack of fit measures. Three-dimensional response surface plots were generated for each production parameter. In this study, predictor variables were permitted to be at any level within the range of the design. In most of the reported RSM applications, several responses were measured for each set of experimental conditions, and a model was fitted for each response. Finding the overall optimal conditions in these multi-response problems is not straight forward or easy, and most researchers use the graphical approach of superimposing the different response surfaces and finding the experimental region that would give the desired values of responses. This method, although visually attractive, requires a large number of graphs to be generated, even for 2 or 3 responses involved.

## OPTIMIZATION

According to the canonical analysis described by Myers (1991), the stationary points were located for the corresponding responses. The search criteria were to find the production conditions that would enhance the statin production. Optimum conditions with respect to statin were glycerol 3.33 %, ammonium nitrate 0.14% and soybean flour 6.71%. Verification experiments, carried out at the predicted conditions showed values reasonably close to those predicted and further confirmed the adequacy of predicted models.

## EXPERIMENTAL DESIGN FOR OPTIMIZATION OF GABA

To study 4 factors at 5 levels would require $5^4$or 625 using conventional experiments, whereas use of standard experimental designs such as CCRD requires only 32 runs or experiments. The center point in the design was repeated 8 times to calculate the reproducibility of the result. Coded values of -2, -1, 0, +1 and +2 corresponded to very low, low, medium, high and very high levels of variables, namely incubation period (days), temperature (°C), inoculum volume (%) and nitrogen level (%). The basic equation for the quantity of GABA (mg/kg) produced as a function of the above mentioned four independent variables involved a second order polynomial of the form shown below:

where Y= GABA yield (mg/kg), $X_1$ = incubation period in days, $X_2$ = temperature in °C, $X_3$ = inoculum volume in % and $X_4$ = nitrogen level in %. STATISTICA software was used to arrive at the significant coefficient terms and to carry out an analysis of variance. The three dimensional surface plots were also obtained using the same software.

As did in the optimization experiment of statin, the above procedures were followed in this experiment.

## EXTRACTION OF GABA

GABA was extracted and estimated by modified method of Zhang and Bown, 1997. One gram of the dried fermented sample was taken in 50 ml Erlenmeyer flask. Ethanol /water mixture 5 ml (60:40 v/v) was added and incubated overnight in an incubator cum shaker set at a temperature of 25 °C and 150 rpm. The incubated content was centrifuged at 5000 rpm for 10 min and the supernatant was collected in a separate Erlenmeyer flask. 0.4 ml of the supernatant was taken in an eppendorf tube. 1.0 ml of $LaCl_3$ was added to the eppendorf tube and shaken for 15 min. The content was centrifuged at 10000 rpm for 10 min and the supernatant was collected. 0.8 ml of the supernatant was taken in a separate tube and 0.16 ml of 1M KOH was added and the content was shaken for 5 min, centrifuged at 10000 rpm for 10 min. From the resultant supernatant fluid of 0.9 ml, 0.55 ml was used in the enzymatic assay. GABA was estimated by UV spectrophotometer (Shimadzu, Japan).

## ESTIMATION OF GABA BY ENZYMATIC ASSAY

One ml of assay system contained 550 µl of a sample containing 10-100 nmol GABA, 150 µl of 4 mM $NADP^+$, 200 µl of 0.5 K pyrophosphate buffer (pH 8.6), and 50 µl of 2 units GABASE per ml. The initial absorbance was recorded at 340 nm before adding 50 µl of 20 mM α-ketoglutaric acid, and the final absorbance was recorded after 1 h. The difference in absorbance values was used to construct a calibration graph **(Figure 16)**. From the calibration graph, the GABA concentration of the test sample was determined. The standard GABASE enzyme preparation was dissolved in 0.1 M K-Pi buffer (pH 7.2) containing 12.5 glycerol and 5 mM 2- mercaptoethanol. The resulting solution was divided into 0.5 ml aliquots each containing 1 unit of activity. These were frozen until used. UV spectroscopic measurements were carried

out using a UV-160A recording spectrophotometer (Shimadzu, Japan) operating at 25°C temperature.

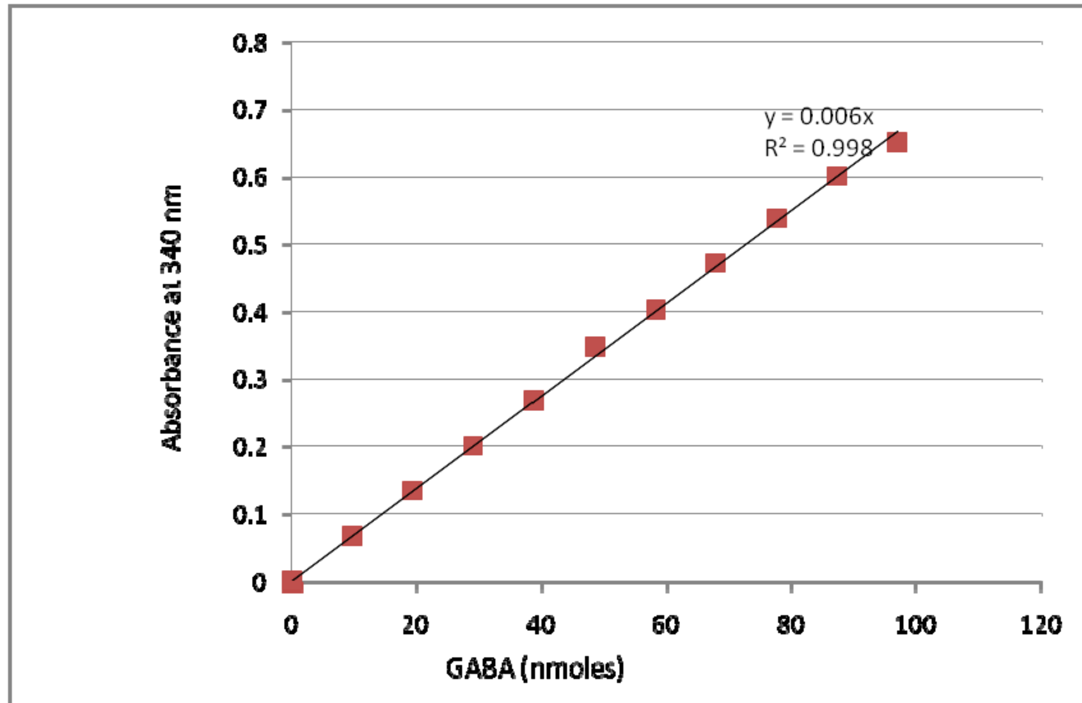



**Figure 16. Calibration graph for GABA determination**

**Calculation**

$$\text{GABA (mg/kg)} = \frac{\text{Absorbance} \times 0.9 \times 1.0 \times 5.0 \times 1000 \times 103.17 \text{ (Molecular wt of GABA)} \times 1000}{0.55 \times 0.96 \times 0.8 \times 0.4 \times 6.75 \times 10^{-3} \text{ (slope of the standard curve)}}$$

**PREPARATION OF LAYER DIET**

The layer diet used in this study was prepared at M/s. Kateel poultry farm, Mysore. The feed composition is shown in **Table 3**.

**Table 3. Composition of basal layer diet**

| Ingredients | (g/kg) |
|---|---|
| Yellow maize | 580.0 |
| Sunflower oilcake | 140.0 |
| De-oiled groundnut cake | 80.0 |
| Soybean oilcake | 90.0 |
| Calcium carbonate | 20.0 |
| Shell grits | 75.0 |
| Dicalcium phosphate | 10.0 |
| Sodium Chloride | 4.0 |
| Mineral mixture* | 0.9 |
| Vitamin mixture** | 0.1 |
| Calculated composition | **(%)** |
| Moisture | 9.48 |
| Protein | 16.74 |
| Fat | 4.53 |
| Fibre | 6.72 |
| Ash | 15.17 |
| Calcium | 3.53 |
| Phosphorus | 0.69 |
| Carbohydrate | 47.36 |
| ME(kcal/kg) | 2593 |

***Mineral Mixture : Calcium 32%, Phosphorus 6%, Iron 0.1%, Magnesium 1000 ppm, Cobalt 60 ppm, Zinc 2600 ppm, Iodine 100 ppm, Copper 100 ppm, Manganese 2700 ppm.**

****Vitamin mixture: Each gram contains Vit. A 82,500 IU; Vit. $D_3$ 12,000 IU; Vit. $B_2$ 50 mg; Vit. K 10mg.**

## PREPARATION OF POULTRY

The poultry experiment was carried after obtaining approval from the Institutional Animal Ethical Committee as per the Guidelines laid by Committee for the Purpose of Control and Supervision of Experiment on Animals (IAEC No:154/09). Twenty four Single Comb White Leghorn layer birds of 32 weeks old, each weighing 1.56 to 1.65 kg which were already laying eggs were used in this experiment. The layer birds were randomly distributed to four treatment groups, each group contained six birds (n=6) and they were individually distributed to cages. They birds were allowed to acclimatize on normal layer diet for one week before test feeding was commenced. First group (control) was fed with standard layer diet. The other three test groups were fed with standard layer diet supplemented with fine powder of germinated *Monascus* fermented finger millet at 2.5%, 5.0% and 10.0 % level.

**Figure 17. Collection of eggs from the caged experimental laying hens**

**DATA COLLECTION**

The quantity of feed left after each week was collected and weighed. Accordingly, daily feed and statin intake were calculated. Eggs were collected at weekly intervals **(Figure 17)** and the egg quality parameters *viz.* egg weight, shape index, Haugh Unit, shell thickness, shell weight, shell (%) and yolk colour were measured using various egg quality parameters measuring devices **(Figure 18)**. At the end of the experiment, two ml blood was collected from jugular vein of each bird **(Figure 19)** after overnight fasting. The blood was centrifuged at 3000 rpm for 10 min, the serum was separated and stored at -20°C for further analysis.

**Vernier calipher**

**Tripod Haugh unit meter**

**Yolk separator**

**Yolk colour Fan**

**Figure 18. Egg quality parameters measuring devices**

**Figure 19. Collection of blood sample from poultry**

## SERUM LIPID PROFILE ANALYSES

Total serum cholesterol and triglycerides were assayed as per the manufacturer's recommendation by using a commercial kit purchased from M/s Span Diagnostics Ltd., Sachin, India. Samples were incubated for 5 min at 37° C and their absorbances were read at 505 nm in a spectrophotometer. High density lipoprotein cholesterol was assayed enzymatically after precipitation of LDL and VLDL by heparin/manganese and their absorbances were read at 505 nm. Low density lipoprotein cholesterol was estimated using Friedewald equation.

## EGG YOLK CHOLESTEROL ANALYSIS

The eggs used for analyses were cooked and allowed to cool. The egg shell was peeled and the albumen was removed carefully without disturbing yolk. Yolk was crumbled and one gram of it was mixed with 15ml of chloroform - methanol 2:1 (v/v), homogenized thoroughly

and filtered. These filtrates were used as egg yolk samples in the estimation of yolk cholesterol as mentioned above using commercial kit purchased from M/s Span Diagnostics Ltd., Sachin, India.

## SERUM ANTIOXIDANT ENZYMES

Serum glutathione reductase activity was assayed according to Carlberg and Mannervik (1985). Glutathione transferase activity was assayed by measuring the conjugate with 1-chloro-2, 4-dinitrobenzene used as substrate as described by Warholm et al. (1985). Glutathione peroxidase activity was determined according to Flohe and Gunzler (1984). Catalase activity was assayed according to Aebi (1984) by following the decomposition of hydrogen peroxide.

## CULTIVATION FUNGAL ISOLATES AND DNA EXTRACTION

Potato dextrose broth was prepared and 35 ml of the same was dispensed in 250 ml conical flasks and sterilized. After cooling, the broth was inoculated with the spores of the parent culture and mutants (albino and hyper) of *Monascus purpureus* MTCC 410 and *Aspergillus terreus* MTCC 2803 (positive control for *lov* genes) separately and incubated at 30°C for 48 h.

Fungal mat was harvested by centrifuging the culture filtrate at 6000 rpm for 10 min. Then 25.0 ml of sterile water was added to the pellet in centrifuge tube. The content was vortexed well and once again centrifuged at 6000 rpm for 10 min. the supernatant was decanted and the pellet was stored at -20 °C for 1 h. The pellet was transferred to sterile pestle and mortar and ground to fine powder using liquid nitrogen. 2.0 ml of TE buffer was added and ground well. The ground content was transferred to the centrifuge tube and 200 µl of 10% SDS, 50 µl of proteinase K was added and mixed well and incubated at 37 °C for 1 h. Then 400 µl of 5M NaCl and 400 µl of 10 % CTAB was added and mixed well. The content was incubated at 65° C for

10-15 min. 2.5 ml Phenol: Chloroform was added, mixed well and centrifuged at 6000 rpm for 10 min at 4 °C. One ml of the top layer was pipetted out into eppendorf tubes and 100 µl of potassium acetate and 600 µl of isopropanol were added, mixed thoroughly and incubated at -20 °C for over night. The supernatant was discarded after centrifuging the content at 13000 rpm for 15 min. at room temperature. 1.5 ml of ethanol was added and centrifuged again at 13000 rpm for 5 min at room temperature. The supernatant was discarded carefully and the pellet was dried in vacuum. The dried pellet was dissolved in 50 µl of TE buffer containing 1.0 µl of RNAse.

**TESTING GENOMIC DNA**

One percent agarose gel was prepared using 0.5X TE buffer. Before casting the gel, 2 µl of ethidium bromide was added. After solidification of gel, it was introduced into a gel electrophoresis tank, which was filled with 0.5X TBE buffer. A known quantity of DNA was mixed with few µl of loading dye. The mixture was loaded in the well and current of 120 volts was passed using power packer. The DNA ran along with dye. When the loading dye reached ¾ of the gel, the same was removed from the tank and placed on the plat form of UV- illuminator and observed for DNA bands and the same was documented using Alpha-imager.

**AMPLIFICATION OF *LOVE* AND *LOVF* GENES FROM GENOMIC DNA**

For amplification of *lovE* and *lovF* genes present in *Monascus purpureus* isolates, the following primers were designed based on the sequences of the *Aspergillus terreus* lovastatin biosynthesis gene cluster in the genebank (AF141925 and were procured from M/s Chromous biotech., Bangalore.

***lovE*** **Fwd: GGGCCATGGCTGCGATCAAGGTATATTCA**

**Rev: GCTGGATCCGTTCATGGAGGAATATTGTTGAGG**

**lovF Fwd: GCGTCGGTACATAAGGGGGG**

**Rev: GTGGTTCCAAGGGTAGGGCGG**

In a 0.5 ml microfuge tube, reagents were mixed in the following order: 2.0 µl of 10x Taq buffer, 0.5 µl of Taq polymerase, 1.0 µl of $MgCl_2$, 2.0 µl of dNTPs, 1.0 µl of forward primer, 1.0 µl of reverse primer, 3.0 µl of genomic DNA and 9.5 µl of sterile water.

**PCR CONDITIONS FOR *LOV E* PRIMERS**

The amplification conditions were as follows: denaturation at 94 °C for 5 minutes; then 40 cycles, each consisting of denaturation (94 °C for 30 seconds), annealing (50 °C for 20 seconds) and extension (72 °C for 1minute and 40 seconds); and finally a single extension at 72°C for 20 minutes and 10 °C for ever.

**PCR CONDITIONS FOR *LOV F* PRIMERS**

The amplification conditions were as follows : denaturation at 95 °C for 5 minutes; then 30 cycles, each consisting of denaturation (95 °C for 1 minute), annealing (50 °C for 1 minute) and extension (72 °C for 1minute); and finally a single extension at 72 °C for 10 minutes and 10 °C for ever.

## ELUTION OF DNA FROM AGAROSE GEL

Maximum quantity of PCR product was run on 1 % agarose gel and placed over the plat form of an UV illuminator. The DNA band of interest was cut by a sharp scalpel. The cut DNA gel was transferred to a pre-weighed 1.5 ml eppendorf tube. The actual weight of the DNA gel was obtained. Based on the weight of the DNA gel, the quantity of gel solubilizer to be added was calculated. 300 µl of gel solubilizer was added to DNA gel and incubated at 50 °C for 5-10 min. with vortexing for every 2-3 min. The pink colour formed was converted to orange colour by addition of 10 µl of sodium acetate pH 5.2. The content was transferred to the Genei pure column with 2.0 ml collection tube.100 µl of iso-propanol was added to the contents of the column and centrifuged at 11000 rpm for 1 min. The flow through was discarded and the Genei pure column was placed back in 2 ml collection tube. 700 µl of diluted wash buffer was added and centrifuged for 1 min at 11000 rpm. The flow through was discarded and the column was placed back in the collection tube. The centrifugation was repeated for 2 min to remove traces of wash buffer. The column was opened and a fresh 1.5 ml vial was placed at the back of column. The column was incubated at 70 °C over a dry bath for 2 min to remove excess ethanol. 30 µl of prewarmed (70 °C) elution buffer was added and incubated for 1 min., centrifuged at 11000 rpm for 1 min and the eluted DNA was stored at -20 °C.

## LIGATION OF INSERT DNA

The DNA eluted from the gel was run in 1% agarose gel to confirm the availability. Ligation of the DNA was carried out as follows:

| | |
|---|---|
| 10x Ligation buffer | -1.0 µl |
| T4DNA Ligase | - 0.5 µl |
| T/A cloning vector | - 1.0 µl |
| Insert DNA | - 8.0 µl |
| Nuclease free water | - 9.5 µl |

The reaction content was incubated over night at 16 °C in an incubator

**TRANSFORMATION OF T/A VECTOR TO *E. COLI* (DH5α)**

One ml of actively growing DH5α was transferred into a 250ml flask containing 30 ml of LB broth. After inoculation, the culture was incubated for 3-4 h at 37 °C. The culture was transferred to sterile ice cold poly propylene tubes. The culture was cooled at 0 °C for 10 min. The content was centrifuged at 5000 rpm for 5 min at 4 °C. The supernatant was decanted and the excess medium was removed by standing the tubes over paper towels in inverted condition for 1 min. The pellet was resuspended in 25 ml 0.1 M ice-cold $CaCl_2$ by gentle vortexing and centrifuged at 5000 rpm for 2 min. The supernatant was discarded and the above step was repeated. The pellet was dissolved in 1.5 ml of 0.1 M ice-cold $CaCl_2$.

100 µl of the competent cell was transferred to a sterile eppendorf tube. Ligated DNA (8 µl) was added to the competent cell in the tube. The content was mixed by gentle swirling. The eppendorf tube was stored on ice for 30 min. Then the tube was transferred to a preheated water bath set at 42 °C for 90 sec. Later the tube was rapidly transferred to an ice bath and allowed to chill for 1-2 min. To the tube, 400 µl of LB broth was added. The culture was incubated at 37 °C for 45 min.

100 µl of culture (transformed competent cells) was transferred on to LB agar (Xgal 50 µl and ITPG 10 µl) medium containing 100 µg/ml of ampicillin. The plates were stored at room temperature. Then the plates under inverted condition were incubated at 37 °C over night. The transformed colonies started appearing in 12-16 h. Plasmid T/A vector (pTZ57R/T) with inserted gene transformed cells appears as white colonies and without insert appears as blue colonies. White colonies and few blue colonies were transferred to new LB agar ampicillin plates and the cells with insert were screened.

**PLASMID ISOLATION**

The bacterial culture and microfuge tubes were chilled on ice for 5 min. Solution I, II and III were prepared freshly and kept on ice. Approximately 1.5 ml of culture was poured into eppendorf tube and centrifuged at 15000 rpm for 30 sec at 4 °C and the supernatant was discarded. The step was repeated once again. The pellet was resuspended in 200 µl of ice cold solution I by vigorous vortexing. 200 µl of freshly prepared solution II was added, mixed thoroughly and stored on ice for 5 min. 200 µl of ice cold solution III was added, mixed thoroughly and stored on ice for 3-5 min. The content was centrifuged at a maximum speed for 10 min at 4 °C and the supernatant was transferred to a fresh eppendorf tube. An equal volume of phenol: chloroform was added and mixed well. The emulsion was centrifuged at maximum speed for 2 min at 4 °C. The aqueous upper layer was transferred to a fresh tube. Two volumes of 100% ethanol or 0.6 volume of iso-propanol was added, mixed by vortexing and then the mixture was allowed to stand at room temperature for 2 min. the content was centrifuged at maximum speed for 10 min at 4 °C. The supernatant was removed by gentle aspiration. One ml of 70% ethanol was added to the pellet and mixed several times. The plasmid DNA was

recovered by centrifugation at 15000 rpm for 5 min at 4 °C. The pellet was dried and dissolved in 50 µl of TE (pH 8.0) containing 20 µg/ml DNAse free RNAse A.

## CONFIRMATION OF PRESENCE OF INSERT DNA BY USING M13 PRIMERS

The presence of insert DNA in the vector was confirmed by PCR amplification technique. The reaction was carried out as follows:

| | |
|---|---|
| Taq buffer | -2.0 µl |
| Taq pol | -0.7 µl |
| dNTP | -2.0 µl |
| $MgCl_2$ | -1.0 µl |
| M13 (F) primer | -0.5 µl |
| M13 (R) primer | -0.5 µl |
| Plasmid DNA | -2.0 µl |
| Sterile water | -11.3 µl |

The *E.coli* culture carrying the plasmid containing insert DNA was sent to Bangalore Genei, Bangalore for sequencing.

## CHARACTERIZATION OF *MONASCUS PURPUREUS* AND ITS MUTANTS FOR GABA PRODUCTION

The precursor of GABA, monosodium glutamate (MSG) was added in the solid state medium with coarsely ground finger millet in 500 ml Erlenmeyer flasks and the media was inoculated with the spores of parent (*Monascus purpureus* MTCC 410) and its albino and hyper mutants. In this experiment, the solid state media was prepared with different concentration of

MSG viz. 0, 2.5, 5.0, 7.5, 10.0 and 12.5 % (w/w) and the moisture content was adjusted to 50% by adding distilled water. The media were sterilized and allowed to cool at room temperature. Then the media was inoculated with 2% (v/w) spore suspension of parent and mutants separately and the content was mixed thoroughly. The inoculated flasks were incubated at 30°C for a period of 10 days. After ten days the flasks were withdrawn and GABA was estimated enzymatically as mentioned above.

## STATISTICAL ANALYSIS

Results are expressed as means ± SEM and statistical analyses were performed using Duncan's Multiple Range Test for comparison of means using SPSS software. *p* values less than 0.05 were considered significant.

# CHAPTER 1

## CHARACTERIZATION OF BIOACTIVE MOLECULES (STATIN AND GABA) PRODUCED BY *MONASCUS PURPUREUS* (MTCC- 410) DURING CEREAL AND MILLET FERMENTATION

Finger millet is a crop of tropics and sub-tropics and can be raised successfully from sea level to an altitude of 2,300 m on hill slopes, as well as plains. It grows best in moist climate. Finger millet *(Eleusine coracana)*, a typical tropical crop belongs to the group millets. It is mainly consumed in India and Africa as *porridge* and *stiff porridge* (*mudde).* In developing countries, finger millet malt is used in weaning foods. This is important millet because of its excellent storage properties of the grains and high nutritive value, which is higher than that of rice and comparable to that of wheat (Shashi et al. 2007).

Ascomycete fungus, *Monascus purpureus*, traditionally known as red yeast rice was reported to produce metabolites like polyketides, antihypercholesterolemic agents, antihypertensive metabolite, lipid lowering fractions and other unsaturated fatty acids like oleic, linoleic, linolenic acids, etc. The culture filtrate of *Monascus purpureus* is also known to contain antioxidant and antibacterial principles.

The production of red yeast rice used as a foodstuff was recorded in old Chinese literature (Hu, 1982). More recently, it has been included and formulated by the Chinese Ministry of Health into their modern food additive standards (National Standard, 1982) to increase the color and delicacy of meat, fish, and soybean products as part of the Chinese diet. The naturally produced pigments from the red yeast give the food its characteristic coloration (Fabre *et al.* 1993), and much research has been performed on identifying the naturally produced

metabolites responsible for this pigmentation (Blanc *et al.* 1994a). Furthermore, pigments of *Monascus purpureus* are authorized for food use in Japan (Blanc *et al.* 1994b). Red yeast rice is used to colour a wide variety of food products, including pickled tofu, red rice vinegar, char siu, Peking Duck, and Chinese pastries that require red food colouring. It is also traditionally used in the production of several types of Chinese wine, Japanese sake (akaisake), and Korean rice wine (hongju), imparting a reddish colour to these wines. Although used mainly for its colour in cuisine, red yeast rice imparts a subtle but pleasant taste to food and is commonly used in the cuisine of Fujian regions of China.

*Monascus purpureus* is normally cultivated on cooked rice to produce a range of secondary metabolite, statin *viz.* lovastatin, monacolin J, pravastatin and mevastatin (Manzoni et al 1999) known as Monacolins. These metabolites inhibit the enzymatic conversion of hydroxymethyl-glutarate to mevalonate by HMG- CoA reductase, which is the important step in the biosynthetic pathway of cholesterol (Manzoni and Rollini 2002; Heber et al 1999). Statin inhibits the action of HMG-CoA reductase by it's the structural similarity with the substrate, hydroxymethyl-glutarate. Statin is commonly used as a medicine in the therapy of hypercholesterolemia (Chen and Johns 1993).

Fermented red rice of *Monascus purpureus* also contains lipid lowering constituents like beta sitosterol and campesterol in addition to statins. Dietary sterol helps in decreasing LDL serum level there by reducing total cholesterol without affecting HDL and triglycerides level (Law 2000; Ostlund 2002). Statin in combination with dietary sterols produced by the fungi has been suggested as a more effective means of lowering cholesterol level than statin alone in the therapeutic use of anticholesterolemia (Plat and Mensink 2001).

Lipid content (%) in red yeast rice constitutes palmitic acid (0.56), stearic acid (0.50), oleic acid (0.62), linoleic acid (0.74), arachidic acid (0.09), linolenic acid (0.36), total unsaturated fatty acids (1.43), and total fatty acid (2.84). Two sets of fatty acids are represented, the saturated acids consisting mainly of palmitic, stearic, and arachidic acids and the unsaturated acids mostly represented by oleic, linoleic, and linolenic acids (Ma *et al.* 2000). RYR also contains B-complex vitamins (niacin) which has role in decreasing serum lipids such as triglycerides and cholesterol**.**

## 1.1. NATURAL COLOURANTS FROM *MONASCUS PURPUREUS*

*Monascus* pigments called azaphilones, with similar molecular structures and chemical properties. These pigments are synthesized by the condensation of acetyl Co-A molecule with malonyl Co-A in the cell cytosol region of the fungus mycelium. The various pigments produced by the fungus are orange, yellow and red. Recently, two pigments which are blue in colour and fluorescent in nature were identified from the *Monascus* spp. Ankaflavine and monascine are yellow pigments, rubropunctatine and monascorubrine are orange and rubropunctamine and monascorubramine are red. These pigments are less water soluble and sensitive to heat and light.

The pigment production capability of the fungal isolate *Monascus purpureus* MTCC-410 was confirmed and the substrate suitability for pigment production was studied by cultivating the fungus on ten different substrates *viz.* Raw rice, Parboiled rice, Njavara rice (Oryza sativa), Finger millet, Germinated finger millet (*Eleusine coracana*) Broken wheat (*Triticum aestivum*), ,Sorghum (*Sorghum vulgare*), Bajra (*Pennisetum thyphoides*) and Maize (*Zea mays*) under solid state fermentation for seven days. The fungus exhibited good growth on all the above substrates. Polyketide pigments were extracted with 70% ethanol and quantified at 500, 475, and 375 nm

wavelength for red, orange and yellow pigment and the same is presented in **Table 4**.*Monascus purpureus* MTCC 410 with raw rice as substrate produced maximum pigment yield of 337.8 OD units with 148.0, 85.8 and 104.0 OD units of yellow, orange and red pigment respectively. Parboiled rice, Maize and germinated finger millet fermented with *Monascus purpureus* exhibited total pigment yield of 248.0, 160.4 and 151.8 OD units respectively. Further, it is observed that the yellow pigment production was high in most of the fermented substrates followed by red and orange pigments. The total pigment yield of different substrates used in the fermentation is portraited in **Figure 20**.

**Table 4. Polyketide pigment production by *Monascus purpureus***

| **Substrates used in fermentation** | **Pigment yield OD unit** | | | | | | **Total Pigment yield** |
|---|---|---|---|---|---|---|---|
| | **Yellow (375 nm)** | **(%)** | **Orange (475 nm)** | **(%)** | **Red (500 nm)** | **(%)** | |
| Raw rice | 148.0±1.23 | | 85.8±0.78 | | 104.0±0.82 | | 337.8±2.11 |
| Parboiled rice | 92.0±0.98 | | 70.0±0.62 | | 86.0±0.65 | | 248.0±1.89 |
| Njavara (Medicinal rice) | 29.5±0.23 | | 13.8±0.08 | | 10.0±0.09 | | 53.3±0.48 |
| Finger millet | 14.8±0.09 | | 9.3±0.05 | | 9.3±0.02 | | 33.4±0.30 |
| Germinated finger millet | 64.3±0.61 | | 41.5±0.21 | | 46.0±0.32 | | 151.8±1.59 |
| Bajra | 30.9±0.28 | | 19.1±0.04 | | 22.2±0.06 | | 72.2±0.68 |
| Sorghum | 14.8±0.07 | | 11.3±0.03 | | 12.8±0.04 | | 38.9±0.31 |
| Maize | 64.3±0.56 | | 43.3±0.31 | | 52.8±0.48 | | 160.4±2.01 |
| Barley | 52.5±0.42 | | 33.5±0.26 | | 42.8±0.39 | | 128.9±1.21 |
| Broken wheat | 43.5±0.32 | | 45.8±0.38 | | 59.8±0.48 | | 149.1±1.69 |

Data represents the mean of three replications

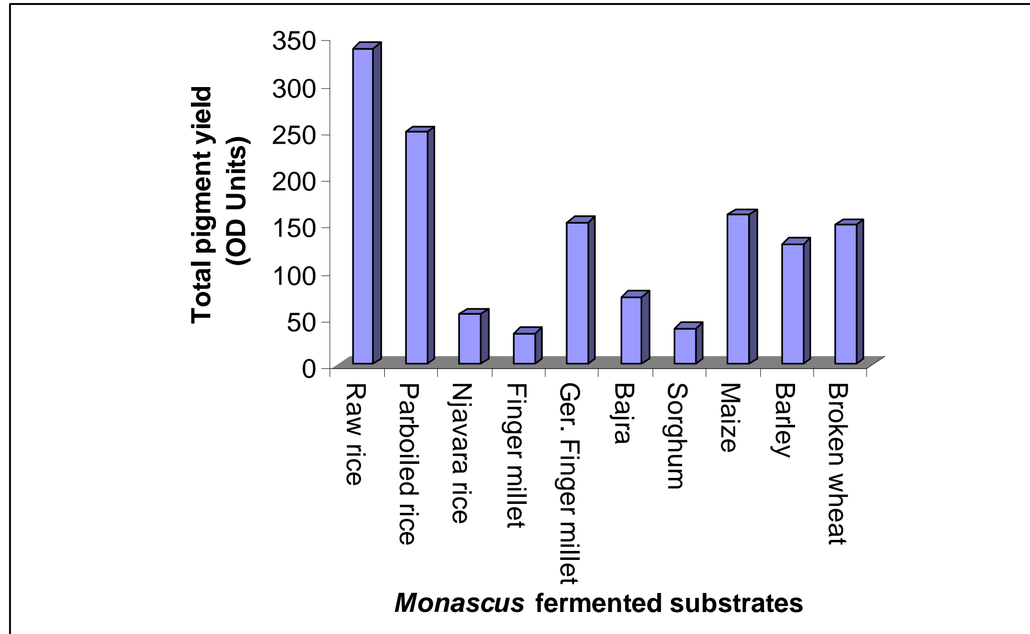



**Figure20. Total pigment yield of *Monascus* fermented substrates**

**Figure 21** shows the *Monascus* fermented germinated finger millet grain which is bright red in colour due to the synthesis of pigment by the fungus.

**Figure 21. Germinated finger millet before and after fermentation with *Monascus purpureus* MTCC 410**

## 1.2. STATIN FROM *MONASCUS PURPUREUS*

Statins are fungal secondary metabolites selectively inhibits HMG-CoA reductase, the first enzyme in cholesterol synthesis. Statins posses a common polyketide portion hydroxy hexa hydro naphthalene ring, to which different side chains are linked at C6 and C8. They are capable of lowering blood cholesterol level up to 60%. They specifically lower LDL cholesterol levels and even produce some increases in HDL cholesterol levels.

*Monascus purpureus* MTCC 410 was grown on PDA slants and incubated for two weeks for good sporulation. The spore suspension was prepared by following the method as furnished in Materials and Methods. The substrates *viz.* raw rice, parboiled rice, finger millet, germinated finger millet, broken wheat, njavara (medicinal rice), sorghum, bajra and maize after adjustment of initial moisture level at 50-60% and sterilization were inoculated with the spores of *Monascus purpureus*. The pH and moisture content of the substrates before fermentation were determined. The fungus was cultivated under solid state fermentation for seven days at 30°C. After seven days of SSF, the pH, moisture content and loss of organic matter (LOM) of the fermented substrates were estimated **(Table 5)**.

**Table 5. pH, moisture content and loss of organic matter of the substrates before and after fermentation**

| S.No. | Substrates | pH of the substrate | | Moisture content (%) | | Loss of organic matter (%) |
|---|---|---|---|---|---|---|
| | | Before fermentation | After fermentation | Before fermentation | After fermentation | |
| 1. | Raw rice | 6.80±0.09 | 5.01±0.04 | 65.2 | 35.2 | 20.6 |
| 2. | Parboiled rice | 6.74±0.11 | 4.81±0.07 | 65.0 | 40.3 | 28.7 |
| 3. | Njavara (medicinal rice) | 6.64±0.10 | 5.12±0.03 | 64.2 | 41.2 | 19.9 |
| 4. | Finger millet | 6.13±0.07 | 5.13±0.02 | 52.1 | 44.2 | 18.9 |
| 5. | Germinated finger millet | 5.85±0.08 | 4.40±0.06 | 53.3 | 30.1 | 32.6 |
| 6. | Bajra | 6.21±0.09 | 5.69±0.05 | 55.0 | 36.6 | 19.8 |
| 7. | Sorghum | 6.52±0.07 | 5.47±0.06 | 58.1 | 40.2 | 19.6 |
| 8. | Maize | 6.79±0.03 | 5.02±0.01 | 56.6 | 38.1 | 28.9 |
| 9. | Barley | 6.03±0.05 | 5.50±0.08 | 61.2 | 45.2 | 19.5 |
| 10. | Wheat (Broken) | 6.74±0.02 | 4.73±0.10 | 58.3 | 32.1 | 30.2 |

Data represents the mean of three replications

The pH of all the substrates before fermentation was in the range of 6.13 to 6.80 except germinated finger millet, which exhibited pH of 5.85. After seven days of fermentation, all substrates except germinated finger millet showed a decrease in pH from 5.3 to 4.8. *Monascus* fermented germinated finger millet exhibited pH of 4.4, which was much lower than other fermented substrates along with the maximum reduction in moisture content, indicating the

growth and metabolism of fungus. Similarly, maximum loss of organic matter (LOM) of 32.6% was observed in *Monascus* fermented germinated finger millet. The production of organic acids during fermentation could be the reason for steep drop in pH. The results are comparable to the observations of Sripriya et al 1997 on pH during germination and fermentation of finger millet, where the initial pH of finger millet (5.8) was reduced to (5.7) due to germination and further reduction was observed from 6 to 12 h of fermentation (5.6 - 4.6), which was attributed to the production of organic acids like lactic acid, acetic acid and citric acids during fermentation.

The statin extracted was quantified by HPLC. The peaks of standard pravastatin and lovastatin were obtained at a retention time of 2.063 min. and 7.097 min. respectively **(Figure 22)**.

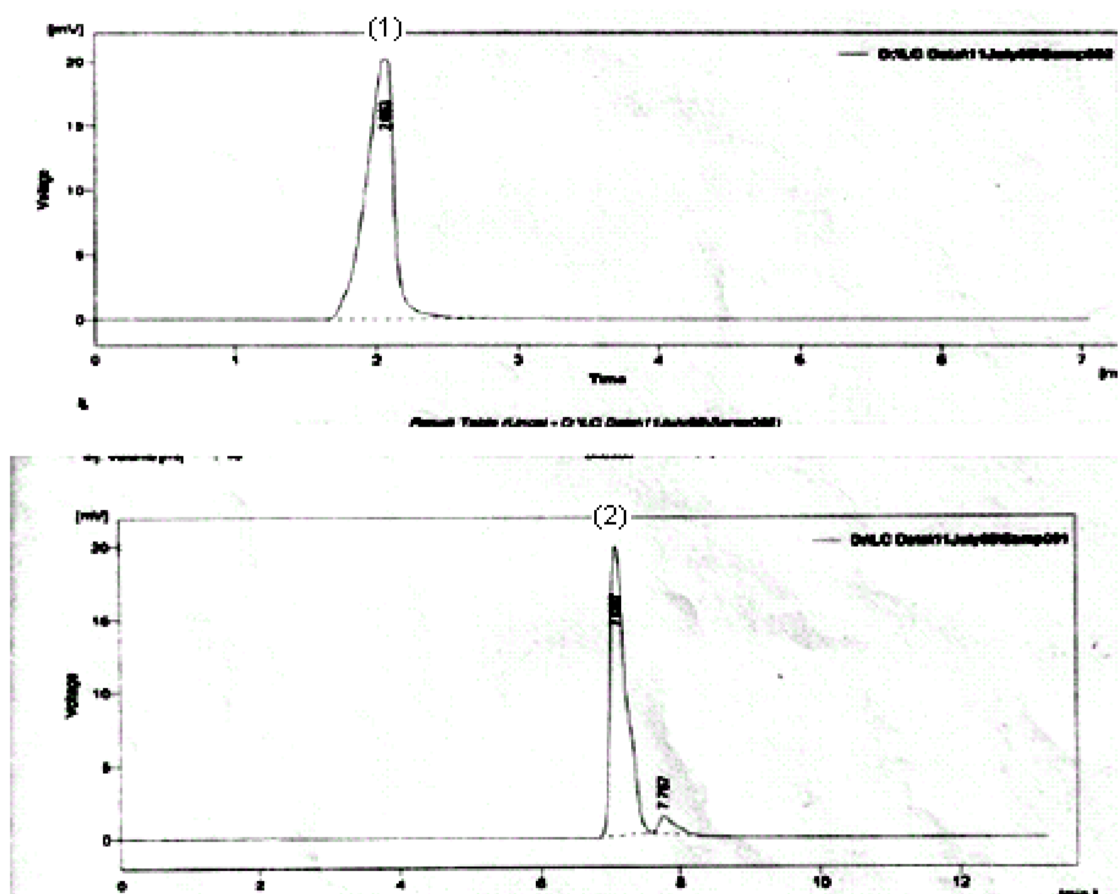



**Figure 22. HPLC profile of standard pravastatin(1) and lovastatin(2)**

The HPLC of statin from the germinated fermented finger millet exhibited pravastatin and lovastatin peaks at 1.850 min and 7.233 min respectively **(Figure 23)**. **Table 6** represents the total yield of statin from different substrates fermented with *Monascus purpureus*. The germinated finger millet fermented with *Monascus purpureus* yielded higher total statin content of 5.24 g/kg (pravastatin and lovastatin content of 4.87 g/kg and 0.37 g/kg respectively) followed by broken wheat (4.41 g/kg) and parboiled rice (4.10 g/kg) **(Table 6)**.

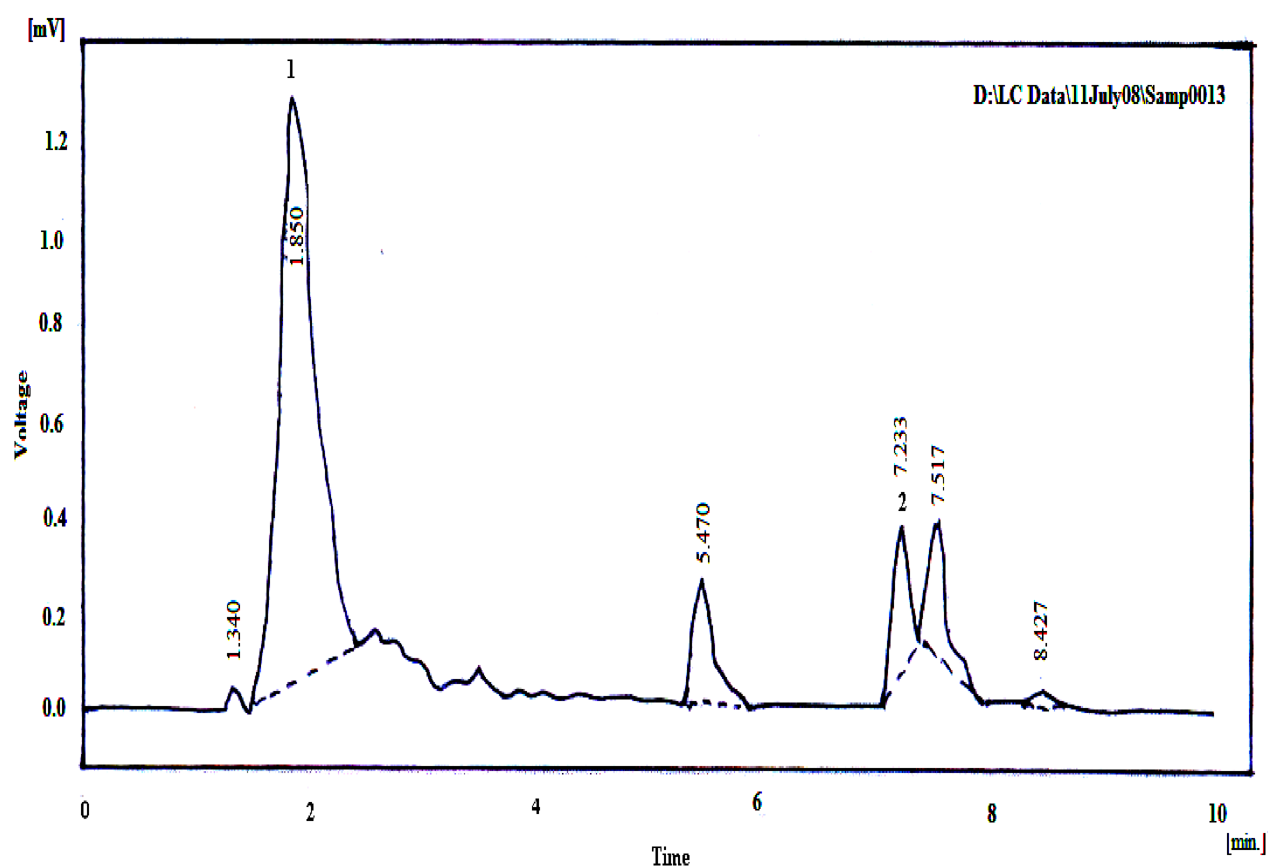



**Figure 23. HPLC profile of pravastatin (1) & lovastatin (2) from *Monascus purpureus* fermented germinated finger millet extract**

**Table 6. Statin yield (g/kg dry wt) by *Monascus purpureus* on different substrates**

| S.No. | *Monascus* fermented substrate | Statin yield g/kg dry wt. | | |
|---|---|---|---|---|
| | | Lovastatin | Pravastatin | Total statin |
| 1. | Raw rice | 0.64 ± 0.01 | 2.65 ± 0.01 | 3.29 ± 0.03 |
| 2. | Parboiled rice | 0.52 ± 0.02 | 3.58 ± 0.02 | 4.10 ± 0.04 |
| 3. | Njavara (Medicinal rice) | 0.16 ± 0.01 | 1.79 ± 0.01 | 1.95 ± 0.02 |
| 4. | Finger millet | 0.08 ± 0.00 | 0.96 ± 0.02 | 1.04 ± 0.02 |
| 5. | Germinated finger millet | 0.37 ± 0.01 | 4.87 ± 0.03 | 5.24 ± 0.09 |
| 6. | Bajra | 0.11 ± 0.02 | 2.88 ± 0.08 | 2.99 ± 0.18 |
| 7. | Sorghum | 0.17 ± 0.01 | 1.79 ± 0.01 | 1.96 ± 0.01 |
| 8. | Maize | 0.47 ± 0.01 | 3.58 ± 0.02 | 4.05 ± 0.02 |
| 9. | Barley | 0.22 ± 0.02 | 1.67 ± 0.02 | 1.89 ± 0.03 |
| 10. | Wheat (Broken) | 0.06 ± 0.01 | 4.35 ± 0.02 | 4.41 ± 0.03 |

Data represent Mean ± SE of duplicate analyses of duplicate samples

Germination helps in the degradation of antinutrient factors like phytic acid and tannins in finger millet grain, hydrolysis of complex carbohydrate to simple sugars and availability of other nutrients and minerals (Mbithi-Mwikya et al 2000) which are required by the fungus for its growth and metabolites production. In this study, the results showed that the yield of statin and sterol are considerably high when germinated finger millet was used as a substrate. Hence, germinated finger millet was used for further studies in the production of statins.The statin yield by *Monascus purpureus* fermented germinated finger millet on different days of fermentation is presented in **Figure 24**. From day 3, the yield of statin showed increasing trend and reached maximum of 5.24 g/kg dry wt on the $7^{th}$ day of fermentation which was significantly higher.

However, the statin yield showed decreasing trend after 7th day of fermentation.

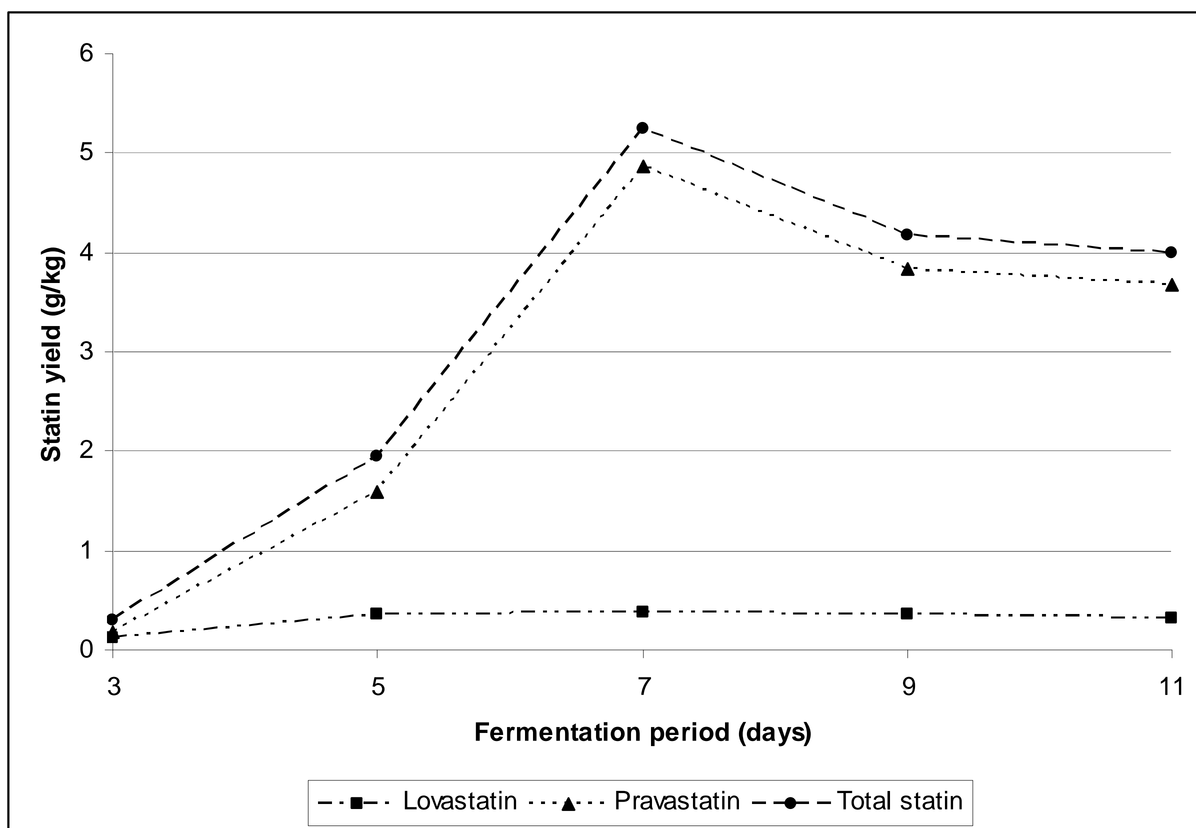



**Figure 24. Statin production by *Monascus purpureus* grown on germinated finger millet**

## 1.3. STEROL FROM *MONASCUS PURPUREUS*

The sterol content of the raw and fermented substrates was derived from the calibration graph drawn using standard cholesrterol **(Figure 25)**. *Monascus* fermented barley, maize and raw rice yielded total sterol of 0.14, 0.09 and 0.08 g/kg dry substrate respectively. Germinated finger millet also exhibited good yield of 0.053 g/kg sterol as against 0.02 g/kg sterol yield of finger millet **(Figure 26).** 7.57, 10.69 and 13.66 folds increase in sterol production was observed in substrates like germinated finger millet, barley and broken wheat respectively, when fermented with *Monascus purpureus* than the control. The remarkable increase in sterol yield is due to the production of sterol by *Monascus purpureus* during SSF. The results obtained in this study were

supported by the report of Heber et al 1999, wherein it was stated that the red yeast rice produces sterols such as β- sitosterol and campasterol in addition to other bioactive molecules. The structural similarity of sterols to cholesterol enables them to compete with cholesterol for incorporation into micelles and thereby lowers the dietary cholesterol and the cholesterol accumulated in the gastrointestinal tract (Lichtenstein 2002, Clifton 2002). Simons (2002) demonstrated that the effect of using a plant sterol ester in combination with statin is equivalent to doubling the dose of statins for the treatment of antihypercholesterolemia.

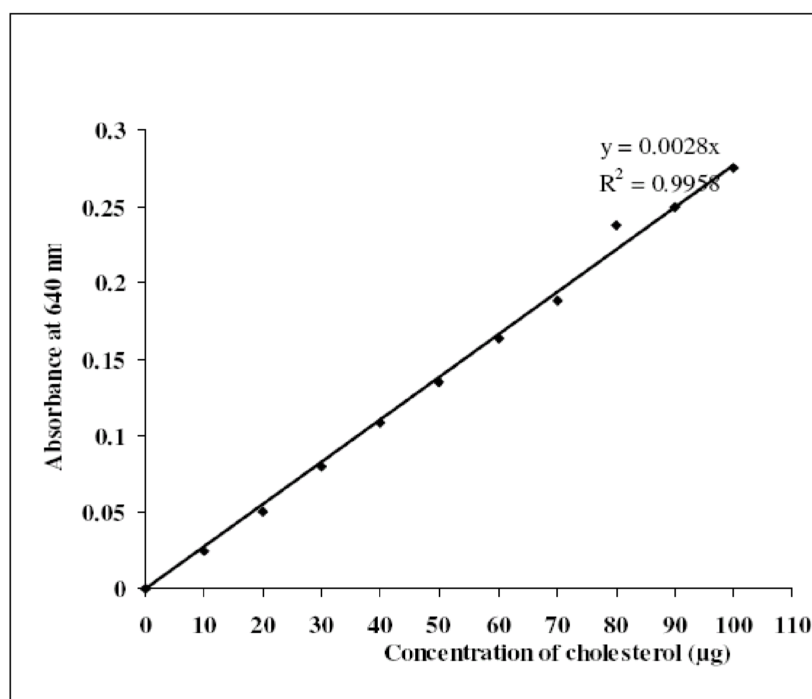



**Figure 25. Standard calibration graph of cholesterol**

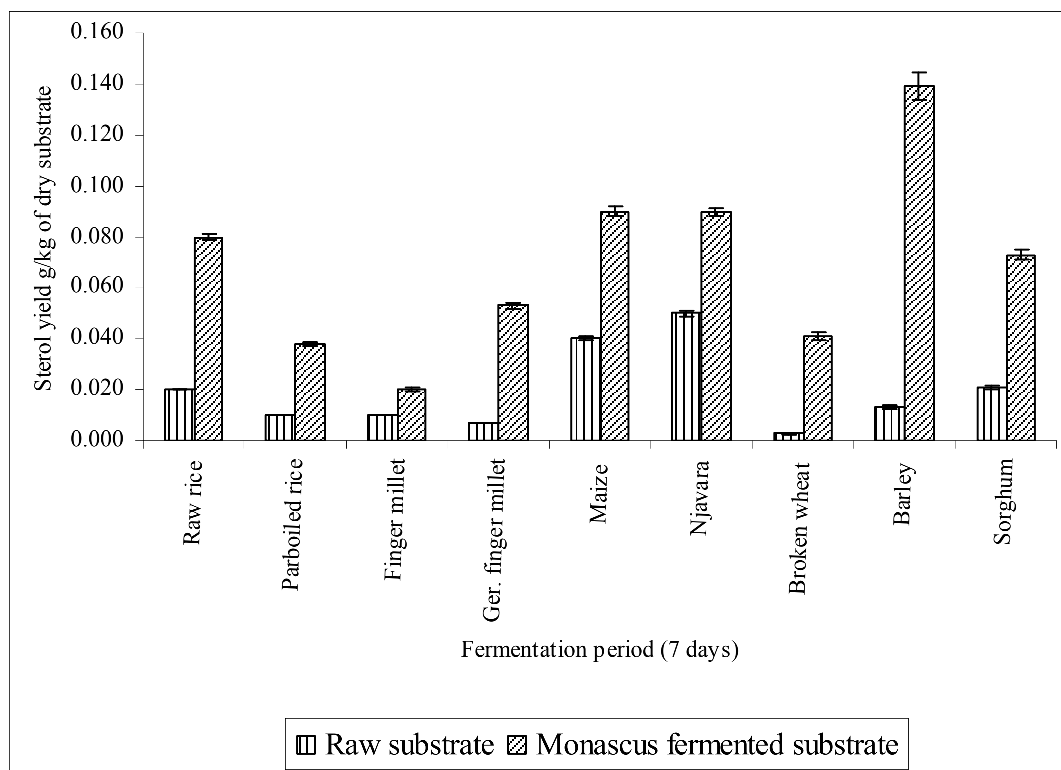



**Figure 26. Sterol production by *Monascus purpureus* on different substrates**

## 1.4. GABA FROM *MONASCUS PURPUREUS*

GABA was extracted from the *Monascus* fermented substrates using ethanol water mixture and estimated by GABAse enzymatic assay as described in Materials and Methods. The yield of GABA for various fermented substrates is presented in **Table 7**. Germinated finger millet exhibited maximum yield of GABA 110.13 mg /kg of dry substrate at the end of 7 days of fermentation. Raw rice, parboiled rice and medicinal rice produced GABA yield of 106.56, 98.87 and 97.49 mg/kg of dry substrate respectively. GABA yield of finger millet was lower (52.58 mg/kg) than other substrates. The increased production of GABA in germinated finger millet might be due to availability of nutrients because of germination.

**Table 7. GABA production by *Monascus purpureus***

| Substrates used in fermentation | GABA yield (mg/kg dry substrate) |
|---|---|
| Raw rice | 106.56±2.11 |
| Parboiled rice | 98.87±1.22 |
| Njavara (Medicinal rice) | 97.49±1.02 |
| Finger millet | 52.58±0.65 |
| Germinated finger millet | 110.13±1.62 |
| Bajra | 62.66±0.92 |
| Sorghum | 60.81 ±0.98 |
| Maize | 67.93±1.01 |
| Barley | 50.31±0.89 |
| Broken wheat | 66.94±1.00 |

Data represents the mean of three replications ±SE

### 1.5. FATTY ACIDS FROM *MONASCUS PURPUREUS*

The fatty acid composition of raw and *Monascus* fermented substrates were determined using Gas Chromatography after extraction of lipid and converting them into their methyl esters as described in Materials and methods. The fatty compositions of the raw and fermented substrates are represented in **Figures 27-32** and the contents are presented in **Table 8**. The fermented raw rice showed an increased unsaturated fatty acid content of 68.33% (linoleic acid - 67.97% and oleic acid- 0.36%) compared to non-fermented (39.78%). Finger millet and bajra fermented by *Monascus purpureus* exhibited 42.16 and 46.96% unsaturated fatty acids over their corresponding control values of 40.06 and 41.09% respectively. In fermented parboiled rice and

maize, the presence of linolenic acid was also observed. From the above results, it may be concluded that the increase of the unsaturated fatty acids in the fermented raw rice, finger millet and bajra was due to the metabolic activity of *Monascus purpureus* on these substrates.

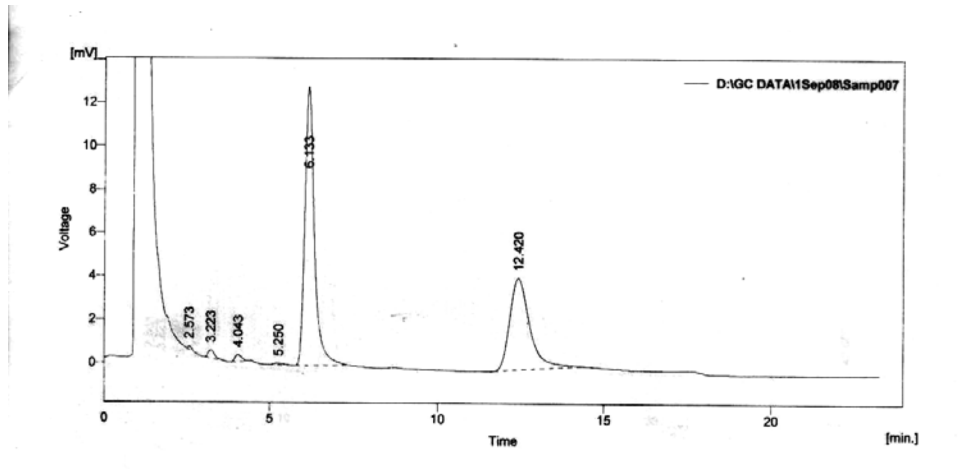



**Figure 27. Fatty acid profile of raw finger millet**

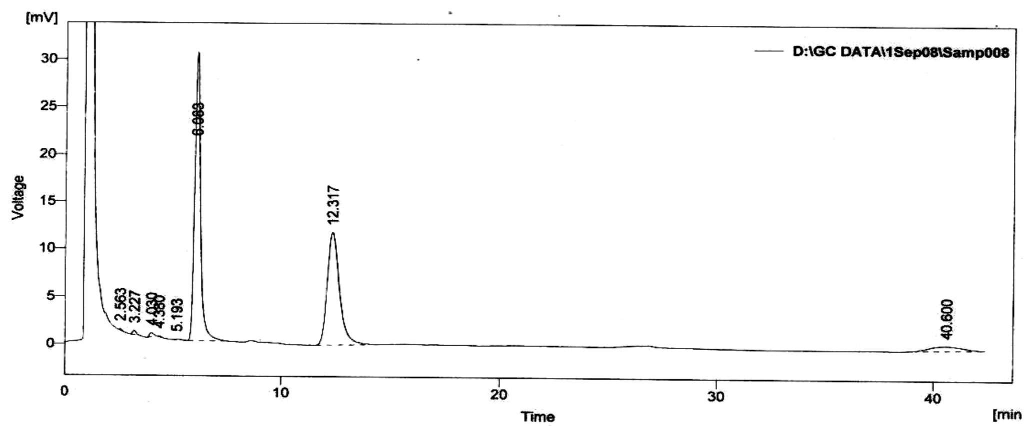



**Figure 28. Fatty acid profile of *Monascus* fermented finger millet**

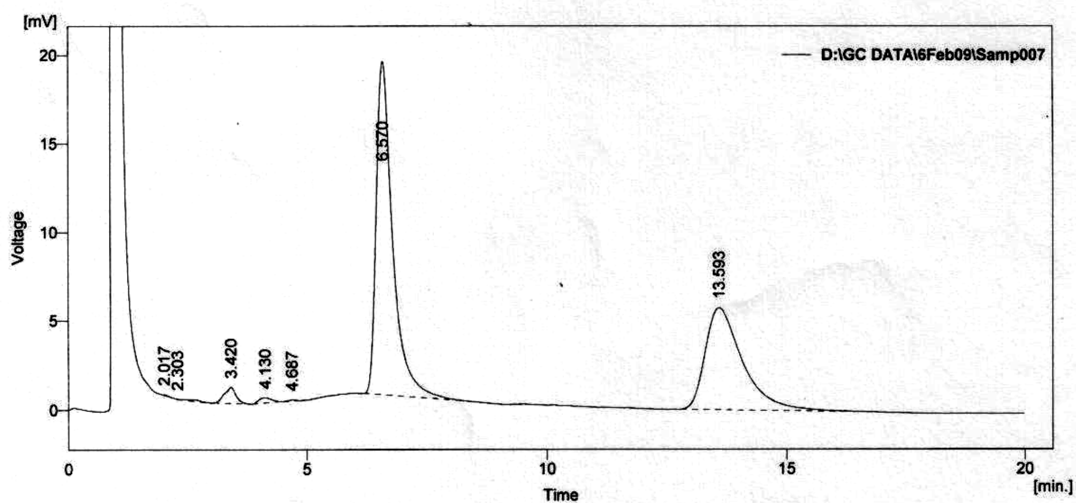



**Figure 29. Fatty acid profile of raw rice**

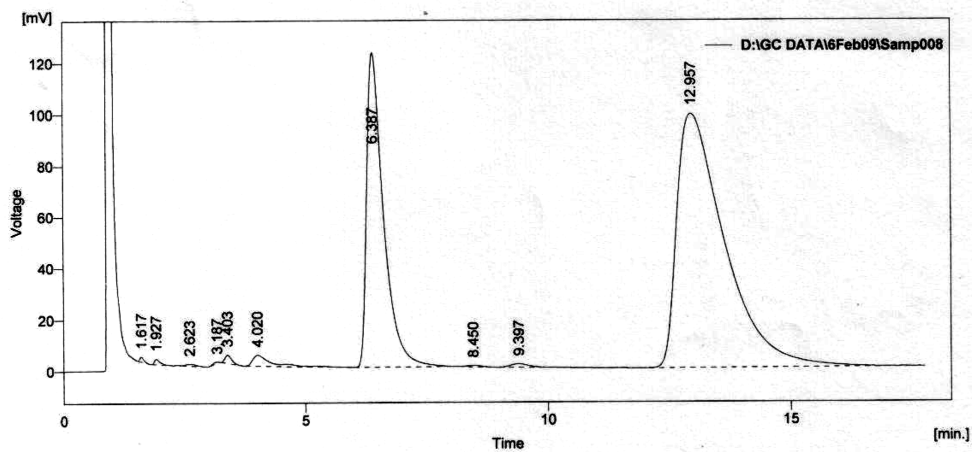



**Figure 30. Fatty acid profile of *Monascus* fermented raw rice**

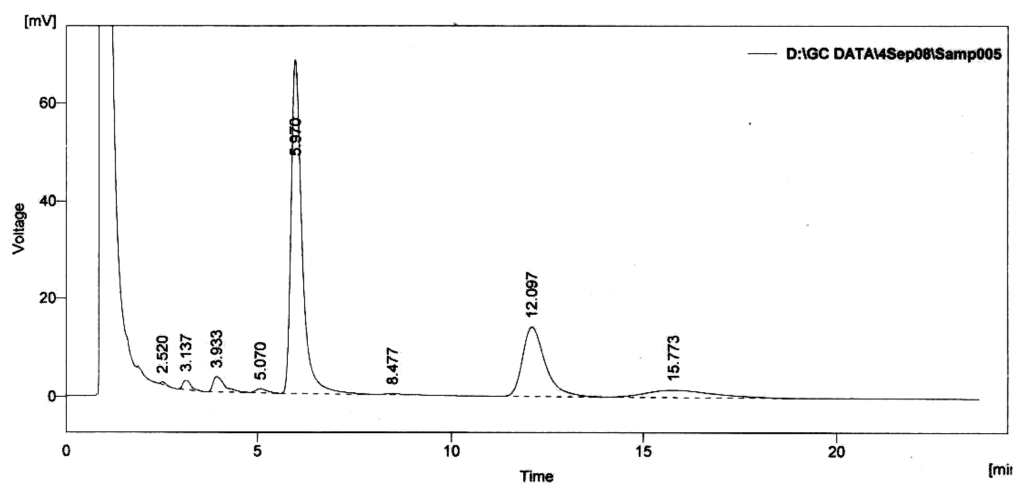



**Figure 31. Fatty acid profile of Sorghum**

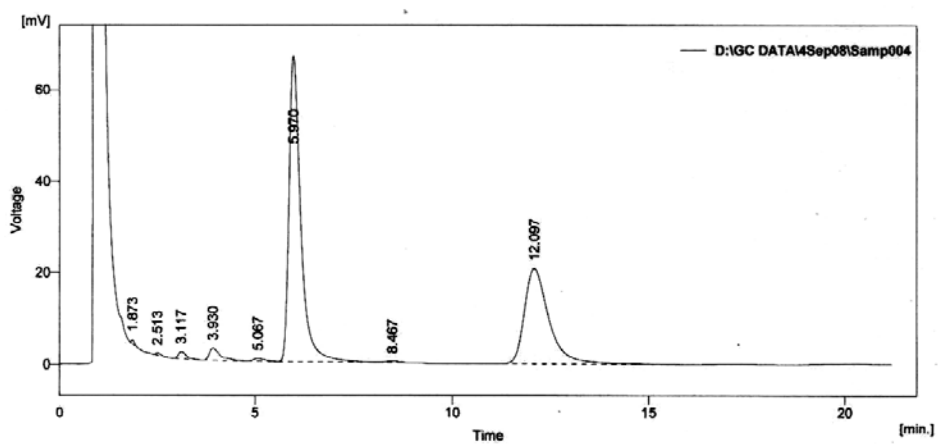



**Figure 32. Fatty acid profile of *Monascus* fermented Sorghum**

**Table 8. Fatty acid production by *Monascus purpureus***

| **Raw substrates** | **Fatty acids (%)** | | | | | | |
|---|---|---|---|---|---|---|---|
| | Lauric acid | Myristic acid | Palmitic acid | Stearic acid | Oleic acid | Linoleic acid | Linolenic acid |
| Raw rice | 2.00 | 0.67 | 57.20 | - | - | 39.78 | - |
| Parboiled rice | 0.78 | 0.57 | 35.81 | 0.46 | - | 62.19 | - |
| Njavara (Medicinal rice) | 1.70 | 1.44 | 51.18 | 0.18 | - | 45.04 | - |
| Finger millet | 1.21 | 1.09 | 56.97 | - | - | 40.06 | - |
| Germinated finger millet | 1.12 | 0.79 | 50.14 | 0.34 | - | 46.56 | - |
| Bajra | 0.98 | 1.01 | 59.18 | - | - | 41.09 | - |
| Sorghum | 0.15 | 1.71 | 70.76 | - | - | 26.30 | - |
| Maize | 0.18 | 2.17 | 42.89 | 0.37 | - | 52.84 | - |
| Barley | 0.63 | 0.39 | 46.70 | 0.81 | - | 51.26 | - |
| Broken wheat | 1.46 | 1.19 | 50.71 | 0.67 | - | 42.64 | - |
| **Substrates fermented with *Monascus purpureus*** | | | | | | | |
| Raw rice | 0.06 | 1.19 | 29.58 | 0.12 | 0.36 | 67.97 | - |
| Parboiled rice | 0.63 | 0.49 | 48.11 | - | - | 47.85 | 2.92 |
| Njavara (Medicinal rice) | 1.24 | 0.89 | 49.08 | - | - | 48.01 | - |
| Finger millet | 0.53 | 0.51 | 51.51 | - | - | 42.16 | - |
| Germinated finger millet | 1.00 | 0.93 | 62.89 | - | - | 34.85 | - |
| Bajra | 0.41 | 0.62 | 50.21 | - | - | 46.96 | - |
| Sorghum | 0.89 | 1.61 | 46.99 | - | 0.33 | 49.33 | - |
| Maize | 1.24 | 2.34 | 58.76 | - | - | 29.54 | 7.62 |
| Barley | 0.81 | 1.35 | 68.59 | - | - | 29.26 | - |
| Broken wheat | 1.40 | 0.89 | 49.08 | - | - | 35.02 | - |

Data represents the mean of three replications

## 1.6. VALUE ADDITION TO FINGER MILLET BY GERMINATION AND FERMENTATION WITH *MONASCUS PURPUREUS* MTCC -410

Minerals play a vital role in human metabolism. Minerals like calcium and phosphorus help in building strong bones and teeth. Calcium is vital to muscle and nerve function. Magnesium stimulates bone growth and is necessary for functioning of muscles and metabolism. Iron is needed for the transport and storage of oxygen. Copper promotes iron absorption and is essential to RBC, connective tissues, nerve fibres and skin pigments. It also acts as co-factor in biochemical reactions. Zinc is necessary for growth and reproduction and it is a coenzyme for many of the enzymes that affect protein synthesis. Potassium and sodium helps in the maintenance of the body's fluid balance and promotes metabolism and muscular functions. Eventhough, finger millet contains all the minerals; their bioavailability is hindered by the antinutritional factors like phytic acid and tannins. These antinutritional factors modify the nutritional value of the individual grains.

Among millets, finger millet was reported to contain high amounts of tannins (Ramachandra et al. 1977) ranging from 0.04 to 3.47 per cent (catechin equivalent). Tannins form complex with proteins and carbohydrates, thereby decreasing nutrient bioavailability (Udayasekhara Rao and Deosthale 1988). Phytate or phytic acid, naturally occurring phosphate in the grain is the other group of antinutritional factor which significantly influences the functional and nutritional properties of foods. Phytic acid has a strong binding capacity, readily forming complexes with divalent and multivalent cations and proteins. Phytate binding renders several minerals biologically unavailable to animals and humans. However, these antinutrients can be removed by processing techniques, germination, fermentation, dehulling, etc. Sankara Rao and Deosthale (1983) reported that malting of the grain significantly reduced the phytate

phosphorus in finger millet. This reduction of phytic acid was accompanied by significant increase in micronutrients.

In this research study, finger millet was used as substrate for cultivating the fungus *Monascus purpureus* to observe major biochemical changes in relation to nutrient availability apart from the antihypercholesterolemic metabolites production. Biochemical changes with respect to carbohydrate, protein, fat, dietary fiber occurred during germination and fermentation of finger millet is presented in **Table 9**.

**Table 9. Composition of raw and processed finger millet (g/100g)**

| Composition | Raw finger millet | Germinated finger millet | *Monascus* fermented finger millet | Germinated – cum-*Monascus* fermented finger millet |
|---|---:|---:|---:|---:|
| Carbohydrate | 70.3 ± 0.7 | 54.2 ± 1.8 | 50.2 ± 0.7 | 35.8 ± 1.0 |
| Reducing sugars | 5.3 ± 0.3 | 9.6 ± 0.8 | 12.1 ± 0.8 | 11.1 ± 0.2 |
| Total Protein | 6.8 ± 0.2 | 5.2 ± 0.4 | 5.4 ± 0.2 | 4.5 ± 0.1 |
| Soluble protein | 0.3 ± 0.1 | 1.3 ± 0.2 | 1.2 ± 0.1 | 1.5 ± 0.3 |
| Fat | 1.5 ± 0.1 | 1.5 ± 0.1 | 1.7 ± 0.1 | 1.9 ± 0.1 |
| Ash | 2.4 ± 0.2 | 2.5 ± 0.1 | 2.7 ± 0.1 | 2.9 ± 0.1 |
| Dietary Fibre | 15.2 ± 1.1 | 20.1 ± 0.3 | 22.6 ± 0.4 | 25.1 ± 0.4 |

Data represents means± SD of three independent processing

## CARBOHYDRATES

The carbohydrate content of raw finger millet decreased from 70.3 % to 53.3% on germination. The carbohydrate content of fermented finger millet was 50.9 %. The sprouted

fermented finger millet showed carbohydrate content of 34.9 %. This reduction in carbohydrate content is attributed to the conversion of starch to reducing sugars during germination followed by fermentation by *Monascus purpureus*.

**PROTEINS**

Raw finger millet exhibited total protein content of 6.8 % with soluble protein content of 0.3%. The soluble proteins exhibited a steep increase of 19.1 % in 72 h of germination, which was further increased to 22.1 % by 10 d of fermentation. The *Monascus* fermented finger millet showed only 17.6 % increase of soluble protein. The reduction in the soluble protein content in the case of sprouted fermented finger millet after 10 d of fermentation may be due to the protein utilization by the fungus for its growth and metabolism.

**FATS**

Germination has no effect on the fat content of the raw finger millet. However, *Monascus purpureus* fermented finger millet and sprouted cum *Monascus purpureus* fermented finger millet showed fat content of 1.7% and 1.9% when compared to control (1.5%). This increase in lipid content might have been attributed by the growth of *Monascus purpureus* on raw finger millet and germinated finger millet.

**DIETARY FIBER**

The dietary fibre content of the processed finger millet observed to increase from raw finger millet < Germinated finger millet < fermented finger millet < germinated cum fermented

finger millet as 15.2 % < 20.13 % < 22.6 % < 25.1% respectively. The increase in dietary fibre is contributed by germination and fermentation by *Monascus purpureus.*

## MINERAL BIOAVAILABILITY, PHYTATE AND TANNINS

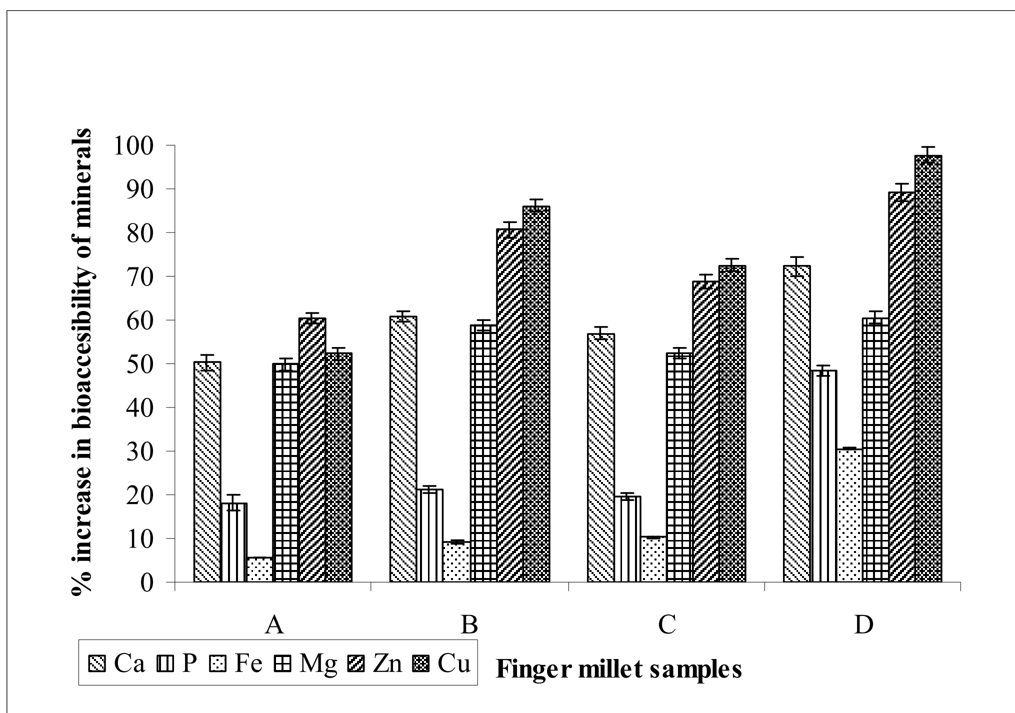
The initial pH (6.13) of finger millet dropped markedly during germination (5.85 at 72 h). The maximum drop of pH from 5.85 to 3.81 was observed after 10 d of fermentation with *Monascus purpureus.* The *Monascus* fermented finger millet exhibited pH of 5.13. The micronutrient composition of raw, germinated, *Monascus* fermented and sprouted cum *Monascus* fermented finger millet is presented in **Table 10**. The bioavailability of calcium content (mg/100g) 218.58 is significantly maximum in sprouted cum *Monascus* fermented finger millet followed by germinated finger millet (183.82) and *Monascus* fermented finger millet (172.33) as compared to the control (152.07). Similarly, the bioavailability of phosphorus and magnesium (mg/100g) is significantly higher in *Monascus* fermented sprouted finger millet (114.71 and 66.45 respectively) followed by germinated finger millet (50.00 and 64.48) and *Monascus* fermented finger millet (46.22 and 57.46) when compared to the control (42.89 and 54.72 respectively). Bioavailability of iron, zinc and copper also observed to be more in the sprouted *Monascus* fermented finger millet when compared to the control, *Monascus* fermented finger millet and germinated finger millet. Germination helped in increasing the extractability of the trace elements like Fe, Zn and Cu from 0.25, 1.27 and 0.27 (mg/100g) in the raw finger millet to 0.39, 1.70, 0.45 (mg/100g) which was further increased to 1.32, 1.88, 0.51(mg/100g) on 10 d of fermentation. However, the *Monascus purpureus* fermented finger millet exhibited bioavailability value of 0.45, 1.45, 0.37 (mg/100g) of Fe, Zn and Cu respectively. It was

obviously observed that the bioavailability of Ca, P, Mg, Fe, Zn and Cu was increased by the combined action of germination (72 h) and fermentation (10 d) as indicated in **Figure 33**.

**Table 10. HCl –extractability of minerals (mg/100g) during germination and fermentation of finger millet by *Monascus purpureus***

| Samples | pH of the substrate | | Minerals (mg/100g) | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Initial | Final | Ca | P | Fe | Mg | Zn | Cu |
| Raw finger Millet[a] | 6.13 | - | 152.07±0.32 | 42.89±0.23 | 0.25±0.09 | 54.72±0.56 | 1.27±0.06 | 0.27±0.01 |
| Germinated (72 h) finger millet | 6.13 | 5.85 | 183.82±0.53 | 50.00±0.28 | 0.39±0.06 | 64.48±0.49 | 1.70±0.04 | 0.45±0.02 |
| *Monascus* Fermented (10 d) finger millet | 6.13 | 5.13 | 172.33±0.62 | 46.22±0.29 | 0.45±0.04 | 57.46±0.38 | 1.45±0.02 | 0.37±0.03 |
| Germinated (72 h) - cum-*Monascus* Fermented (10 d) finger millet | 6.13 | 3.81 | 218.58±0.59 | 114.71±0.36 | 1.32±0.06 | 66.45±0.29 | 1.88±0.03 | 0.51±0.00 |

Values are mean± SD of three independent processing
[a]Total mineral analysis mg/100g dry matter: Ca-302.33±0.57, P- 237±1.0, Fe- 4.33±0.08, Mg-109.66±0.57, Zn- 2.11±0.08, Cu- 0.52 ± 0.00




A - Raw finger millet
B - Germinated finger millet
C - *Monascus purpureus* fermented finger millet
D - Germinated-cum- *Monascus purpureus* fermented finger millet

**Figure 33. Per cent increase in bioavailability of minerals by processing of finger millet**

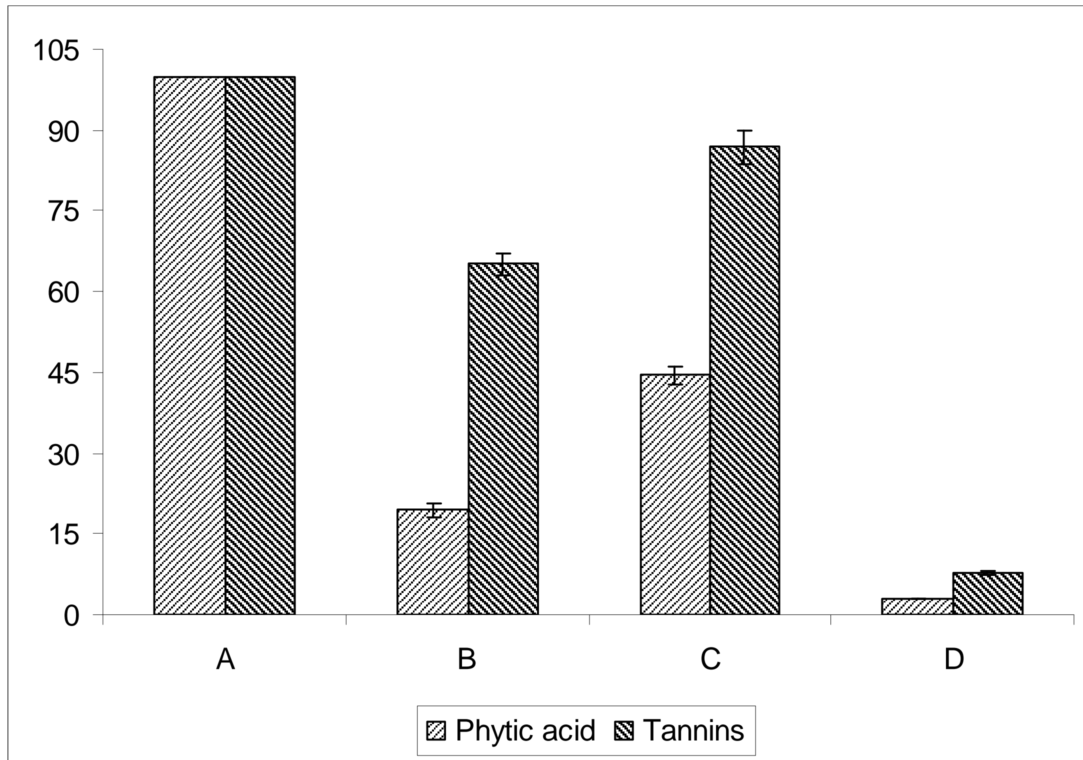
Phytic acid and tannin reduces the bioavailability of minerals by forming complexes with them. Germination reduced the phytate content from 285.0 to 55.3 mg/100g which was further reduced to 18.6 mg/100g dry matter on fermentation. Fermentation alone reduced the phytate content from 285.0 to 126.7 mg /100 g dry matter. Similarly, tannin content was significantly reduced from 441.3 to 287.0 mg/100 g on germination (72 h), which was further reduced to 43.7 mg/100 g on 10 d of fermentation **(Table 11)**.

**Table 11. Antinutritional composition of raw and processed finger millet**

| Samples | Antinutritional factors (mg/100g) | |
|---|---|---|
| | Phytic acid | Tannins |
| Raw finger millet | 285.0 ± 2.6 | 441.3 ± 3.1 |
| Germinated finger millet | 55.3 ± 3.0 | 287.0 ± 7.5 |
| *Monascus* fermented finger millet | 126.7 ± 2.5 | 383.0 ± 2.0 |
| Germinated & *Monascus* fermented finger millet | 32.0 ± 1.2 | 43.7 ± 2.1 |

Values are mean± SD of three independent processing

Phytic acid and tannin content of finger millet was reduced by 88.8% and 91.1% respectively by germinating (72 h) followed by fermenting with *Monascus purpureus* (10 d). Germination and fermentation of finger millet decreased the phytate and tannin content **(Figure 34)**. The degradation of phytates and tannins by germination and fermentation increases the HCl extractability of minerals thereby increasing their bioavailability. The results of this experiment suggest that the germinated (72 h) finger millet fermented (10 d) with *Monascus purpureus* showed reduction in phytic acid and tannin contents by 88.8 and 91.1% respectively with an increase of 61.5 % HCl- extractable minerals.




A - Raw finger millet
B - Germinated finger millet
C - *Monascus purpureus* fermented finger millet
D - Germinated-cum- *Monascus purpureus* fermented finger millet

**Figure 34. Per cent reduction in antinutrients by processing of finger millet**

# CHAPTER -2

## OPTIMIZATION OF CULTURAL CONDITIONS FOR STATIN AND GABA PRODUCTION

Microbial fermentations are widely explored in industries for the production of an array of metabolites like antibiotics, enzymes, pigments, organic acids, vitamins, amino acids and pharmacologically important bioactive molecules. In these processes, optimization of cultural conditions is essential for large scale production of metabolites. Various parameters that affect microbial physiology and yield of metabolite are inoculum level, pH, temperature, incubation period, aeration and media composition (Wang et al.1999) Therefore, it is necessary to design the fermentation conditions favourable for the microbial metabolite production.

Lovastatin, a hypocholesterolemic agent, competitively inhibits the rate-limiting enzyme of cholesterol biosynthesis 3-hydroxy-3-methyl glutaryl coenzyme A (HMG-CoA) reductase, which catalyzes the reduction of HMG-CoA to mevalonate during cholesterol biosynthesis (Alberts et al. 1980; Hajjaj et al. 2001). In addition to this lovastatin has a clear evidence of benefit on stroke (Hebert et al. 1997) and it also shows in vivo tumor suppression by inhibiting the synthesis of nonsterol isoprenoid compounds (Valera et al. 2005). Lovastatin was the first statin to obtain approval from the United States Food and Drug Administration in August1987 (Tobert 2003, Demain 1999; Manzoni and Rollini 2002). Lovastatin can be produced from numerous fungi, including *Monascus purpureus* (Su et al. 2003; Wang et al. 2003).

*Monascus purpureus*, *Monascus ruber* and *Monascus pilosus* are three traditional fermentation fungi used in food for thousands of years in East Asia (Hawksworth and Pitt, 1983). Recently, scientific researches revealed the prominent decreasing blood pressure effect of *Monascus*, and demonstrated its antihypertensive substance, γ-aminobutyric acid (GABA) (Kono

and Himeno 2000; Rhyu *et al.* 2002; Tsuji *et al.* 1992). It is well known that GABA, with two receptors-$GABA_A$ and $GABA_B$, is the main suppressive nerve transmitter of the central nervous system (Lauder, 2005). Moreover, GABA-rich food has multiple physiological functions such as antihypertensive (Aoki *et al.* 2003b; Nakamura et al. 2000; Hayakawa *et al.* 2004), liver protective and tranquilizer effects (Okada *et al.* 2000). Therefore, GABA has been applied to clinical medicine for hypertension therapy (Lacerda *et al.* 2003), Parkinson's disease therapy (Nandi *et al.* 2002), hypochondria (Petty *et al.* 1997), and epilepsy therapy (Löscher *et al.* 1998), etc. With a view to the source of GABA, it was reported that it could be produced by various fungi other than *Monascus* (Kono and Himeno, 2000) including *Rhizopus* (Akoi *et al.* 2003a), *Saccharomyces cerevisiae* (Kishimoto and Sodeyama, 2003) and *Aspergillus* (Kato *et al.* 2002). GABA production of *M. purpureus* was reportedly as high as 5,004 mg/kg (Wang *et al.* 2003). However, there is a disadvantage with some of high GABA producing strains of *M. purpureus*, because of citrinin production. Citrinin contamination has become a pressing issue in fungi fermented GABA product due to its severe nephrotoxicity and hepatototoxicity (Kitabatake *et al.* 1993). Blanc et al. (1995) reported that citrinin was definitely detected in the product fermented by *M. purpureus* and *M. ruber*. Fortunately, as to fungus *M. pilosus*, few citrinin contamination cases were reported in fermented products; these findings undoubtedly doomed the commercial potential of *M. pilosus*. Moreover, GABA-abundant red yeast rice, a product of solid state fermentation (SSF) by *M. pilosus*, has already proved a good market as supplements in Japan (Kato *et al.* 2002). Since the optimization of the solid-state fermentation conditions with *M. pilosus* for the GABA production has not been extensively studied yet, we aimed at optimizing the condition of solid fermentation by *Monascus* species, to improve the yield of GABA.

Process optimization is a tedious process due to involvement of multivariable process parameters. In this process, screening of important factors is initially carried out and these selected factors are then optimized by different techniques (Box and Hunter 1957; Lewis et al. 1999). Plackett-Burman design (PBD) is a well-established, widely used statistical design technique for key factors screening, out of a large number of solid-state fermentation (SSF) parameters, without numerous experiments. Response surface methodology (RSM) is a three factorial design that gives relationship between one or more measured dependent responses with a number of input (independent) factors. RSM has some advantages that include less experiment numbers, suitability for multiple factor experiments, search for relativity between factors, and finding of the most suitable condition and forecast response (Chang et al. 2006). Response surface methodology (RSM) is very useful to test multiple process variables because fewer experimental trials are needed compared to the study of one variable at a time. In addition, interactions between variables could be identified and quantified by such a technique.

Another design, central composite design (CCD), was conducted in the optimum vicinity to locate the true optimum values of the multiple variables. Therefore, RSM and CCD are increasingly used for optimization of many fermentation conditions. In this, linear or quadratic effects of experimental variables construct contour plots and a model equation fitting the experimental data. This facilitates the determination of optimum value of factors under investigation and prediction of response under optimized condition (Chakravarti and Sahai 2002). RSM, as an efficient experimental strategy, was used to seek the optimal conditions for multivariable system and factors affecting the objects were evaluated with the PBD and the CCD.

## 2.1. ENHANCEMENT OF STATIN PRODUCTION BY *MONASCUS PURPUREUS* MTCC 410 UNDER SOLID STATE FERMENTATION WITH FINGER MILLET AS SUBSTRATE

In the past, optimization of cultural conditions for fungal metabolite production was done by varying each factor one by one. This type of optimization is tedious as a large number of factors are involved and interaction effect between two interrelated factors becomes difficult for interpretation. Statistical methodologies have been applied in biotechnological process to define fermentation process and to deal with a large number of variables (Manuel and Antonio 1997). Out of various statistical methods, Response Surface Methodology (RSM) is an efficient tool and can be used to test multiple process variables with fewer experimental runs.

RSM designs provide a systematic and efficient means of studying several control factors in fermentation process. It is widely used to examine operational variables in designing an experiment by predicting a model based on factors and conditions used for optimization (Buchanan and Philips 1990).

Optimization of enzyme production in solid state fermentation by using RSM design was carried out by Zhu *et al*. (1996). Sanjay et al. (2009) have optimized xanthin production by *Aspergillus carbonarius* mutant using RSM. Chang *et al*. (2002) used RSM to optimize culture medium for production of lovastatin by *Monascus ruber*. In the present study, an attempt was made to enhance the production of statin by *Monascus purpureus* grown on finger millet under solid state fermentation which mainly dependent on medium constituents and concentrations of carbon and nitrogen source through a response surface methodological approach.

A Response Surface Methodology study as described by Box and Wilson was conducted to determine the effect of three predictor variables (soybean flour, glycerol and ammonium

nitrate) on the total statin production by *Monascus purpureus*. A CCRD was constructed using the software package Statistica (1999). The limits for the each independent variable were fixed based on the prior trials conducted in the laboratory. Table 1 shows levels of predictor variables and shows the combination of predictor variable levels used in CCRD. To study 3 factors at 5 levels would require $5^3$or 125 using conventional experiments, whereas use of standard experimental designs such as CCRD requires only 22 runs or experiments. The center point in the design was repeated 6 times to calculate the reproducibility of the result. The coded and the actual values of variables are presented in **Table 12.**

**Table 12. Coded levels and actual values of the variables for RSM of statin**

| **Variables** | **Coded levels and actual values** | | | | |
|---|---|---|---|---|---|
| | **-1.682** | **-1** | **0** | **1** | **+1.682** |
| Glycerol (%) | 1 | 1.81 | 3 | 4.18 | 5 |
| Ammonium nitrate (%) | 0.1 | 0.14 | 0.2 | 0.26 | 0.3 |
| Soybean flour (%) | 5 | 7.02 | 10 | 12.98 | 15 |

For each of the response variables, model summaries and lack of fit tests were analyzed for linear or quadratic models. From this information, the most accurate model was chosen by the sequential F tests-lack of fit measures. Three-dimensional response surface plots were generated for response variable. In this study, predictor variables were selected for the experiment to be at any level within the range of the design. In most of the reported RSM

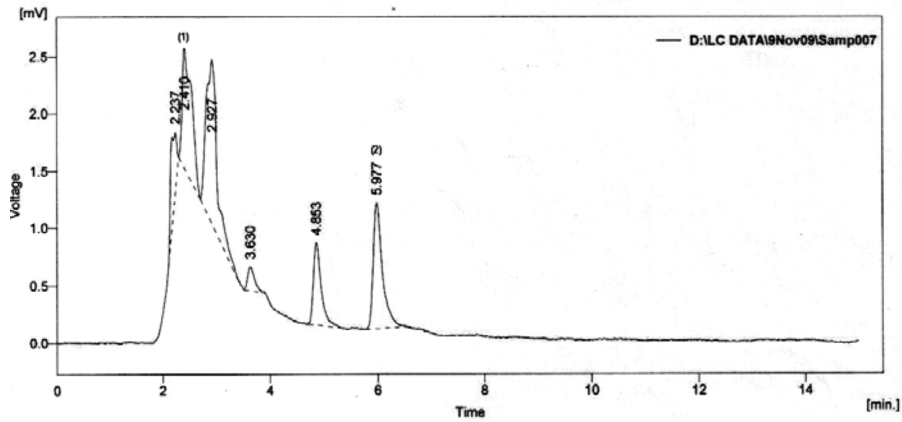
applications, several responses were measured for each set of experimental conditions, and a model was fitted for each response. Finding the overall optimal conditions in these multi-response problems is not straight forward or easy, and most researchers use the graphical approach of superimposing the different response surfaces and finding the experimental region that would give the desired values of responses. This method, although visually attractive, requires a large number of graphs to be generated, even for 2 or 3 responses involved. Optimization

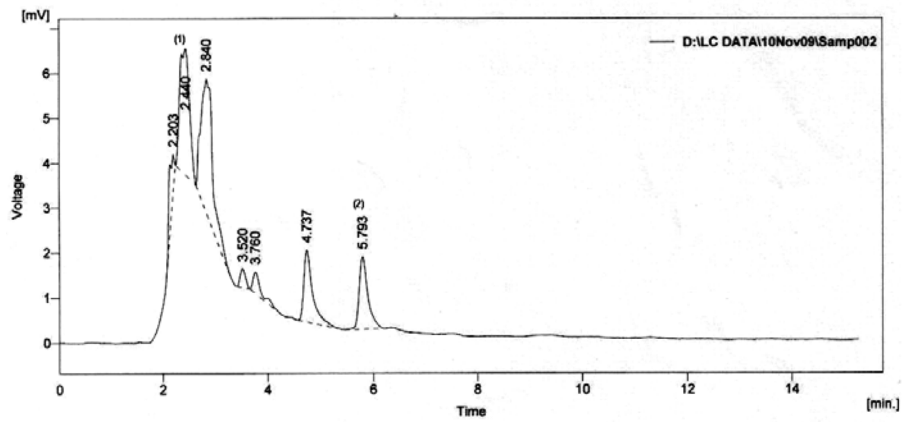
According to the canonical analysis described by Myers (1991), the stationary points were located for the corresponding responses. The search criteria were to find the production conditions that would enhance the statin production. Optimum conditions with respect to maximum statin production of 8.025 g $kg^{-1}$ were found out with glycerol 3.12 %, ammonium nitrate 0.30% and soybean flour 15% by RSM experiment. Verification experiments, carried out at the predicted conditions showed values close ($P<0.05$) to those predicted and further confirmed the adequacy of predicted models.

To optimize the levels of key nutrients influencing statin production, a solid state fermentation with the above mentioned design was carried out. Three constituents (soybean flour, glycerol and ammonium nitrate) were chosen for study. An experimental design of 22 runs containing 6 central points was made according to CCR design. The individual and interactive effects of these nutrient variables were studied by conducting the fermentation run at randomly selected different levels of all three parameters. The response was measured in terms of statin production. The HPLC peaks of the experimental samples exhibiting the pravastatin and lovastatin is presented at **Figure 35**. The results of experimental data and the simulated values are listed in **Table 13**. Data collected for statin concentration in each experiment was analyzed

using STATISTICA (1999) and fitted into a multiple non-linear regression model proposes following equation for statin production.

**Statin (g/kg) = $-8.59 + 3.45A + 29.96B + 0.84C - 0.49A^2 - 68.33B^2 - 0.04C^2 - 2.80AB + 0.03AC + 1.28\,BC$** where A, B and C represent glycerol, ammonium nitrate and soybean flour respectively in % in the solid state medium.



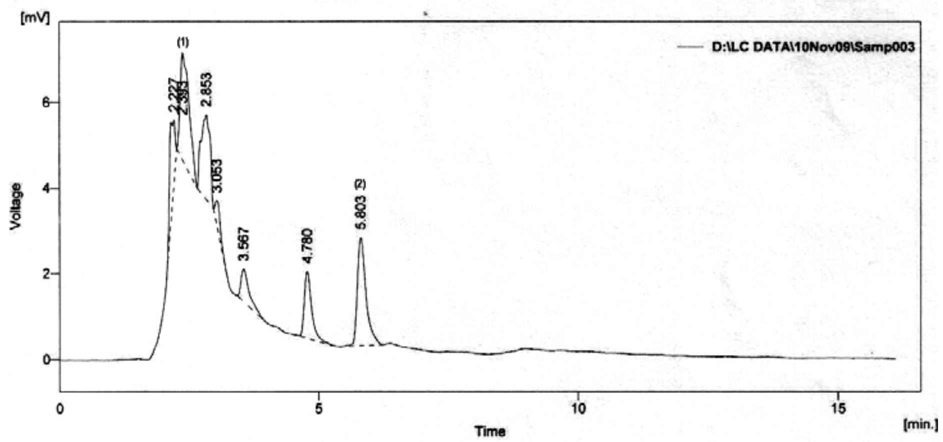




**Figure 35. HPLC peaks of samples (*Monascus purpureus* MTCC 410 fermented finger millet) exhibiting pravastatin (1) and lovastatin (2)**

**Table 13 CCRD experimental design for statin production by *Monascus purpureus***

| Experi-mental design point | Glycerol (%) | Ammonium nitrate (%) | Soybean flour (%) | Statin yield (g/kg) | | Total statin yield (g/kg) | |
|---|---|---|---|---|---|---|---|
| | | | | Lovastatin | Pravastatin | Theoreti-cal | Actual |
| 1 | -1(1.81) | -1(0.14) | -1(7.02) | 0.67 | 3.31 | 4.33 | 3.98 |
| 2 | -1(1.81) | -1(0.14) | 1(12.98) | 0.91 | 4.70 | 5.64 | 5.61 |
| 3 | -1(1.81) | 1(0.26) | -1(7.02) | 0.81 | 3.07 | 4.99 | 3.88 |
| 4 | -1(1.81) | 1(0.26) | 1(12.98) | 0.38 | 6.43 | 6.93 | 6.81 |
| 5 | 1(4.18) | -1(0.14) | -1(7.02) | 0.89 | 4.07 | 4.91 | 4.96 |
| 6 | 1(4.18) | -1(0.14) | 1(12.98) | 1.22 | 6.33 | 6.52 | 7.55 |
| 7 | 1(4.18) | 1(0.26) | -1(7.02) | 1.06 | 3.90 | 5.02 | 4.96 |
| 8 | 1(4.18) | 1(0.26) | 1(12.98) | 0.93 | 6.60 | 7.26 | 7.53 |
| 9 | -1.682(1.0) | 0( 0.2) | 0(10) | 1.28 | 3.68 | 4.19 | 4.96 |
| 10 | +1.682(5.0) | 0(0.2) | 0(10) | 0.52 | 3.90 | 5.10 | 4.42 |
| 11 | 0(3.0) | -1.682(0.1) | 0(10) | 0.81 | 4.04 | 5.24 | 4.85 |
| 12 | 0(3.0) | +1.682(0.3) | 0(10) | 1.15 | 5.97 | 6.65 | 7.12 |
| 13 | 0(3.0) | 0(0.2) | -1.682(5.0) | 0.80 | 3.70 | 3.81 | 4.50 |
| 14 | 0(3.0) | 0(0.2) | +1.682(15) | 0.51 | 6.24 | 7.36 | 6.75 |
| 15 | 0(3.0) | 0(0.2) | 0(10) | 1.29 | 5.08 | 6.63 | 6.37 |
| 16 (C) | 0(3.0 ) | 0(0.2) | 0(10) | 1.14 | 5.14 | 6.63 | 6.28 |
| 17 (C) | 0(3.0) | 0(0.2) | 0(10) | 1.09 | 5.40 | 6.63 | 6.49 |
| 18 (C) | 0(3.0) | 0(0.2) | 0(10) | 1.36 | 5.74 | 6.63 | 7.10 |
| 19 (C) | 0(3.0) | 0(0.2) | 0(10) | 1.42 | 5.47 | 6.63 | 6.89 |
| 20 (C) | 0(3.0) | 0(0.2) | 0(10) | 1.02 | 5.77 | 6.63 | 6.79 |
| 21 (C) | 0(3.0) | 0(0.2) | 0(10) | 1.31 | 5.61 | 6.63 | 6.92 |
| 22 (C) | 0(3.0 ) | 0(0.2) | 0(10) | 1.29 | 4.97 | 6.63 | 6.26 |

**(C) represents the central point of experimental design**

The effects of nutrient parameters on statin production were depicted by response surface graphs. Two three-dimensional surface plots of calculated model for total statin production are shown in Figure 34 and 35. The analysis of variance and the regression for total statin production were summarized in **Tables 14 and 15**. In case of total statin production, this calculated model is able to explain 81% of the results.

**Table 14. Analysis of variance for statin RSM**

| **Independent production variables** | **Sum of squares** | **Degrees of freedom** | **Mean sum of squares** | **F value** | **P value** |
|---|---|---|---|---|---|
| Glycerol (L) | 1.06 | 1 | 1.06 | 2.52 | 0.14 |
| Glycerol (Q) | 6.07 | 1 | 6.07 | 14.38 | 0.00* |
| Ammonium nitrate (L) | 1.76 | 1 | 1.76 | 4.16 | 0.06 |
| Ammonium nitrate (Q) | 0.44 | 1 | 0.44 | 1.04 | 0.33 |
| Soybean flour (L) | 13.35 | 1 | 13.35 | 31.61 | 0.00* |
| Soybean flour (Q) | 1.35 | 1 | 1.35 | 3.20 | 0.10 |
| Interaction of Glycerol (L) and Ammonium nitrate (L) | 0.16 | 1 | 0.16 | 0.37 | 0.55 |
| Interaction of Glycerol (L) and soybean flour (L) | 0.05 | 1 | 0.05 | 0.11 | 0.75 |
| Interaction of Ammonium nitrate (L) and soybean flour (L) | 0.20 | 1 | 0.20 | 0.48 | 0.50 |
| Error | 5.07 | 12 | 0.42 | | |
| Total sum of squares | 29.21 | 21 | | | |
| R square | 0.81 | | | | |

L – Linear effect; Q- Quadratic effect, * = $P<0.05$

**Table 15. Regression coefficients of predicted quadratic polynomial model for selected parameters**

| Independent production variables | Coefficient | Standard error | t value | P value |
|---|---|---|---|---|
| Intercept | -8.59 | 6.64 | -1.29 | 0.22 |
| Glycerol (L) | 3.45 | 1.58 | 2.19 | 0.05* |
| Glycerol (Q) | -0.49 | 0.13 | -3.80 | 0.00* |
| Ammonium nitrate (L) | 29.96 | 31.96 | 0.94 | 0.37 |
| Ammonium nitrate (Q) | -68.33 | 52.02 | -1.31 | 0.21 |
| Soybean flour (L) | 0.84 | 0.64 | 1.32 | 0.21 |
| Soybean flour (Q) | -0.04 | 0.02 | -2.01 | 0.07 |
| Interaction of Glycerol (L) and Ammonium nitrate (L) | -2.80 | 4.80 | -0.58 | 0.57 |
| Interaction of Glycerol (L) and soybean flour (L) | 0.03 | 0.10 | 0.31 | 0.76 |
| Interaction of Ammonium nitrate (L) and soybean flour (L) | 1.28 | 1.92 | 0.67 | 0.52 |

L – Linear effect; Q- Quadratic effect, * = P<0.05

The optimum values of glycerol, ammonium nitrate and soybean flour (% w/w) in solid state medium were determined **(Table 16)**. These values predict 8.025 g $kg^{-1}$ of total statin production, which was the highest when compared to earlier reports. These optimized values of nutrient parameters were validated in a duplicate flask study.

**Table 16. Optimum conditions for maximum statin production**

| Variables | Critical Value |
|---|---|
| Glycerol (%) | 3.12 |
| Ammonium nitrate (%) | 0.30 |
| Soyabean flour (%) | 15.00 |
| Predicted total statin (g/kg) | 8.025 |

**Figure 36** shows the effect of glycerol and ammonium nitrate on statin production. The level of glycerol on stain production was studied from 1.0 to 5.0 %. The level of glycerol from 1.0% to 3.5 % exhibited increase in statin production and beyond 3.5%, a steady decrease in the statin production was observed. Similarly ammonium nitrate addition from lower level 0.1% to 0.3% revealed steady increase in statin production. The interaction effect of glycerol and ammonium nitrate exhibited maximum increase in statin production at 3.5% of glycerol and 0.3% of ammonium nitrate.

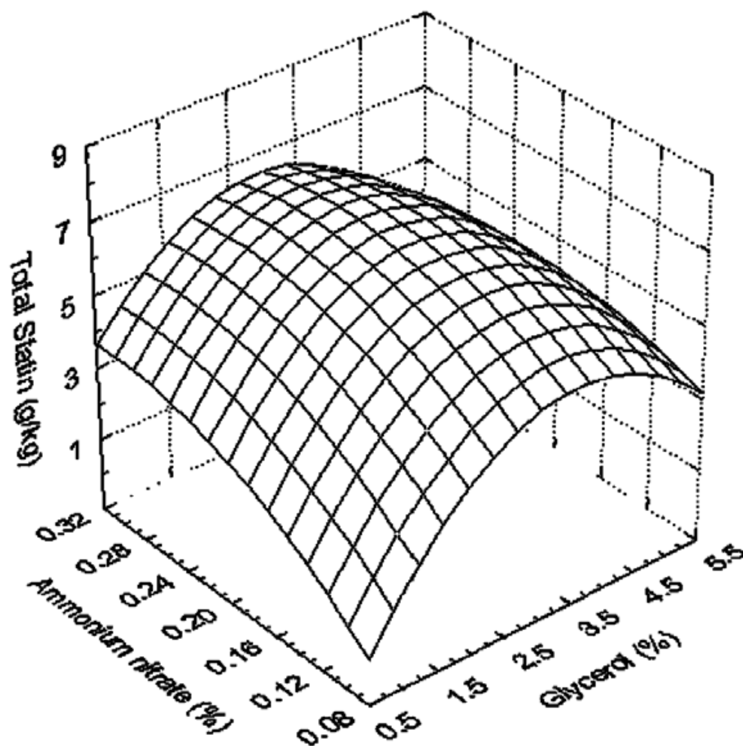



**Figure 36. Three dimensional surface plot showing the effect of glycerol and ammonium nitrate on statin yield with 10% soybean flour**

**Figure 37** reveals the effect of ammonium nitrate and soybean flour on total statin production. The level of ammonium nitrate from 0.1% to 0.3% showed steep increase in the statin production. Increase in concentration of soybean flour from 5% to 15% in the solid

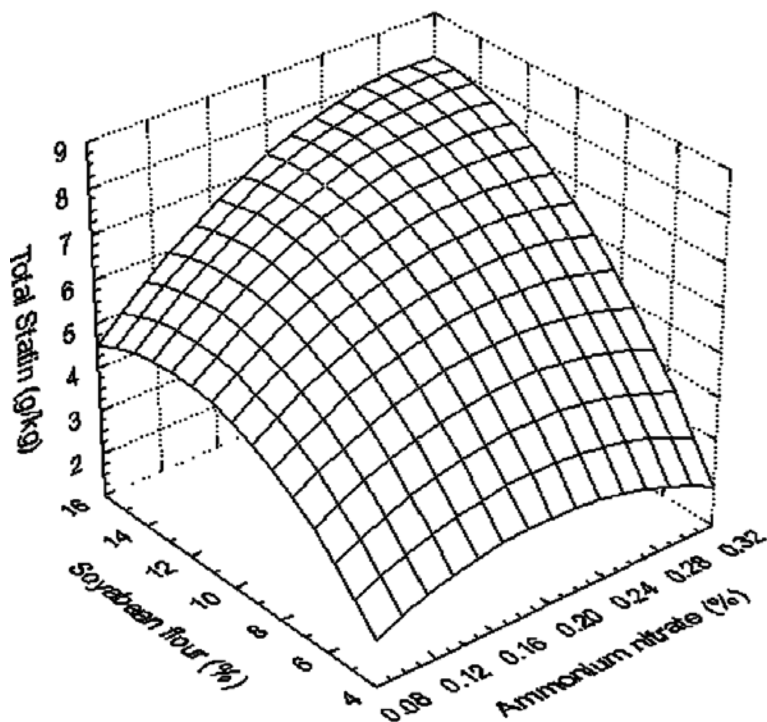



**Figure 37. Three dimensional surface plot showing the effect of ammonium nitrate and soybean flour on statin yield at 3% glycerol**

state medium enhanced the statin production considerably. The interaction effect of ammonium nitrate and soybean flour showed maximum increase in total statin production at high levels of ammonium nitrate and soybean flour.

It is evidenced from the above that the 3.12% glycerol, high level (0.3%) of ammonium nitrate and high level (15%) of soybean flour addition in the finger millet substrate enhanced the total statin production upto 8.025 g $kg^{-1}$ of the substrate used in the solid state fermentation.

## 2.2 OPTIMIZATION OF GABA PRODUCTION BY *MONASCUS PURPUREUS* MTCC 410 UNDER SOLID STATE FERMENTATION WITH FINGER MILLET AS SUBSTRATE

Gamma amino butyric acid (GABA) produced by *Monascus purpureus* is an effective antihypertensive metabolite which has several physiological functions such as neurotransmitting, diuretic and hypotensive effect (Su *et al.* 2003). GABA is formed by the decarboxylation of glutamic acid by a key enzyme, glutamate decarboxylase produced by the fungus. *Monascus purpureus* fermented rice contains good amount of GABA which exhibits an anti-hypertensive effects. Therefore, GABA has been applied to clinical medicine for treatment of hypertension, Parkinson's disease, hypochondria and epilepsy (Petty *et al.* 1997; Loscher *et al.* 1998; Nandi *et al.* 2002; Lacerda *et al.* 2003).

RSM is very useful to test multiple process variables. In addition, interactions between variables could be identified and quantified by such a technique. Central composite design (CCD) is conducted in the optimum vicinity to locate the true optimum values of the multiple variables. Therefore, RSM and CCD are mainly used for optimization of many fermentation conditions (Chang *et al.* 2006; Sadik *et al.* 2007; Karuppaiya *et al.* 2009). In this research study, an attempt was made to optimize the production of GABA by *Monascus purpureus* grown on finger millet under solid state fermentation which is mainly dependent on incubation period, temperature, inoculum volume and nitrogen level, through a response surface methodological approach.

The Response surface methodology (RSM) used in the present study is a central composite design (CCD) involving four different factors. The limits for the each independent variable were fixed based on the prior trials conducted in the laboratory. **Table 17** shows level of

predictor variables and the combination of predictor variable levels used in CCRD. Experiments were conducted in a randomized fashion. The dependent variable selected for this study was the GABA yield (mg/kg) dry substrate. The independent variables chosen were incubation period (days) $X_1$, temperature in °C $X_2$, inoculum volume in % $X_3$, and nitrogen level in % $X_4$.

**Table 17. Coded levels and actual values of the variables employed in RSM of GABA**

| Variables | Coded levels and actual values | | | | |
|---|---|---|---|---|---|
| | **-2** | **-1** | **0** | **+1** | **+2** |
| Incubation period (days) | 4 | 7 | 10 | 13 | 16 |
| Temperature (°C ) | 16 | 21 | 26 | 31 | 36 |
| Inoculum volume (%) | 0.8 | 2.4 | 4.0 | 5.6 | 7.2 |
| Nitrogen level (%) | 0.1 | 0.375 | 0.65 | 0.925 | 1.2 |

An experimental design of 30 runs containing 6 central points was made according to CCR design. The individual and interactive effects of these nutrient variables were studied by conducting the fermentation run at randomly selected different levels of all three parameters. The response was measured in terms of GABA yield. The GABA produced under the actual conditions of experimentation with the predicted and experimental yields are shown in **Table 18**.

**Table 18. CCRD experimental design for GABA production by *Monascus purpureus***

| Experimental design point | Incubation period (days) | Temperature (°C) | Inoculum level (%) | Nitrogen level (%) | GABA yield (mg/kg) | |
|---|---|---|---|---|---|---|
| | | | | | Theoretical | Actual |
| 1 | -1(7) | -1(21) | -1(2.4) | -1(0.375) | 9.01 | 12.30 |
| 2 | -1(7) | -1(21) | -1(2.4) | +1(0.925) | 32.75 | 32.55 |
| 3 | -1(7) | -1(21) | +1(5.6) | -1(0.375) | 61.80 | 72.69 |
| 4 | -1(7) | -1(21) | +1(5.6) | +1(0.925) | 81.20 | 78.23 |
| 5 | -1(7) | +1(31) | -1(2.4) | -1(0.375) | 77.47 | 80.10 |
| 6 | -1(7) | +1(31) | -1(2.4) | +1(0.925) | 92.34 | 98.16 |
| 7 | -1(7) | +1(31) | +1(5.6) | -1(0.375) | 110.33 | 104.24 |
| 8 | -1(7) | +1(31) | +1(5.6) | +1(0.925) | 120.85 | 122.25 |
| 9 | +1(13) | -1(21) | -1(2.4) | -1(0.375) | 54.84 | 61.23 |
| 10 | +1(13) | -1(21) | -1(2.4) | +1(0.925) | 59.06 | 66.23 |
| 11 | +1(13) | -1(21) | +1(5.6) | -1(0.375) | 50.96 | 46.22 |
| 12 | +1(13) | -1(21) | +1(5.6) | +1(0.925) | 50.86 | 56.02 |
| 13 | +1(13) | +1(31) | -1(2.4) | -1(0.375) | 167.43 | 171.47 |
| 14 | +1(13) | +1(31) | -1(2.4) | +1(0.925) | 162.77 | 159.68 |
| 15 | +1(13) | +1(31) | +1(5.6) | -1(0.375) | 143.62 | 151.63 |
| 16 | +1(13) | +1(31) | +1(5.6) | +1(0.925) | 134.63 | 132.38 |
| 17 | -2(4) | 0(26) | 0(4) | 0(0.65) | 36.32 | 33.34 |
| 18 | +2(16)) | 0(26) | 0(4) | 0(0.65 | 95.92 | 90.03 |
| 19 | 0(10) | -2(16) | 0(4) | 0(0.65) | 37.76 | 29.68 |
| 20 | 0(10) | +2(36) | 0(4) | 0(0.65) | 189.99 | 189.19 |
| 21 | 0(10) | 0(26) | -2(0.8) | 0(0.65) | 72.8 0 | 64.23 |
| 22 | 0(10 ) | 0(26) | +2(7.2) | 0(0.65) | 97.44 | 97.16 |
| 23 | 0(10) | 0(26) | 0(4) | -2(0.1) | 79.99 | 72.19 |
| 24 | 0(10) | 0(26) | 0(4) | +2(1.2) | 94.74 | 93.65 |
| 25(C) | 0(10) | 0(26) | 0(4) | 0(0.65) | 61.27 | 62.10 |
| 26(C) | 0(10 ) | 0(26) | 0(4) | 0(0.65 | 61.27 | 61.06 |
| 27(C) | 0(10) | 0(26) | 0(4) | 0(0.65) | 61.27 | 60.98 |
| 28(C) | 0(10) | 0(26) | 0(4) | 0(0.65) | 61.27 | 59.69 |
| 29(C) | 0(10) | 0(26) | 0(4) | 0(0.65) | 61.27 | 62.56 |
| 30(C) | 0(10 ) | 0(26) | 0(4) | 0(0.65) | 61.27 | 61.23 |

(C)- Central points of the experimental design

The statistical significance of the coefficient terms and ANOVA data are shown in **Table 19.** Experiments were performed according to the CCD experimental design given in **Table 18** in order to search for the optimum combination of components of the medium.

**Table 19. Analysis of variance for GABA RSM quadratic model**

| Independent production variables | Coefficient factor | Sum of squares | Degrees of freedom | Mean sum of squares | F value | p value Prob>F |
|---|---|---|---|---|---|---|
| Model | | 53143.31 | 14 | 3795.95 | 80.80 | <0.0001* |
| Intercept | 61.27 | | | | | |
| Incubation period (L) | 14.90 | 5331.82 | 1 | 5331.82 | 113.50 | <0.0001* |
| Incubation period (Q) | 1.21 | 40.32 | 1 | 40.32 | 0.86 | 0.3689 |
| Temperature (L) | 38.06 | 34767.05 | 1 | 34767.05 | 740.08 | <0.0001* |
| Temperature (Q) | 13.15 | 4743.02 | 1 | 4743.02 | 100.96 | <0.0001* |
| Inoculum level (L) | 6.16 | 910.20 | 1 | 910.20 | 19.38 | 0.0005 |
| Inoculum level (Q) | 5.97 | 975.94 | 1 | 975.94 | 20.77 | 0.0004 |
| Nitrogen level (L) | 3.69 | 326.64 | 1 | 326.64 | 6.95 | 0.0187 |
| Nitrogen level (Q) | 6.52 | 1166.45 | 1 | 1166.45 | 24.83 | 0.0002 |
| Interaction of Incubation period (L) and Temperature (L) | 11.03 | 1946.57 | 1 | 1946.57 | 41.44 | <0.0001* |
| Interaction of Incubation period (L) and Inoculum level (L) | -14.17 | 3210.92 | 1 | 3210.92 | 68.35 | <0.0001* |
| Interaction of Incubation period (L) and Nitrogen level | -4.88 | 381.23 | 1 | 381.23 | 8.12 | 0.0122 |
| Interaction of Temperature (L) and Inoculum level (L) | -4.99 | 397.60 | 1 | 397.60 | 8.46 | 0.0108 |
| Interaction level of Temperature (L) and Nitrogen level (L) | -2.22 | 79.03 | 1 | 79.03 | 1.68 | 0.2142 |
| Interaction of inoculum level (L) and Nitrogen level (L) | -1.09 | 18.97 | 1 | 18.97 | 0.40 | 0.5347 |
| Residual | | 704.66 | 15 | 46.98 | | |
| Lack of fit | | 699.68 | 10 | 69.97 | 70.26 | <0.0001* |
| Pure Error | | 4.98 | 5 | 1.00 | | |
| Cor.Total | | 53847.98 | 29 | | | |

**L – Linear effect; Q- Quadratic effect; * Significant**

**SD: 6.85, Mean: 82.75, C.V. %: 8.28, $R^2$: 0.9869, Adj $R^2$: 0.9747, Pred $R^2$: 0.9250, AdeqPrecision:37.346**

The Model F-value of 80.80 implies the model is significant. The Lack of Fit F-value of 70.26 implies that the Lack of Fit is significant. There is only a 0.01% chance that a large “Lack of Fit F-value” could occur due to noise. The Fisher F-test with a very low probability value (p model >F = 0.0001) demonstrates a very high significance for the regression model. The goodness of fit of the model was checked by the determination coefficient ($R^2$). The coefficient of determination ($R^2$) was calculated as 0.9869 for GABA production. This implies that 98.69% of experimental data of the GABA production was compatible with the data predicted by the model (Table I) and only 1.31% of the total variations are not explained by the model. The $R^2$ value is always between 0 and 1, and a value >0.75 indicates aptness of the model. For a good statistical model, $R^2$ value should be close to 1.0. The adjusted $R^2$ value corrects the $R^2$ value for the sample size and for the number of terms in the model. The value of the adjusted determination coefficient (Adj $R^2$ = 0.9747) is also high to advocate for a high significance of the model. In this case the adjusted $R^2$ value is 0.9747, which is lesser than the $R^2$ value of 0.9869. The predicted $R^2$ of 0.9250 is in reasonable agreement with the adjusted $R^2$ of 0.9747. The value of CV is also low as 8.28 % indicates that the deviations between experimental and predicted values are low. Adequacy precision measures the signal to noise ratio. A ratio greater than 4 is desirable. In this work the ratio is 37.346, which indicates an adequate signal. This model can be used to navigate the design space. The mathematical expression of relationship to the GABA production with variables is shown below:

$$\mathbf{GABA(mg/kg)=61.27+14.90X_1+38.06X_2+6.16X_3+3.69X_4+11.03X_1X_2-14.17X_1X_3-4.88X_1X_4-4.99X_2X_3-2.22X_2X_4-1.09X_3X_4+1.21X_1^2+13.15X_2^2+5.97X_3^2+6.52X_4^2} \text{--------}\mathbf{(A)}$$

The results of multiple linear regressions conducted for the second order response surface model and the significance of each coefficient was determined by *p*-values, which are listed in

**Table 19**. Values of "Prob > F" less than 0.05 indicate model terms are significant. In this case $X_1$, $X_2$, $X_3$, $X_4$, $X_1X_2$, $X_1X_3$, $X_1X_4$, $X_2X_3$, $X_2^2$, $X_3^2$ and $X_4^2$ are significant model terms. Values greater than 0.10 indicate the model terms are not significant. This implies that all linear and quadratic terms corresponding to incubation period, temperature, inoculum level and nitrogen level were found to be significant. Except the two cross product terms involving nitrogen level with temperature and inoculum level, all other interaction effects were significant. These suggests that the incubation period, temperature, inoculum level and nitrogen level have a direct relationship with the production of GABA and interactive effect of incubation period and temperature in this experiment influence GABA production positively. The coefficients of the equation were determined by employing Design-Expert 8.0.1.0 software. Analysis of variance (ANOVA) for the final predictive equation was done using Design-Expert 8.0.1.0 software **(Table 19)**. The response surface equation was optimized for maximum yield in the range of process variables using Design-Expert 8.0.1.0 software. Three dimensional surface plots for the interaction of one parameter with another parameter were obtained using the same software and from these surface plots, the interaction effects were studied.

**Figure 38** shows the effect of incubation period and temperature on GABA production at 4% inoculum volume and 0.65% of nitrogen level. As the incubation period increased at all the temperatures, increase in GABA production was observed. Maximum GABA production (140 mg/kg) was obtained at a temperature of 31°C over a period of 13 days of incubation. As the incubation period increases, the fungus at stationary phase, results in the production of series of metabolites. This clearly indicates that the increase in fermentation period would lead to appreciable production of GABA in the solid state medium. At a temperature of 36°C, the metabolic activity of the fungus is more resulting in high GABA production.

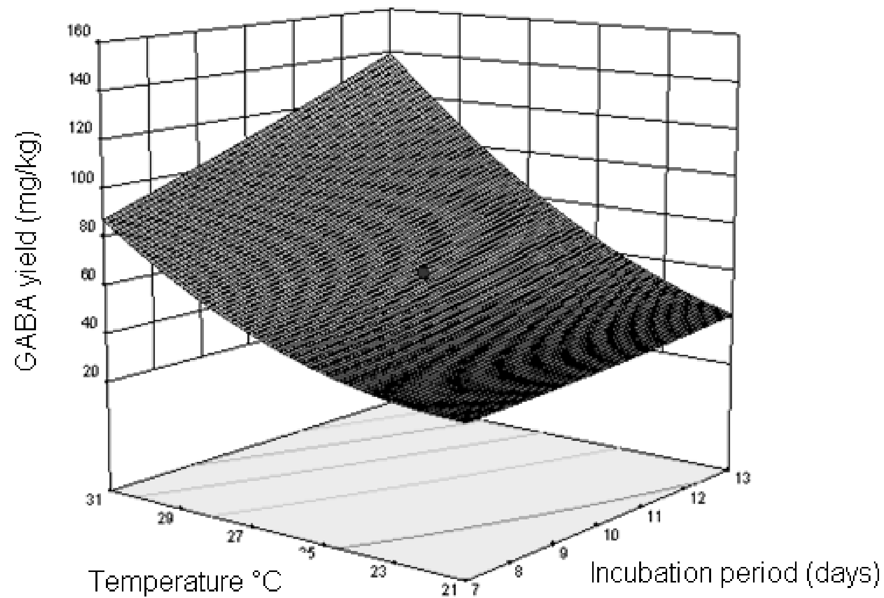



**Figure 38. Three dimensional surface plot showing the effect of incubation period and temperature on GABA yield at 4% inoculum volume and 0.65% of nitrogen level**

The effect of incubation period and inoculum level in SSF for GABA production at a temperature of 26°C and nitrogen level of 6.5% is shown in **Figure 39**. At lower levels of inoculum with the increase in incubation period, the yield of GABA seems to be significantly high. However, at 5.6% of inoculum level, GABA production was 70 mg/kg. Incubation period has positive influence on GABA production.

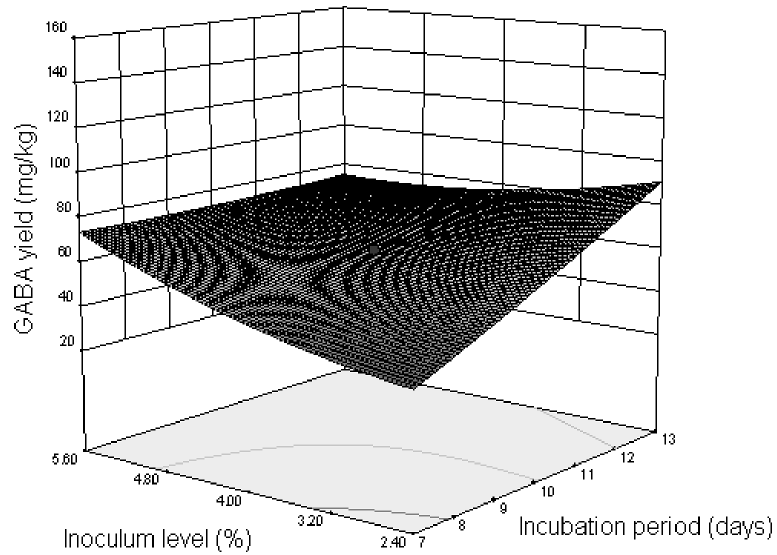



**Figure 39. Three dimensional surface plot showing the effect of incubation period and inoculum level on GABA yield at a temperature of 26° C and 0.65% nitrogen level**

**Figure 40** depicts the effect of incubation period and nitrogen level in SSF for GABA production at a temperature of 26 °C with an inoculum level of 4%. Nitrogen level at the low range of 0.38 to 0.65 % exhibited increase in GABA production. Maximum GABA production is pronounced at low levels of nitrogen with longer period of incubation.

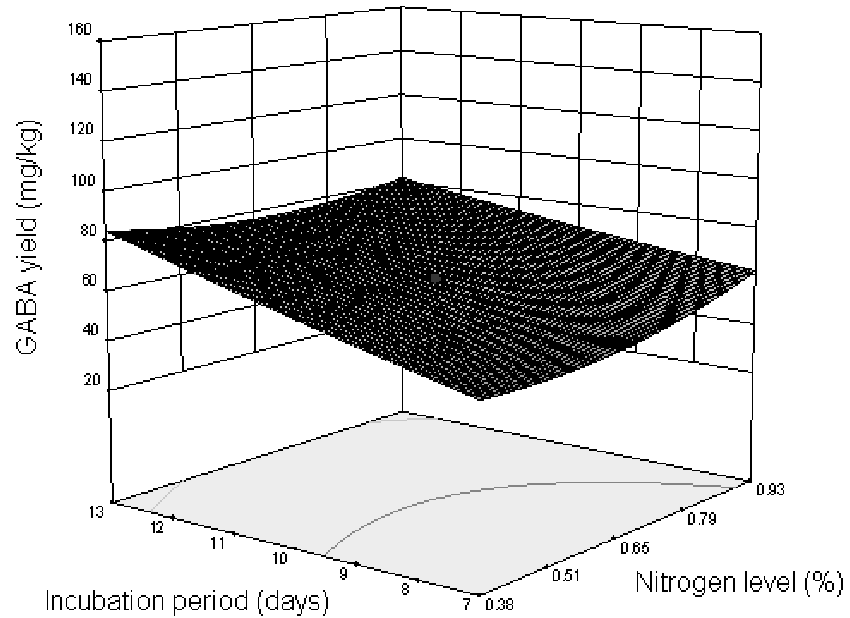



**Figure 40** **Three dimensional surface plot showing the effect of incubation period and nitrogen level on GABA yield at a temperature of 26° C and 4% inoculum level**

**Figure 41** portraits the effect of temperature and inoculum level on GABA production at 0.65% nitrogen level for an incubation period of 10 days. As the temperature increases beyond 21°C, GABA yield is also increased. Inoculum level independently showed increase in GABA production as its level increases. The interaction effect of temperature and inoculum level exhibited maximum GABA production (120 mg/kg) at 31°C and 2.4% respectively. Optimum temperature for GABA production is 36°C. From this, it is clear that temperature higher than the normal required for the fungal growth influences GABA production in the medium.

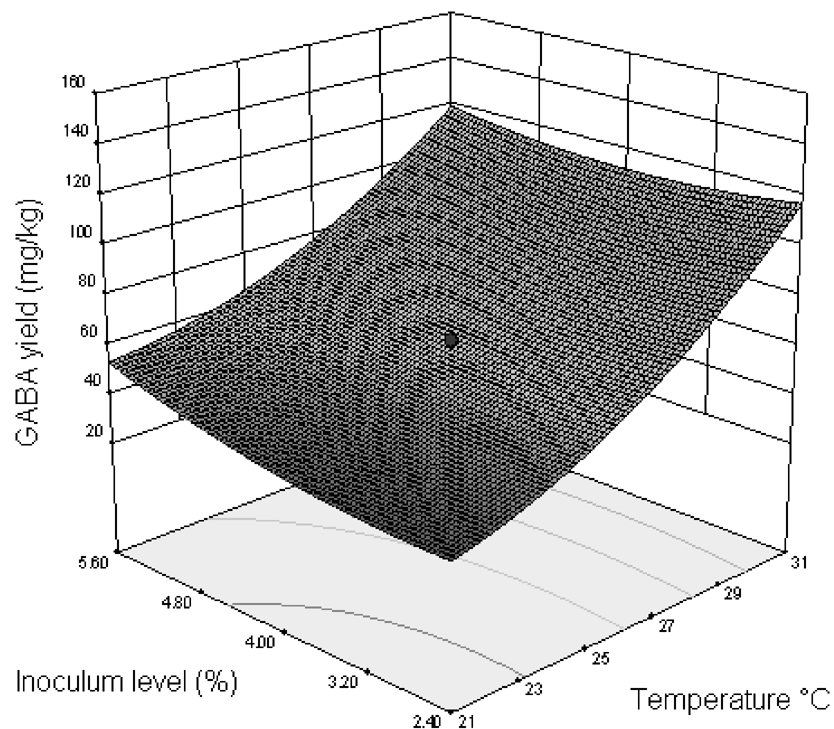



**Figure 41** **Three dimensional surface plot showing the effect of temperature and inoculum volume on GABA yield at 0.65% of nitrogen level for 10 days of incubation**

The effect of temperature and nitrogen level at 0.56 % inoculum level for a period of 10 days is shown in **Figure 42**. It is observed that the low levels of nitrogen and increase in temperature have positive effect on GABA production. At higher levels of nitrogen, GABA yield is low.

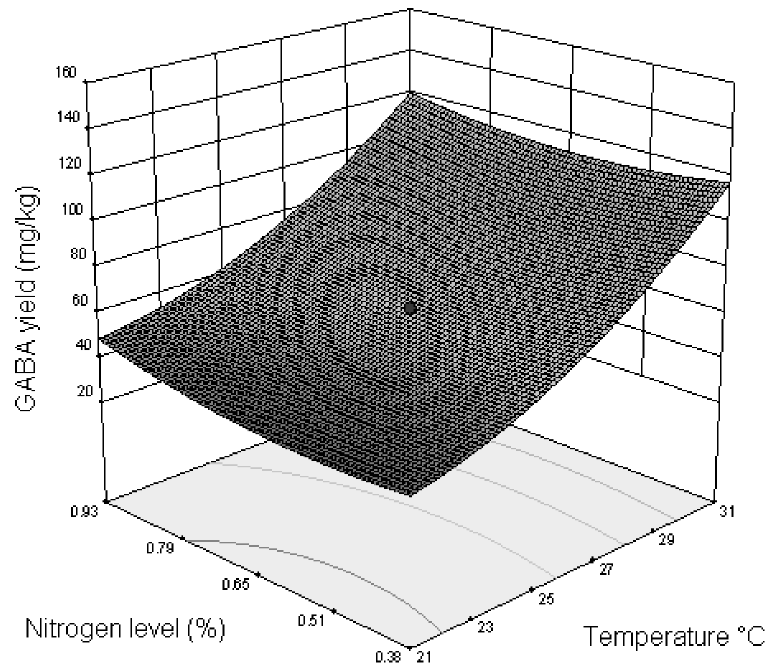



**Figure 42**. **Three dimensional surface plot showing the effect of temperature and nitrogen level on GABA yield at 4% inoculum level for 10 days of incubation**

Effect of inoculum volume and nitrogen level in SSF is shown in **Figure 43** at a temperature of 26°C and 10 days of fermentation. It is observed that the low levels of inoculum and nitrogen favours GABA production. At intermediate levels of 4% inoculum volume and 0.93 % nitrogen level, a drop in GABA production is observed.

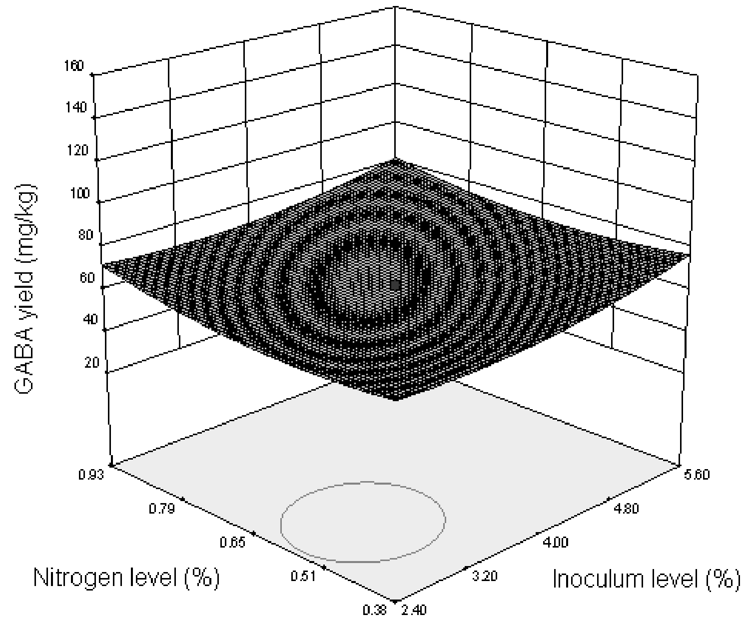



**Figure 43. Three dimensional surface plot showing the effect of inoculum level and nitrogen level on GABA yield at a temperature of 26 °C for 10 days of incubation**

From the **figures 38-41**, it was observed that the higher levels of incubation period and temperature had resulted in higher GABA yields. The **figures 42 and 43**, exhibits low levels of GABA at higher levels of inoculum and nitrogen. The shape of the response surface curves showed moderate interaction between the variables.

From equations derived by differentiating equation (A), the optimum values for the independent variables investigated are worked out as followed in earlier optimization experiments (Vijayalakshmi *et al.* 1999; Rajasimman and Subathra, 2009). Under the optimum values of temperature 36°C, 0.8 % (v/w) inoculum volume and 0.1% (w/w) nitrogen level over an incubation period of 16 days resulted in GABA yield of 399.31 mg/kg. Results were confirmed by carrying out fermentation by *Monascus purpureus* using shake falsk experiments.

The maximum concentration of GABA obtained experimentally was found to be 395.06 ± 5.11 mg /kg (No. of runs = 3) **(Table 20)**.

**Table 20. Optimum conditions for maximum GABA production**

| Variables | Prediction | Experimental |
|---|---|---|
| Incubation period (days) | 16 | 16 |
| Temperature (°C ) | 36 | 36 |
| Inoculum volume (%) | 0.8 | 0.8 |
| Nitrogen level (%) | 0.1 | 0.1 |
| GABA production (mg/kg) | 399.31 | 395.06±5.11 |

The good correlation between the experimental and predicted results verified the goodness of fit of the model ($R^2$ = 0.9869). Validation experiments also showed 98.94% correlation between experimental and predicted yields. Hence, experimental predictive equation for GABA production by *Monascus purpureus* with finger millet as a substrate under SSF appears to be significant.

## 2.3 EFFECTS OF *MONASCUS PURPUREUS* FERMENTED FINGER MILLET ON EGG QUALITY, SERUM LIPID PROFILE AND EGG YOLK CHOLESTEROL IN LAYING HENS

Egg is highly nutritious food which provides protein, vitamins and lipids that contain high levels of cholesterol. Thus, eggs are considered as high cholesterol food. Considering the high lipid content of egg and health consciousness of consumers, attempts were made to reduce the cholesterol content of egg. Genetic selection of layer birds and diet alterations were carried out in modifying whole-egg cholesterol in the past. Nowadays, considering the higher incidence of cardiovascular diseases due to hypercholesterolemia, much attention has been focused on the use of hypocholesterolemic agents to the layer hen's feed to regulate the egg yolk cholesterol. Ascomycetes fungus, *Monascus sp* traditionally known as red yeast rice has been used in the diet and as folk remedies. Endo (1979) discovered lipid lowering monacolin K in the broth of *Monascus ruber*. The fungus produces a range of secondary metabolite, statins *viz.* lovastatin, monacolin J, pravastatin and mevastatin (Manzoni *et al.* 1999) known as Monacolins. These metabolites are antihypercholesterolemic in nature and inhibit the enzymatic conversion of hydroxymethyl-glutarate to mevalonate by HMG- CoA reductase, which is the important step in the biosynthetic pathway of cholesterol (Manzoni and Rollini, 2002; Heber *et al.* 1999).

Finger millet is rich in minerals (mg/100g) Ca-302.33, P-237, Fe-4.33, Mg-109.66, Zn-2.11 and Cu - 0.52 (Sripriya *et al.* 1997). Considering the high nutritional property and economic advantage of finger millet over rice, the former has been selected as a substrate for cultivating the fungus, *Monascus purpureus* under solid state fermentation and the fermented product was used in layer feed supplementation for the first time to study its lipid lowering properties .

The effect of *Monascus purpureus* fermented finger millet supplementation with diet at different concentrations on egg quality, laying performance, serum lipid profile, egg yolk cholesterol and serum antioxidant enzymes activity in Single-Comb White Leghorn layer hens was studied.

## DAILY FEED AND STATIN INTAKE

The daily feed and statin intake for four weeks among different groups is presented in **Table 21.** The average statin content of *Monascus purpureus* fermented finger millet used in the study was 2944±108 mg/kg of dry substrate. Accordingly, the daily intake of statin with the corresponding feed intake was worked out for different weeks. It was observed that the intake of feed by the layer birds increased with increased levels of supplementation with red mold fermented finger millet.

**Table 21. Daily feed and statin intake by layer birds fed with *Monascus* fermented finger millet supplemented diet**

| Experimental Diets | 0 Week | I Week | | I I Week | | III Week | | IV Week | |
|---|---|---|---|---|---|---|---|---|---|
| | Feed (g) | Feed (g) | Statin (mg) | Feed (g) | Statin (mg) | Feed (g) | Statin (mg) | Feed (g) | Statin (mg) |
| CON | 124.13±0.86 | 125.62 ±0.93 | - | 126.43 ±2.05 | - | 128.85 ±1.89 | - | 128.92 ±1.67 | - |
| M25 | 124.23±0.98 | 125.45 ±1.05 | 0.092 | 127.28 ±1.76 | 0.094 | 129.73 ±1.54 | 0.095 | 130.18 ±1.49 | 0.096 |
| M50 | 124.19±1.16 | 126.26 ±0.87 | 0.186 | 127.54 ±1.23 | 0.188 | 128.64 ±1.09 | 0.089 | 127.54 ±0.98 | 0.188 |
| M100 | 124.15±1.08 | 127.67 ±1.21 | 0.376 | 128.35 ±1.09 | 0.378 | 129.27 ±1.36 | 0.380 | 128.97 ±1.63 | 0.379 |

Data represents mean of six values ± SEM in each treatment group

## EGG QUALITY

The effect of fermented red mold finger millet at different dosages on egg quality parameters like egg weight, shape index, Haugh Unit, shell thickness, shell weight, shell % and yolk colour were calculated and presented in **Table 22**. The results revealed that supplementation levels of red mold finger millet had no significant effect on Haugh Unit. Supplementation of germinated-cum-*Monascus* fermented finger millet 50 g/kg and 100 g/kg exhibited significant improvement in egg weight, shell weight, shell % and yolk colour. The eggs obtained from 100 g/kg supplemented group exhibited maximum yolk colour score of 12.75 ± 0.14 **(Figure 44)** when compared to control group eggs (9.25 ± 0.14).

**Table 22. Effect of *Monascus purpureus* fermented finger millet supplementation on the egg quality in layer birds**

| Experimental Diets | Egg weight (g) | Shape Index | Haugh Unit | Shell thickness (mm) | Shell weight (g) | Shell (%) | Yolk colour |
|---|---|---|---|---|---|---|---|
| CON | 55.28 $\pm1.88^{a}$ | 71.83 $\pm0.55^{a}$ | 103.46 $\pm1.58^{a}$ | 0.44 $\pm0.00^{a}$ | 5.35 $\pm0.05^{a}$ | 9.71 $\pm0.40^{a}$ | 9.25 $\pm0.14^{a}$ |
| M25 | 59.00 $\pm0.09^{b}$ | 73.63 $\pm1.11^{b}$ | 104.28 $\pm0.73^{a}$ | 0.46 $\pm0.00^{a}$ | 5.75 $\pm0.13^{b}$ | 9.75 $\pm0.20^{a}$ | 10.25 $\pm0.32^{ab}$ |
| M50 | 60.43 $\pm0.69^{b}$ | 73.43 $\pm0.24^{b}$ | 104.52 $\pm0.27^{a}$ | 0.46 $\pm0.01^{a}$ | 5.90 $\pm0.12^{b}$ | 9.77 $\pm0.31^{a}$ | 11.50 $\pm0.20^{bc}$ |
| M100 | 61.40 $\pm0.35^{b}$ | 73.72 $\pm0.46^{b}$ | 104.54 $\pm0.29^{a}$ | 0.47 $\pm0.01^{a}$ | 6.13 $\pm0.07^{bc}$ | 9.98 $\pm0.14^{c}$ | 12.75 $\pm0.14^{c}$ |

Data represents mean of six values ± SEM in each treatment group
Means bearing same superscripts within the column do not differ significantly ($P<0.05$)

**a) Egg yolk colour**

c) **Egg yolk colour of hen fed with diet supplemented with 5.0% *Monascus purpureus* fermented finger millet**

**b) Egg yolk colour of hen fed with diet supplemented with 2.5% *Monascus purpureus* fermented finger millet**

d) **Egg yolk colour of hen fed with diet supplemented with 10% *Monascus purpureus* fermented finger millet**

**Figure 44.** **Egg yolk colour of hen fed with diet supplemented with different concentrations of *Monascus purpureus* fermented finger millet**

## PRODUCTION PERFORMANCE

The effect of *Monascus* fermented finger millet content in the layer diet on body weight, egg production, egg weight and yolk weight is presented in **Table 23**. The results of the 25, 50 and 100 g/kg supplemented treatment groups exhibited reduction in the body weight (25.2 ± 11.5 g, 27.5 ± 14.9 g and 14.8 ±1 2.1 g respectively) when compared to the weight gained in control group (49.8±16.1 g). The per cent egg production in 50 and 100 g/kg supplemented groups significantly increased when compared to control and 25 g/kg supplemented groups.

## EGG YOLK CHOLESTEROL

The degree of reduction of egg cholesterol varied with the content of germinated-cum-*Monascus* fermented finger millet in the feed. Egg cholesterol of control group is 193.21±2.87 mg/egg. After four weeks of feeding, reduction in cholesterol content by 11.96% and 14.08% were observed for the 25 and 50 g/kg supplemented groups respectively. However, the group fed with 100 g/kg supplementation exhibited maximum reduction of cholesterol by 16.45%.

## SERUM CHOLESTEROL, HDL, LDL AND TRIGLYCERIDES

Supplementation of *Monascus purpureus* fermented finger millet in the layer diets exhibited positive effects on serum cholesterol, HDL, LDL and triglycerides level (Table 23). The results indicated that the reduction of cholesterol, LDL and triglycerides was observed in all the treatment groups. However, after four weeks of experimental period, the treatment group fed with 100 g/kg supplementation revealed significant reduction in serum cholesterol, LDL and triglycerides by 36.30%, 33.39% and 21.70% respectively and increase in HDL level by 33.43% when compared to control **(Figure 45)**.

**Table 23. Effect of *Monascus purpureus* fermented finger millet on serum lipids in layers**

| Experimental Diets | Total cholesterol (mg/dl) | Triglycerides (mg/dl) | HDL Cholesterol (mg/dl) | LDL Cholesterol (mg/dl) |
|---|---|---|---|---|
| CON | 167.17±2.07[a] | 1648.20±11.83[a] | 39.48±1.29[a] | 43.93±0.88[a] |
| M25 | 160.17±4.62[a] | 1511.61±16.06[ab] | 49.39±1.16[a] | 40.67±0.55[b] |
| M50 | 133.05±1.90[b] | 1377.09±13.13[bc] | 51.45±0.99[a] | 33.07±0.34[c] |
| M100 | 106.47±3.13[c] | 1290.48±12.24[c] | 52.68±2.02[b] | 29.26±0.38[c] |

Data represents mean of six values ± SEM in each treatment group
Means bearing same superscripts within the column do not differ significantly (P<0.05)

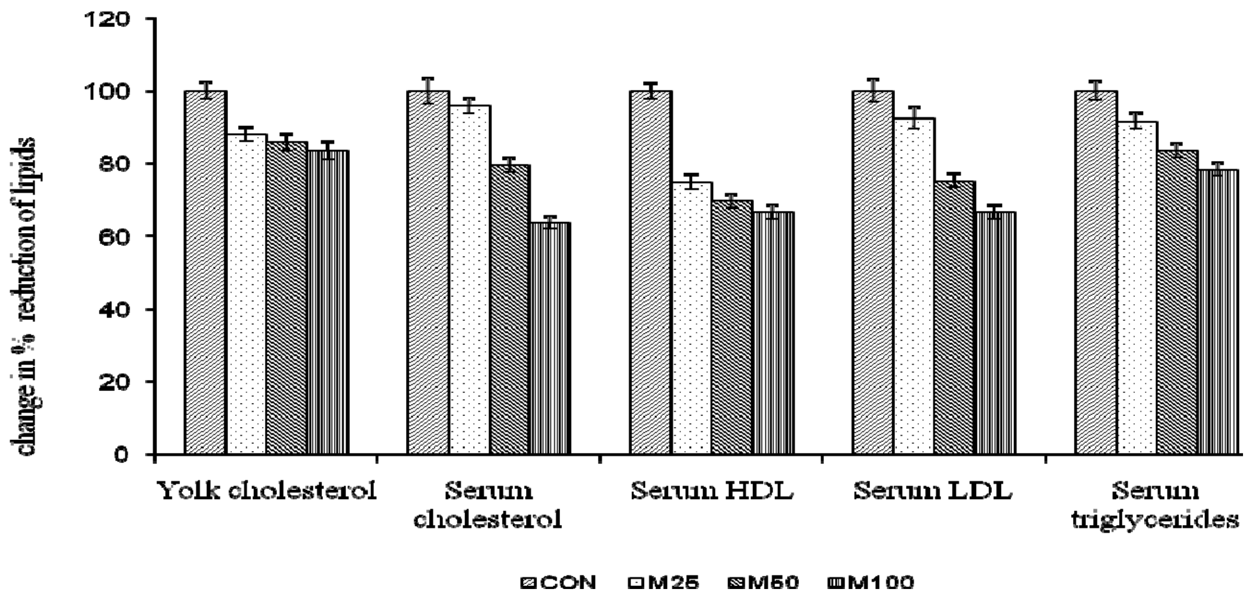



**Figure 45. Percentage reduction of yolk and serum lipids among different treatment groups**

## SERUM ANTIOXIDANT ENZYMES ACTIVITY

The antioxidant Activities of glutathione reductase, glutathione peroxidase, glutathione transferase and catalase in serum of treatment groups and control group are presented in Table 24. Serum glutathione reductase activity was not affected by any of the red mold finger millet supplemented group. The activity of glutathione peroxidase was generally increased by the supplementation of red mold finger millet with the basal layer diet. The increase in the enzyme activity produced in the layer hens by 25, 50 and 100 g/kg red mold finger millet supplemented diet were 34.3%, 53.4% and 71.3% respectively.

Serum glutathione transferase activity was enhanced by red mold finger millet supplemented layer diet **(Table 24)**. The increase in the enzyme activity produced in the layer hens by 25, 50 and 100 g/kg red mold finger millet supplemented diet were 36.4%, 76.5% and 104% respectively. Catalase activity was also found to be reduced by the action of germinated-cum-*Monascus* fermented finger millet.

**Table 24. Effect of *Monascus purpureus* fermented finger millet on serum antioxidant enzyme activity**

| Experimental Diets | Glutathione reductase (mmol/min/dl) | Glutathione peroxidase (mmol/min/dl) | Glutathione transferase (mmol/min/dl) | Catalase (mmol/min/dl) |
|---|---|---|---|---|
| CON | 1.98±0.28[a] | 34.50±1.18[a] | 1.92±0.35[a] | 10.01±0.16[a] |
| M25 | 2.06±0.12[a] | 46..32±1.26[b] | 2.62±0.62[ab] | 9.88±0.75[a] |
| M50 | 2.13±0.20[a] | 52.93±1.92[bc] | 3.39±.0.54[bc] | 8.12±0.61[b] |
| M100 | 1.97±0.16[a] | 59.11±2.16[c] | 3.92±0.48[c] | 7.31±0.23[bc] |

Data represents mean of six values ± SEM in each treatment group
Means bearing same superscripts within the column do not differ significantly ($P<0.05$)

# CHAPTER 3

## CHARACTERIZATION OF *MONASCUS PURPUREUS* AND MUTANT IN RELATION TO STATIN AND GABA PRODUCTION

Red mold rice fermented using *Monascus spp* is effective in producing statins and gamma amino butyric acid. Polyketide synthase (PKS) enzyme is involved in the biosynthesis of statins. The activity of PKS enzyme is regulated by PKS gene. Studies of polyketide synthase on lovastatin biosynthesis were carried out in *Aspergillus terreus*, the main species used in industrial production of lovastatin. PKS gene structure was investigated and sequence analysis found that in the cloned 64 kb DNA there were 18 open reading frames (ORF), 13 of which had been functionally determined by blasting. The gene cluster consists of the lovastatin nonaketide synthase gene (*lov B*), lovastatin diketide synthase gene (*lovF*), enoyl reductase gene (*lovC*), transferase gene (*lov D*), HMG-CoA reductase gene (*ORF 8*), regulatory genes (*lovE* and *ORF13*) and cytochrome P450 mono-oxygenase gene. Molecular studies have been carried out to establish the causes of the superior lovastatin productivity of a novel solid state fermentation process (Gonzalez *et al.* 2008).

*Monascus spp. viz. purpureus, pilosus* and *ruber* are also involved in the production of statin. In *Monascus pilosus*, the characterization of monacolin K biosynthetic gene cluster was carried out. The gene cluster consists of polyketide synthase genes (*mokA and mokB*), P450 monooxygenase gene (*mokC*), oxidoreductase (*mokD*), dehydrogenase (*mokE*), transferase (*mokF*), HMG-CoA reductase (*mokG*), transcription factor (*mokH*) and efflux pump (*mokI*) which are identical (homologous) to the *lovB, lovF, lovA, lovG,lovC, lovD, lvrA, lovE* and *lovI* genes respectively of the lovastatin biosynthetic gene cluster of *Aspergillus terreus*. In the present study, characterization of *Monascus purpureus* MTCC 410 wild type and its mutants in

relation to statin production was carried out to explore the possibility of the presence of gene similar to *lov* genes.

Hyper pigment producing and albino mutants were developed by spreading one week old spore suspension on PDA plates and exposed to germicidal UV to a time that resulted in 30-40% kill. After incubation for 16 h at 4°C in the dark, temperature selection was carried out by keeping the plates at 42°C for 24 h. the plates were transferred to 30 °C and colonies emerging after 5 d incubation were screened for phenotypic changes by visual observation. The albino and hyper mutant so isolated were used in this study.

*Monascus purpureus* wild type, albino and hyper pigment producing mutants were grown under SSF with germinated finger millet as substrate for production of statin. The statin yield of all the isolates was analyzed using HPLC and the results are presented in **Table 25.**

**Table 25. Statin yield (mg/kg) dry substrate of *Monascus purpureus* wild type, albino and hyper mutants**

| **Culture isolate** | **Statin yield (mg /kg) at different days of fermentation** | | | | |
|---|---|---|---|---|---|
| | **3** | **5** | **7** | **9** | **11** |
| Wild type | 296 ± 2.11 | 1934 ± 9.22 | 5237 ± 23.09 | 4180 ± 18.02 | 4002 ± 15.23 |
| Albino mutant | - | 12 ± 0.01 | 58 ± 1.20 | 71 ± 0.08 | 64 ± 0.09 |
| Hyper mutant | 30±0.07 | 76 ± 0.14 | 158 ± 1.28 | 240 ± 2.69 | 238 ± 2.01 |

Data represents mean of three values ± SEM

The wild type has yielded highest amount of statin 5237 ± 23.09 mg/kg dry wt compared to the mutants. In order to find out the reason for low production of statin by the mutants, an attempt using biotechnological procedure was carried out to identify the gene responsible for statin production as mentioned below.

## ISOLATION OF DNA

The DNA of *Aspergillus terreus* MTCC 2803 (positive control for *lov* genes), *Monascus purpureus* MTCC 410 wild type and its albino and hyper pigment producing mutants was isolated using the standard procedure as indicated in Materials and Methods. The isolated DNA was tested for its quality using 1.5% agarose gel with 0.5x TBE buffer. After half way run in the agarose gel, the DNA was examined under UV illuminator **(Figure 46)**.

## AMPLIFIED TARGET FRAGMENT

The PCR product for *lovE* showed non-specific bands. However, for *lovF* gene the PCR product of 750 bp was amplified with primers *lovF*-F and *lovF*-R which coincided with the expected product of 750 bp. **Figure 47** indicates the target fragment amplified successfully in *Monascus purpureus* MTCC 410 wild type, albino and hyper pigment producing mutants with corresponding amplification in the positive control (*Aspergillus terreus* MTCC 2803).

## CONFIRMATION OF THE PRESENCE OF DNA INSERT USING M13 PRIMERS

Plasmid was isolated from the transformed DH5α cells. The DNA of plasmid was used in PCR amplification with M13 primers. The corresponding bands including the size of the insert DNA was found in *Aspergillus terreus* (positive control), wild type, albino and hyper mutant of *Monascus purpureus*.

## SEQUENCING

After elution and ligation of the PCR product with T/A vector, the transformation of vector into DH5α *E.coli* cells was carried out. The *E.coli* culture carrying the plasmid containing insert DNA was sent to M/s Bangalore Genei, Bangalore for sequencing. Sequences are portraited in the **Figure 48.** The sequence (unrelated plasmid sequence was removed) was identical with a part of *Aspergillus terreus* lovastatin biosynthesis gene cluster from genebank.

## NUCLEOTIDE AND AMINO ACID SEQUENCE ANALYSIS

The sequencing results were analysed using Molecular Evolutionary Genetics Analysis software **MEGA 4.0** (http://www.megasoftware.net/megahtml). The newly identified gene sequence with 750 bp size of *Monascus purpureus* MTCC 410 (wild type) is identical to the *Aspergillus terreus lovF* gene (AF141925), which led to the identification of the presence of *lovF* gene in *Monascus purpureus,* which is involved in polyketide (Lovastatin diketide synthase- LDKS) synthesis during lovastatin biosynthesis.

## MOLECULAR ANALYSIS OF HYPER PIGMENT PRODUCING AND ALBINO MUTANTS OF *MONASCUS PURPUREUS*

The results revealed that the *Monascus purpureus* (MTCC410) and *Aspergillus terreus* (MTCC2803) had two nucleotide variations in at 275 and 331 bp positions (**Figure 48**). At position 275, T was replaced by A and at 331, A was replaced by G. When the hyper-and albino- mutants were compared with the wild strain, the change was in reverse order. In hyper pigment producing mutant, A was replaced by T (at 275 bp) and G was replaced by A (at 331 bp) and no change occurs at albino-mutant. The experiment confirmed the occurrence of two point mutations in *lovF* gene of hyper pigment producing mutant, which is not observed in albino-mutant.

It was confirmed that the mutation has not affected the lovF gene responsible for statin biosynthesis (LDKS) in both the wild as well as the mutants. However, it might have affected the other regulatory (*lovE*) gene and nonaketide synthase (*lovB*) gene, which are also responsible for lovastatin biosynthesis. As a result, enhanced yield of statin is not observed in mutant strains of *Monascus purpureus*.

Having confirmed the above on statin biosynthesis, experiments were carried out to study the effect of this mutation on GABA production in the presence of precursor, monosodium glutamate.

## 3.2 CHARACTERIZATION OF *MONASCUS PURPUREUS* MTCC 410 AND ITS MUTANTS IN RELATION TO GABA PRODUCTION

Gamma aminobutyric acid (GABA), a four carbon non protein amino acid, acts as a major inhibitory neurotransmitter in the central nervous system (Krnjevic 1974). GABA has several physiological functions such as neurotransmission, induction of hypotensive effects, diuretic effects, treatment of epilepsy and transquilizer effects (Jakobs *et al*.1993; Cohen *et al*. 2002; Komatsuzaki *et al*. 2005). Some recent studies showed that GABA is also a strong secretagogue of insulin from the pancreas, effectively preventing diabetes (Adeghate and Ponery 2002; Hagiwara *et al*. 2004). To date, GABA has been used extensively in pharmaceuticals and functional foods such as gammalone, cheese, gabaron tea and shochu.

Due to the increasing commercial demand for GABA, various chemical and biological methods for GABA have been studied. Biosynthesis of GABA may be a much more promising method due to simple reaction procedure, high catalytic efficiency, mild reaction conditions and environmental compatibility. Glutamate decarboxylase (GAD) is the unique enzyme to catalyse the conversion of L-glutamate or its salts to GABA through the single-step α-decarboxylation.

The production of GABA by batch fermentation has been reported using various microorganisms, but the recovery of GABA from such complex fermentation broth is generally difficult and expensive to perform. *Monascus purpureus* CCRC 31615, on addition of sodium nitrate during solid-state fermentation produced 1.267 g/kg of GABA. *Lactobacillus brevis* were entrapped into Ca-alginate gel beads and the biotransformation of sodium L-glutamate (L-MSG) to GABA (Huang *et al*. 2007).

The molecular studies carried out confirmed the presence of *lovF* gene responsible for lovastatin production. It also suggested that mutation in *lovF* gene alone cannot be responsible for the lower production of lovastatin. Further, it was attempted to study the effect of mutation on the other important metabolite, GABA produced by *Monascus purpureus* using the precursor Monosodium glutamate in the growth medium. The GABA yield by the isolates of *Monascus purpureus* after 10 days fermentation at different concentrations of monosodium glutamate is presented in **Table 26.**

**Table 26. GABA yield (mg/kg) from *Monascus purpureus* wild type and its mutants with different concentrations of MSG**

| Culture isolate | GABA yield (mg/kg) at different concentrations of MSG | | | | | |
|---|---|---|---|---|---|---|
| | 0% | 1% | 2% | 3% | 4% | 5% |
| Wild type | 89.16±2.11 | 108.61±1.12 | 316.23±5.61 | 432.61±6.14 | 621.65±11.11 | 520.14±12.12 |
| Albino mutant | 38.67±1.14 | 68.32±2.13 | 110.13±2.42 | 169.72±4.12 | 210.18±6.18 | 168.56±7.11 |
| Hyper mutant | 51.62±1.66 | 81.11±1.02 | 98.62±4.12 | 153.61±5.16 | 182.16±6.84 | 180.37±4.99 |

Data represents mean of three values ± SEM

The wild type, albino and hyper mutants of *Monascus purpureus* showed the increasing trend of GABA yield at 0-4% level of MSG. Among the isolates, wild type produced 621.65±11.11mg /kg GABA compared to others. Hyper pigment producing mutant and albino mutant yielded GABA of 182.16±6.84 mg/kg and 210.18±6.18 mg/kg respectively at a concentration of 4% MSG.

# DISCUSSION

Finger millet, also known as *ragi*, is valued as staple food in south India (Karnataka, Tamil Nadu and Andhra Pradesh) and hilly regions of the country. The straw has immense utility as fodder. Silage is also made from *ragi* forages at flowering stage. It is a rich source of calcium (0.344%) for growing children and aged people. It is usually converted into flour, which is used for preparation of cake/puddings/porridge. Straw makes valuable fodder for both draught and milch animals. It is wholesome food for diabetics.

The cytomorphological studies indicate that finger millet (*E. coracana*) might have originated from *E. africana* through selection in Ethiopia and highlands of Africa. It was introduced into India about 3,000 years ago, and became the secondary center of finger millets diversity.

Finger millet is a rich source of minerals. However, the nutritional availability of these is hindered due to their bound form with phytic acid. Germination of finger millet is known to degrade phytic acid, thereby releasing the minerals in the available form. Fermentation of finger millet apart from degrading phytic acid and releasing minerals from the phytic acid-mineral complex also degrades tannin-carbohydrate and tannin-complexes into simple sugars and amino acids.

*Monascus purpureus* was reported to produce metabolites like polyketides, antihypercholesterolemic agents, antihypertensive metabolite, lipid lowering fractions and other unsaturated fatty acids like oleic, linoleic, linolenic acids, etc. Microbial growth in addition, contributes to dietary fibre and lipid content. Thus fermentation of germinated finger millet by

*Monascus purpureus* in relation to nutritional quality and value addition formed the subject of the present investigation.

## BIOACTIVE MOLECULES FROM *MONASCUS PURPUREUS*

Fermentation of germinated finger milet by *Monascus purpureus* resulted in increasing the nutritional availability, converting complex carbohydrates and proteins to simple sugars and amino acids and supported the production of bioactive metabolites like statins **(Table 6)**, γ-amino butyric acid **(Table 7)** dietary sterols **(Figure 24)** and unsaturated fatty acids **(Table 8)**.

After seven days of SSF, the germinated finger millet fermented with *Monascus purpureus* yielded higher total statin of 5.24 g/kg (pravastatin and lovastatin content of 4.87 g/kg and 0.37 g/kg respectively) when compared to other substrates used such as broken wheat (4.41 g/kg) and parboiled rice (4.10 g/kg) **(Table 6)**. *Monascus pupureus* 'MTCC 369' when grown on synthetic medium under shake flask culures yielded lovastatin of 351 g/l (Sayyad et al 2007). *Monascus pupureus* 'NTU601' when grown on SSF with rice as substrates, the production of monocolin K at 30 °C was 530 mg/kg (Wang et al 2003). Su et al (2003) reported that *Monascus pupureus* 'CCRC 31615' exhibited monocolin K yield of 378 mg / kg under SSF with the long grain rice. *Monascus purpureus* 'NTU 301' with *Dioscorea* as the substrate has resulted in 2584 mg monocolin k which is 5.37 times more when rice is used as a substrate (Lee et al 2006). It is inferred from the above literature that the yield of statin was very high (5.24 g/kg) when *Monascus purpureus* was cultivated on germinated finger millet by SSF. This may be due to availability of essential nutrients and growth factors, promoted by germination of finger millet, for the growth of *Monascus purpureus*. However, from 9$^{th}$ day of fermentation, the statin yield showed decreasing trend **(Figure 22)**. Manzoni et al (1999) reported that *Monascus paxii*

‘AM12M’ spontaneous mutant yielded 127 mg lovastatin per litre and 53 mg pravastatin per litre at 21 days when whole soybean flour medium was used. The results obtained in the present research exhibited higher production of total statin 5.24 g/kg dry wt (lovastatin 0.37 g /kg and pravastatin 4.87 g/kg dry wt.) in a short fermentation period of 7 days.

The *Monascus* fermented barley, maize and raw rice yielded total sterol of 0.14, 0.09 and 0.08 g/kg respectively. Germinated finger millet exhibited highest yield of 0.53 g/kg sterol as against 0.02 g/kg sterol yield of finger millet **(Figure 24)**. The substrates like germinated finger millet, barley and broken wheat when fermented with *Monascus purpureus* resulted in 7.57, 10.69 and 13.66 folds significantly higher sterol production than the control. The remarkable increase in sterol yield is due to the production of sterol by *Monascus purpureus* during SSF. The results obtained in this study were supported by the report of Heber et al 1999, wherein it was stated that the red yeast rice produces sterols such as β- sitosterol and campasterol in addition to other bioactive molecules. The structural similarity of the sterols to cholesterol enables them to compete with cholesterol for incorporation into micelli and thereby lowers the dietary cholesterol and also the cholesterol accumulated in the gastrointestinal tract (Lichtenstein 2002, Clifton 2002). Simons (2002) demonstrated that the effect of using a plant sterol ester in combination with statin is equivalent to doubling the dose of statins for the treatment of antihypercholesterolemia.

**Value addition to finger millet**

The decrease in carbohydrate content of finger millet on germination, fermentation and both compared to raw finger millet is presented in **Table 9**. This change in carbohydrate content was attributed to the conversion of starch to reducing sugars during germination followed by

fermentation by *Monascus purpureus*. During germination, mobilization and hydrolysis of seed polysaccharides occur (Khetarpaul and Chauhan 1990; Sripriya et al. 1997; Mbithi-Mwikya et al. 2000). The unhydrolysed polysaccharides can be further hydrolysed to simple sugars by the saccharifying enzymes produced by fermenting microbes (Bernfeld 1962; Parvathy and Sadasivam 1982). A marked drop in reducing sugar in the *Monascus* fermented sprouted finger millet and *Monascus* fermented finger millet may be due to microbial utilization of sugars for its growth and metabolism.

The soluble protein content of the processed finger millet exhibited a steep increase due to the increased microbial enzyme activity and protein hydrolysis during fermentation (Hamad and Fields 1979). The reduction in the soluble protein content in the case of sprouted fermented finger millet after 10 d of fermentation may be due to the protein utilization by the fungus for its growth and metabolism.

An increase in the lipid content of fermented finger millet and sprouted fermented finger millet is attributed to the growth of *Monascus purpureus* on raw finger millet and germinated finger millet. These results are comparable to the observations of Mugula and Lyino (1999) wherein, it was reported that the fermented tempe made of finger millet-mung bean- groundnuts exhibited crude fat content of 3.1% when compared to control value of 2.9 %. The difference in the lipid content was not significant.

Increase in the dietary fiber content of the finger millet during processing is contributed by germination and fermentation by *Monascus purpureus*. Malleshi and Klopfenstein (1998) reported that the dietary fiber content of native sorghum, pearl millet and finger millet increased when they were malted for 24 to 96 h, which appears to be similar to the results obtained in the present study.

Germination and fermentation of finger millet showed reduction in pH. The results are comparable to the observations of Sripriya et al. (1997) wherein, it was reported that the initial pH of finger millet 5.8 was reduced to 5.7 during germination and 3.87 after 48 h of fermentation, which was attributed to the production of organic acids like lactic acid, acetic acid and citric acids during fermentation. This reduced pH is responsible for eliminating undesirable microflora in fermented foods (Chavan and Kadam 1989).

The bioavailability of minerals was increased by germination and fermentation of finger millet by *Monascus purpureus* **(Table 10)**. This is in agreement with earlier reports on processing (germination and fermentation) of finger millet, wherein the increase in bioavailability of minerals (Sripriya et al. 1997; Malleshi and Klopfenstein 1998) was reported.

The processing of finger millet by germination and fermentation with *Monascus purpureus* reduced the antinutrient factors viz. phytic acid and tannins **(Table 11)**. Similar findings have been reported in Faba beans, where phytate levels decreased by up to 77% during a 10 d germination period (Eskin and wiebe 1983). The observed reduction in tannin content in germinated seeds has been attributed to the formation of hydrophobic associations of tannins with seed proteins and not due to actual loss or degradation of tannins (Butler et al. 1984). A decrease in tannin content in sorghum has been attributed to leaching in the sprouting medium and increased activity of polyphenol oxidase and other catabolic enzymes as observed by Kruger (1976) in wheat. This degradation of phytates and tannins by germination and fermentation increases the HCl extractability of minerals thereby increasing their bioavailability. Sripriya *et al*. 1997 have reported the reduction of phytate and tannin or total phenols by germination and fermentation would lead to increase in bioavailability of minerals.

**Optimization of statin production**

Statistical methodologies have been applied in biotechnological process to define fermentation process and to deal with a large number of variables (Manuel and Antonio 1997). Out of various statistical methods, Response Surface Methodology (RSM) is an efficient tool and can be used to test multiple process variables with fewer experimental runs. RSM was used to enhance the yield of statin and GABA.

From the ANOVA (**Table 14)**, it is observed that the linear effects of ammonium nitrate and soybean flour and quadratic effect of glycerol exhibited significant effect ($p<0.05$) with respect to statin production. It is also observed from the regression coefficient **Table 15** that the linear and quadratic effects of glycerol and quadratic effect of soybean flour exhibited significant effect ($p<0.05$) with respect to statin production and are important factors in determining the statin production by *Monascus purpureus* in this study.

The experimental yields fitted the second order polynomial equation as indicated by high R squared value (0.81). The regression coefficient of all the linear and quadratic terms of glycerol were significant at $P<0.05$. ANOVA suggested that the model to be significant at $P<0.05$. The P- values used as a tool to check the significance of each of the coefficients indicated the pattern of interactions between the variables. Smaller value of P was more significant to the corresponding coefficient (Liu et al. 2003).

The three dimensional response surface plots **(Figures 35&36)** described by the regression model were drawn to illustrate the effects of the independent variables, and effects of interactions of independent variables, on the response variable. From the three dimensional

response surface plots, the optimal values of the independent variables were determined and the interaction between each independent variable pair described.

It is evidenced from the above that the 3.12% glycerol, high level (0.3%) of ammonium nitrate and high level (15%) of soybean flour addition in the finger millet substrate enhanced the total statin production upto 8.025 g/kg of the substrate used in the solid state fermentation.

Fungus *Monascus purpureus* MTCC 410 produced 8.025 g/kg of total statin under optimized parameters by solid state fermentation. This is much higher than that of lovastatin produced by *Monascus purpureus* MTCC 369 (Sayyad et al. 2007), *Aspergillus terreus* BST and *Monascus paxii* AM12M reported by Su et al. (2003) and Manzoni et al. (1999) in submerged fermentation. The outcome of this study tremendous scope in scaling up of industrial statin production.

**Optimization of GABA production**

**Table 17** shows the considerable variation in the yield of GABA under different cultural conditions along with the predicted values. The GABA yield varied from 12.30 to 189.19 mg/kg of the dry finger millet substrate. The run #1 and run #20 had the minimum and maximum GABA yield respectively. The results of ANOVA **(Table 19)** for the GABA yield by *Monascus purpureus* demonstrated that the model is highly significant, as it is evident from the calculated F value (80.80 %), low probability value (Pmodel>F 0.0001) and high R squared (0.9869) value. The regression coefficient data along with the corresponding P-values **(Table 19)** for GABA yield by *Monascus purpureus* showed that the regression coefficients of the linear terms of incubation period and temperature, quadratic terms of temperature and interaction effects of incubation period with temperature and inoculums level were significant at $P<0.0001$.

The response analysis revealed that maximum yield of GABA (399.31 mg/kg) could be achieved when *Monascus purpureus* was cultivated on germinated finger millet at a temperature of 36 °C with 0.8% inoculum volume and 0.1% nitrogen level for an incubation period of 16 days. The three dimensional surface response for independent variables, incubation period, temperature, and inoculum volume and nitrogen level on the yield of GABA **(Figures 36-41)** suggested that biomass yield was affected by all the variables. The validation experiment conducted at the optimized conditions yielded GABA of 395.06 mg/kg of the germinated finger millet substrate **(Table 20)**.

The four level central composite rotatable design (CCRD) applied with the aim of optimizing the cultural conditions for GABA production by *Monascus purpureus* resulted in enhanced yield of GABA. Evaluation was carried out with four parameters in 30 experiments **(Table 18)** on GABA yield by *Monascus purpureus* when cultivated on germinated finger millet. From three dimensional response surface plots, the optimal values of the independent variables and the interaction between each independent variables pair are described in **Figures 36-41**. This experiment was the first attempt for statistical experimental design to optimize the GABA production from *Monascus purpureus* using finger millet as substrate. These facts are important in making the whole process economically more feasible for production of GABA from *Monascus purpureus*.

In conclusion, it can be stated that the production of GABA by *Monascus purpureus* using finger millet as substrate is new to literature.

**Effect of red yeast finger millet on laying hens**

The hypocholesterolemic and antioxidant activity of Monascus purpureus fermented finger millet was confirmed by poulry experiment as elaborated in Chapter 2.The increase in feed intake **(Table 21)** among the supplemented treatment groups was observed with increase in statin content of the feed (Mori *et al*. 1999; Wang and Pan 2003). The increased yolk colour **(Figure 42)** may be due to carotenoid and polyketide production by *Monascus purpureus* fermented finger millet. there are reports on the significant increase in shell weight in layer hens fed with 8 per cent supplemented red mold rice (Wang and Pan, 2003). In the present study, the improvement in egg weight, shell weight and shell percentage must have been contributed by the calcium present in the finger millet used in solid state fermentation. Increase in the egg weight of the supplemented groups **(Table 22)** may be due to the mineral rich finger millet, used as substrate in *Monascus* fermentation. As per the reports of Engberg *et al.* (2009) the use of fermented feed increased egg weight and shell weight. Significant decrease in yolk weight was observed in *Monascus* fermented germinated finger millet when compared to control. The reduction in the body and yolk weight in the treated birds might have been attributed by the hypolipidemic effect of statin present in the *Monascus* fermented finger millet supplemented diet. However, the earlier study of Wang and Pan (2003), wherein the rice was used as a substrate in the fermentation of *Monascus* and fed as dietary supplement to layer birds had no significant effect on the yolk weight.

Reduction in yolk cholesterol **(Figure 43)** by red mold finger millet supplemented diet agree closely with the reports of Wang and Pan (2003), wherein it is stated that the yolk cholesterol reduction by 13.89% was achieved by feeding the layer hens with 2.0% red mold rice

supplemented layer diet. Yolk cholesterol reduction was also observed by feeding the layer birds with statin drugs supplemented diet (Elkin and Rogler, 1990; Elkin *et al*. 1999; Mori *et al*. 1999).

The changes in the percentage of serum lipids of layer hens fed with 10% Monascus fermented finger millet supplemented layer diet at **Table 23** cprroborates with the results reported by Mori *et al*. (1999), wherein 0.001% lovastatin drug reduced the plasma triglycerides level by 38.5% and total cholesterol by 30.4%. Wang and Pan (2003) reported a significant reduction in LDL by 21.73% in the layer birds fed with 8% red mold rice supplemented feed containing 0.056% monocolin K. Similar results were reported by Elkin and Rogler (1990) and Luhman *et al*. (1990).

Serum catalase activity was negatively influenced by red mold finger millet at different supplementation levels in the layer birds, when compared to control. There is very scanty literature on the antioxidant enzymes activity in layer birds in relation to red mold rice. However, the results of the present study corroborates with the work of Wang and Pan (2003) wherein it was reported that 6-14% inhibition rate of serum lipid peroxidation was achieved by red mold rice, which proved it as an ameliorating substance in reducing lipid peroxidation in serum. *Monascus* fermented finger millet exhibited antioxidant property which is on par with the reports of Lee *et al*. (2008). The increased level of serum glutathione peroxidase and transferase **(Table 24)** might have been attributed to the supplementation of *monsacus* fermented finger millet, which contains antioxidant principles and the result corroborates with the findings of Tuzcu *et al*. (2008).

In conclusion, the present study showed the efficacy of *Monascus purpureus* fermented finger millet content in diet on improvement of egg quality, reduced lipid levels of the serum and

egg yolk. Maximum reduction in serum cholesterol, triglycerides, LDL-cholesterol and significant reduction in yolk cholesterol were observed in layer hens fed with diet supplemented with 10% *Monascus purpureus* fermented finger millet, resulting in low cholesterol eggs, which is of health importance. The outcome of this study can be used in developing hypolipidemic designer eggs. These low cholesterol eggs may form as a better diet for the patients affected by hypercholesterolemia than the normal egg.

**Molecular analysis of hyper pigment producing and albino mutants of *Monascus purpureus* in relation to statin production**

Lovastatin is one of the secondary metabolite of few fungi with great commercial exploitation. Understanding the biosynthetic pathway of lovastatin is the main theme of research in this field for expression in *E. coli* like simple bacterial systems and for over-production through mutations (Askenazi *et al.* 2003; Sorensen *et al.* 2003; Xie *et al.* 2006; Barrios-Gonzalez *et al.* 2008). Several works of polyketide synthase on lovastatin biosynthesis are mainly conducted in *Aspergillus terreus*, which is currently the main species for industrial production of lovastatin (Hendrickson *et al.* 1999; Kennedy *et al.* 1999; Sutherland *et al.* 2001). *Aspergillus terreus* lovastatin biosynthesis gene cluster consists of 18 open reading frames (ORF), 13 of which had been functionally determined by basic local alignment search tool (BLAST). The gene cluster consists of the lovastatin nonaketide synthase gene (*lovB*), lovastatin diketide synthase gene (*lovF*), enoyl reductase gene (*lovC*), transesterase gene (*lovD*), HMG-CoA reductase gene (ORF8), regulatory genes (*lovE* and ORF13) and cytochrome P450 monooxygenase gene.

*lovE* is one of the regulatory genes in lovastatin biosynthesis and encodes a GAL4-like transcriptional factor with a zinc finger domain. No lovastatin or any intermediate products

could be detected by the deletion of the *lovE* gene, while an additional copy of *lovE* transformed into *Aspergillus terrus* resulted in overproduction of lovastatin by 5–7 fold (Todd 1997; Kennedy *et al.* 1999). Similarly, *lovF* is one of the two polyketide synthases involved in lovastatin biosynthesis, whose expression can be controlled by fermentation conditions (Barrios-González *et al.* 2008). In the present study, an attempt was made to compare the *lovF* gene of wild and mutants of *Monascus purpureus* along with the positive control (*Aspergillus terreus*). The main objectives of this work are to compare the *lovF* of *Aspergillus terrus* and *Monascus purpureus* and also to find the cause for low accumulation of lovastatin in hyper pigment producing mutant of *Monascus purpureus*. In the present study, the primers designed from *A. terreus* to amplify the *lovF* was used for *M. purpureus* as there are no reports with respect to lovastatin biosynthesis gene in *Monascus purpureus*. The PCR could amplify uniform single band at about 750 bp of nearly full-length of *lovF*, which can be used as a rapid simple molecular tool for detection of lovastatin producing organisms. The present experiment also confirmed the presence of *lovF* in *M. purpureus* with nucleotide variability in two positions. However, when searching the cause for low accumulation of lovastatin in hyper pigment producing mutant, the change in bases occurred in two positions of this gene may not be the reason as it looks identical to that of *A. terreus lovF* gene. Similarly, the lower production of lovastatin cannot be attributed to lovF gene only as the nucleotide sequence of the both albino mutant and the wild type is identical **(Figure 48)**. Hence, the present molecular approach confirmed the presence of *lovF* in *M. purpureus* and mutation in *lovF* may not be the only reason for low production of lovastatin in hyper pigment producer and albino mutants.

**Physiological study of hyper pigment producer and albino mutants of *Monascus purpureus* in relation to GABA production**

The wild type Monascus purpureus MTCC 410 had the capacity to grow well and produce maximum GABA when compared to the mutants **(Table 27)**. However, among mutants, albino produced more GABA than the hyper pigment producer. From the result, we hypothesize that the wild type has the ability to utilize the MSG (precursor of GABA) to yield GABA, but the mutants are not efficient to convert the MSG to GABA successfully. The lower yield of GABA by mutants may be attributed to the mutation effect caused by the UV radiation, which has scope for further research on GABA synthesis.

## SUMMARY

Finger millet (*Eleusine coracana*), also known as ragi, is valued as staple food in Asia and Africa. In India, an appreciable area is under cultivation of ragi. It is a rich source of minerals (especially calcium), dietary fibre and well balanced protein. Germination of finger millet is known to degrade phytic acid, thereby releasing the minerals in the available form. Fermentation of finger millet apart from degrading phytic acid releasing minerals from the phytic acid-mineral complex also degrades tannin-carbohydrate and tannin-protein complexes into simple sugars and amino acids. In addition, microbial growth contributes to dietary fiber and lipid content. Thus fermentation of germinated finger millet by *Monascus purpureus* in relation to nutritional quality and value addition formed the subject of the present investigation.

The production of metabolites like pigments, statins, sterols, unsaturated fatty acids and γ- amino butyric acid (GABA) by *Monascus purpureus* MTCC 410 during its growth in various starch rich cereals and millets under solid state fermentation was studied. Germination of finger millet resulted in increased availability of essential nutrients like Ca, P, Mg, Zn, Fe, etc and thereby supports the growth of *Monascus purpureus* and metabolites production. Germinated (72 h) finger millet fermented (10 d) with *Monascus purpureus* showed reduction in phytic acid and tannin contents by 88.8 and 91.1% respectively with an increase of 61.5 % HCl- extractable minerals. Use of germinated finger millet as substrate for solid state fermentation increased statin production (5.24 g $kg^{-1}$), dietary sterols (0.053 g $kg^{-1}$), pigment (151.8 OD units), unsaturated fatty acids (linoleic acid- 34.85%) and GABA (110 g $kg^{-1}$). Of 5.24 g $kg^{-1}$ of total statin, pravastatin and lovastatin constituted 4.87g kg-1 and 0.37g $kg^{-1}$ respectively. Germinated finger

millet was also identified as a cheap substrate for metabolite production using *Monascus purpureus* under solid state fermentation.

Fermentation conditions using Response Surface Methodology (RSM) for enhancing total statin and GABA production was optimized. For this study, *Monascus purpureus* MTCC 410 was grown on germinated finger millet substrate supplemented with additional nutrients. The procedure resulted in a protocol for obtaining 8 g $kg^{-1}$ statin and 395 mg $kg^{-1}$ GABA. This appeared a new report in relation to use of finger millet as substrate in SSF for culturing *Monascus purpureus* for production of statin and GABA.

Poultry experiments with layer hens were carried to study the effect of *Monascus* fermented finger millet on production performance, yolk cholesterol and serum lipid. Feed supplementation with 10% *Monascus purpureus* fermented finger millet improved egg quality parameters and there was a reduction of 16.45% cholesterol in the egg. The lipid analyses showed significant decrease in serum cholesterol (36.30%), triglycerides (21.70%), LDL (33.39%) and increase in HDL (33.43%) in the group fed with 10% *Monascus purpureus* fermented finger millet compared to the control.

An albino and hyper pigment producing mutants of *Monascus purpureus* have been isolated in this laboratory. These mutants were characterized for statin and GABA production as compared to the wild type *Monascus purpureus*. The wild type yielded higher statin content of 5237 mg $kg^{-1}$ compared to the mutants on $7^{th}$ day of fermentation. In the hyper pigment mutant, point mutations were identified at $275^{th}$ and $331^{st}$ bases in the DNA sequence. This apparently

reasoned reduction in statin production by the mutants. Even though, the albino mutant had sequence identical to that of wild type, its ability for statin production was low. The wild type *Monascus purpureus* exhibited higher GABA yield of 621.65 mg $kg^{-1}$ when the solid state medium of finger millet was supplemented with 4% monosodium glutamate.

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## Publications

1. **Venkateswaran, V.** and G.Vijayalakshmi (2010). Value addition to finger millet (*Eleusine coracana*) by germination and fermentation with *Monascus purpureus*. Accepted on 08.03.2010 for publication in the *International Journal of Food Sciences and Nutrition*.

2. **Venkateswaran, V.** and G. Vijayalakshmi (2010). Finger millet (*Eleusine coracana*) - an economically viable source for antihypercholesterolemic metabolites production by *Monascus purpureus* accepted on18.03.2010 for publication in the *Journal of Food Science and Technology*.