CHAPTER 2

LITERATURE REVIEWS

2.1 Botanical data of *Monascus* species

Based on morphology and taxonomy, *Monascus* spp. belong to the Kingdom of Fungi, Division Amastigomycota, Class Ascomycetes, Order Eurotiales, and particularly to the family of Monascaceae (Hawksworth and Pitt, 1983). The genus *Monascus* includes four species which are M. *pilosus, M. purpureus, M. ruber* and *M. floridanus* (Juzlova *et al.*, 1996). The common names for fermentation products from *Monascus* spp. are Red Rice, Red Yeast Rice, Red Mold Rice, Red Fermented Rice, Chinese Red Rice, Red leaven, *Monascus* Fermented Rice, beni-koji (Japanese), hung-chu, zhitai (Chinese), anka, ankak, ang-kak, or Hongquor. *Monascus* is distinguished by its ascospores. These are usually spherical, having 5 microns in diameter, or ovoid (6 × 5 microns) as shown in Fig 2.1 -2.4.

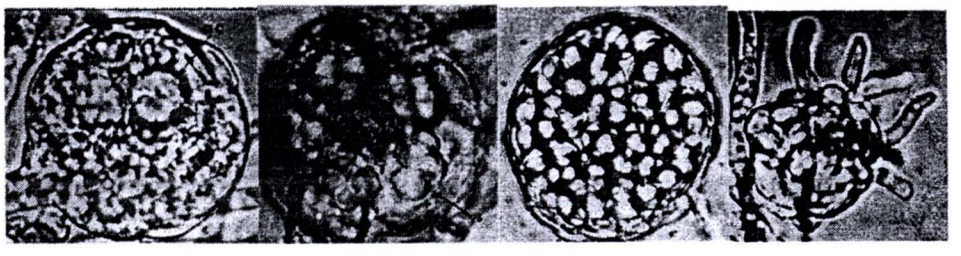


Fig 2.1 Fig 2.2 Fig 2.3 Fig 2.4

Fig 2.1 cleistothecium with developing asci (ac). The spores are not yet visible

Fig 2.2 well-developed asci with clearly visible spores (s)

Fig 2.3 mature cleistothecium. The asci have broken down, releasing the spores that now fill the whole structure.

Fig 2.4 Hyphae continue to grow around the ascogonium, forming a dense layer of hyphae. (source: Carels and Shepherd, 1975)

At the early stages, the young part of the mycelium is white. However, it rapidly changes to a rich pink and later to a distinctly yellow-orange color, reflecting the increasing acidity of the medium and the production of yellow orange hyphae. A deep crimson color is found at the substratum as the culture ages (Juzlova *et al.*, 1996).

2.2 Monacolin K

Monacolin K, with the molecular formula $C_{24}H_{36}O_5$ and a molecular weight of 404.5, contains two polyketide chains, (4- and 18-carbons long). Fig 2.5 describes biosynthesis of monacolin K. Its biosynthetic pathway starts from acetate units coupled to each other in head-to-tail fashion. The methyl group presented in the side chain or at C6 derived from methionine, as frequently occurs in fungal metabolism, and is inserted in the structure before the closure of the rings. This mechanism demonstrates that Mevastatin or Compactin , which lacks of the 6α-methyl group at C6, is not an imtermediate in Monacolin K biosynthesis. The main chain is then cyclized while bound to the polyketide synthase or immediately after dissociation from the enzyme and esterified by a side chain at C8. (Manzoni and Rollini, 2002; Juzlova *et al.*, 1996)

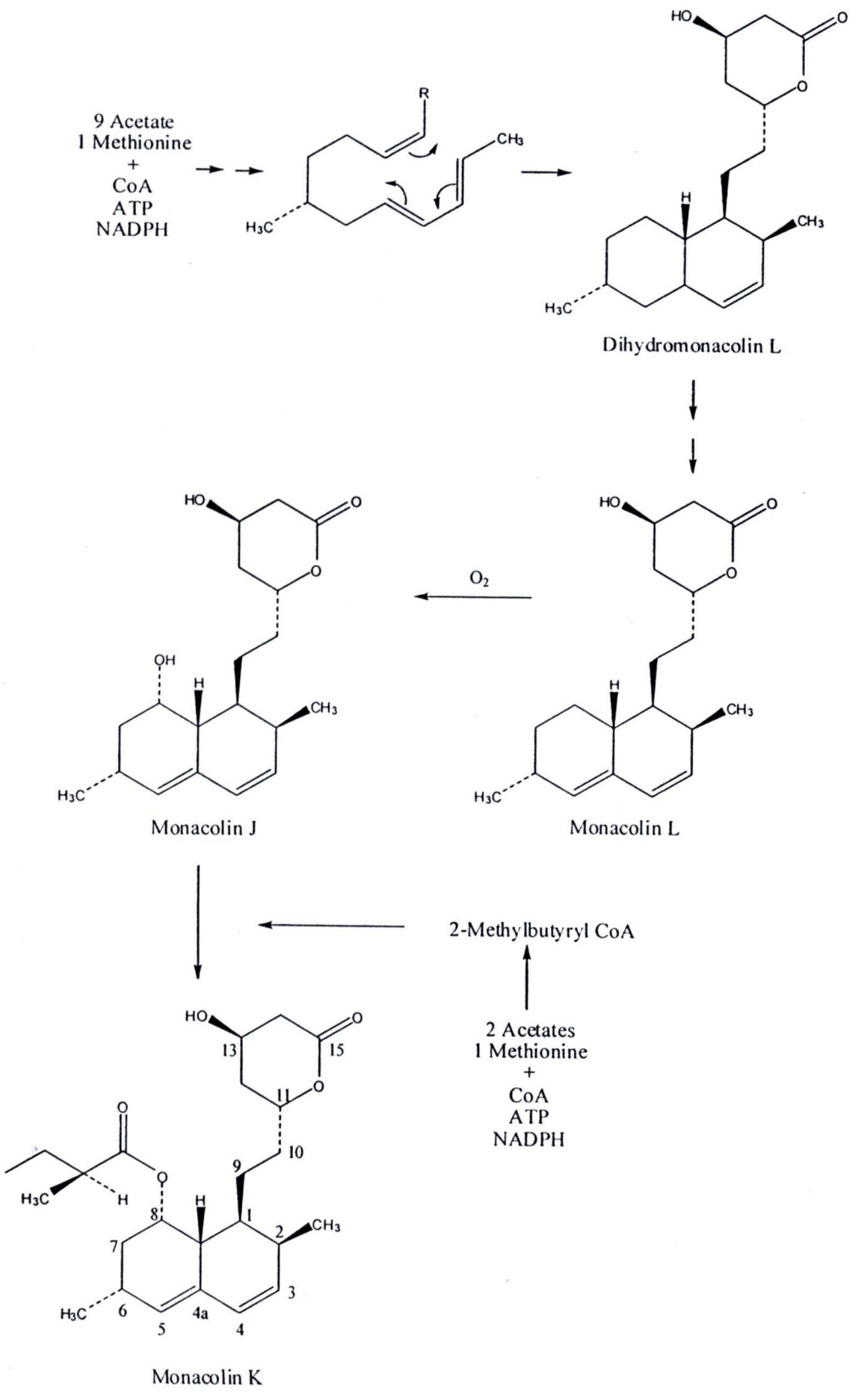


Figure 2.5 Monacolin K biosynthetic pathway (source: Manzoni and Rollini, 2002)

Endo (1979) had studied fungal secondary metabolite, monacolin K, that exhibits hypocholesterolemic effect from the culture of *Monascus* which was isolated from a food sample collected in Thailand, and was classified as *Monascus ruber*. It was grown aerobically at 28°C in a medium containing 6% glucose, 2.5 peptone, 0.5% corn steep liquor and 0.5% ammonium chloride for 10 days. Monacolin K has molecular formula $C_{24}H_{36}O_5$ (Mw 404) and melted at 157-159°C. The UV spectrum (methanol) showed maximum absorption at 229, 237 and 246 nm. IR spectrum (KBr) showed absorption bands at 3550, 2970, 1696 and 1220 cm^{-1}. Monacolin K was soluble in methanol, ethanol, acetone, chloroform and benzene but not soluble in *n*-hexane and petroleum ether. In addition, the study also suggested that the LD_{50} of monacolin K in mice was over 1,000 mg/kg.

Li *et al.* (2004) had identified structures of monacolin compounds in red yeast rice by using liquid chromatography with photodiode array detector and mass spectrometry (LC/PDA/MS). Red Yeast Rice powder was extracted with 75% ethanol and filtered through a 0.45-μm membrane before injection. The separations by LC/PDA/MS were performed on a narrow-bore reversed-phase Zorbax SB-C_{18} HPLC column (2.1mm × 100mm i.d., 5μm, Agilent Scientific,CA, USA) with a gradient elution consisting of acetonitrile (eluent A) and aqueous 0.2% acetic acid (eluent B) at a flow rate of 300 μl per minute. Linear gradient increasing from 35 to 75% A in 20 min and keeping 75% A from 20 to 28 min. The molecular ion of the predominant peak at t_R = 16.65 min was 405 (M+1), identified as monacolin K, which was confirmed by injection of monacolin K standard. The second strong peak (t_R 13.15 min) displayed the molecular ion 423 (*M*+1) belonging to the hydroxy acid form of monacolin K. The SIC of 321(t_R 7.09 min) and 339 (t_R 5.22 min) were deduced to be

monacolin J and its hydroxyl acid form, respectively. The SIC of 305 was observed for two peaks : the peak at t_R 14.55 min may be assumed to be monacolin L, supported by its characteristic mountain-like UV absorption at λmax 230, 237, 247 nm, and the peak at t_R 6.44 min may be its isomer displaying a UV λmax 260 nm. Similarly, SIC at 323 nm was also observed for two peaks at t_R 10.99 and 4.54 min, and the UV spectrum supported the former (t_R 10.99 min) to be the hydroxyl acid form of monacolin L. The SIC at 407 and 425 were contributed by monacolin M and its hydroxy acid form, respectively. Monacolin X and monacolin X hydroxy acid were deduced from the SIC at 419 and 437, respectively. Dehydromonacolin K appeared in SIC at 387, and compactin existed by evidence of the peak of SIC at 391. Other two components, dihydromonacolin L and 3α-hydroxy-3,5-dihydromonacolin L, which showed no peaks in LC chromatogram at 237 nm, still were found in SIC at 307 and 341, respectively. Conclusively, the monacolins were found both in lactone and hydroxy acid forms.

Optimizing environmental conditions for *Monascus* on Red Yeast Rice is crucial for production of monacolins from *Monascus* species. Wang *et al.* (2003) had investigated monacolin K, γ-aminobutyric acid (GABA) and citrinin production ratio from *Monascus purpureus* NTU 601 on solid state medium. After the rice was sterilized and cooled, extra chemical compounds were added individually. The effect of the carbon source (glucose, acetate and ethanol), nitrogen source [ammonium chloride, monosodium glutamate (MSG), methionine and urea], and/or fatty acid and oil (octanoic acid, dodecanoic acid, soybean oil and corn oil), temperature, water supplemented, and cultivation time on monacolin K, γ-aminobutyric acid (GABA) and citrinin production of *Monascus purpureus* NTU 601 on solid-state culture was

analyzed. The opimal condition was that 500 g substrate inoculated with a 5% (w/v) spore suspension of *Monascus* and the inoculated substrate was cultivated at 30°C, with 0.3% ethanol as carbon source and water addition at 145 ml. Production of monacolin K, citrinin and GABA started to increase slowly between day 2 and 4. Monacolin K and citrinin are secondary metabolites derived from the polyketide pathway, and are therefore produced together in Red Yeast Rice, with their growth rate doubling on days 5–8 day. Monacolin K reached maximum production on day 8. Addition of 0.5% ethanol decreased production of citrinin from 813 ppb to 561 ppb, but increased production of monacolin K from 136 mg/kg to 383 mg/kg, as well as GABA from 1,060 mg/kg to 7,453 mg/kg. Addition of 1% glucose increased production of monacolin K from 136 mg/kg to 178 mg/kg. The only nitrogen source that had a positive effect on monacolin K production was 0.5% NH_4Cl, whereas other nitrogen sources decreased monacolin K production. Addition of octanoic acid, dodecanoic acid, soybean oil and corn oil decreased production of monacolin K.

Chen and Hu (2005) had investigated for a mutant strain with low-producing citrinin and high-producing monacolin K capabilities and its solid state fermentation conditions. The study had revealed that the mutant strain, *Monascus pilosus* M12-69, acquired by treatment with mutagenic agents from a wild strain M12 of *Monascus* screened from Red Yeast Rice samples gathered around China possessed the ability to produce a higher ratio of monacolin K to citrinin than other strains. The optimization condition of solid state fermentation of strain M12-69 was 100 g pre-immersed rice, 1 g wheat bran, 50 ml H_2O, 20 ml liquid nutritional broth (40 g glucose, 4 g sodium glutamate, 3 g protease peptone, 3 g NH_4NO_3, 5 g NaH_2PO_4, and 1000 ml distilled water), and incubation for 16 days at 25 °C. Under these fermentation conditions, the

concentrations of monaclin K and citrinin of Red Yeast Rice by Strain M12-69 were 2.52 mg/g and 0.13 ng/g, respectively. Therefore strain M12-69 is a potential strain, which can be used to obtain Red Yeast Rice with high concentration of monacolin K and low concentration of citrinin.

Su *et al.* (2003) had studied the production of secondary metabolites γ-aminobutyric acid (GABA) and monacolin K from *Monascus* species in both submerged culture and solid culture. By screening sixteen different strains of *Monascus* with various results in monacolin K production, they found that cultivation of *Monascus purpureus* CCRC 31615 by solid state fermentation at 30 °C, using rice as solid medium, had yielded the highest amount of monacolin K which was 378 mg/kg. Furthermore, inoculum size had a significant effect on the productivity of monacolin K, and solid state cultivation always produced more monacolin K and GABA than submerged cultivation in any condition.

For any product used as a drug or food supplement, stability is imperative in both extraction and storage order to comply with official regulations. For the fact that monacolins are able to be in both lactone and hydroxyl acid forms, Li *et al.* (2005) had designed the stability stress testing for Red yeast rice powder. Well-resolved peaks of seven main compounds of monacolin family (monacolin K, L, J and their hydroxyl acid forms as well as dehydroxymonacolin K) were profiled on a C_{18} reverse-phase column using a linear gradient of 0.1% trifluoroacetic acid and acetonitrile as the mobile phase, and the detection wavelength was set at 237 nm. The results exhibited that monacolins decreased significantly under the conditions of high humidity at high temperature (75% RH, 60 ◦C) and sunlight. Monacolin K and its hydroxyl acid form would be dehydrolyzed and turned to dehydromonacolin K at high temperature (80

◦C) while the monacolin K, J and L would be transformed into their corresponding hydroxyl acid forms under the condition of high humidity (92.5% RH, 25 ◦C). The indication is that monacolins in red yeast rice powder are light-sensitive and thermal-sensitive. Therefore, it has been suggested that the preparations containing monacolins should be stored in the place of cool and lightproof.

Heber *et al.* (2001) had analyzed nine Chinese Red yeast rice preparations to test for chemical constituents. Monacolins were measured by high performance liquid chromatography (HPLC) that separates the various monacolins in Chinese red yeast rice. Citrinin concentration, a toxic fermentation byproduct, was measured by radioimmunoassay. The results of the analyses exhibited that the contents of total monacolin K ranged from 0.15 to 3.37 mg per capsule, and only one preparation had the complete profile of monacolins. Seven of nine supplements had detectable levels of citrinin.

Miyake *et al.* (2005) have found that both red light and blue light affect development in *Monascus*, influencing the processes of mycelium and spore formation, and the production of secondary metabolites such as γ-aminobutyric acid, red pigments, monacolin K and citrinin. *M. pilosus* IFO4520 was incubated in GGP medium (7% glycerol, 3% glucose, 3.8% peptone, 0.1% $MgSO_4 \cdot 7H_2O$, 0.2% $NaNO_3$) at 25°C for 14 days at 120 rpm and either kept in the dark or exposed to red light or blue light at 0.16 mW/cm^2. Monacolin K produced by *M. pilosus* IFO4520 after 7, 10 and 14 days of culture in GGP medium in the dark and under light exposure was determined by HPLC (Agilent 1100 system; Agilent Technologies, Palo Alto, Calif.) connected to TOFMS (JMS-T100LC "AccuTOF"; JEOL, Tokyo, Japan) under established conditions [HPLC column, COSMOSIL 5C18-MS (4.6x150 mm; Nakarai

Tesque, Kyoto, Japan); linear gradient, acetonitrile-water containing 0.1% formic acid (60:40, v/v) to acetonitrile-water containing 0.1% formic acid (100:0, v/v) in 20 min; flow rate, 0.5 mL/min; oven temperature, 40°C; injection volume, 10 μL]. Standards of monacolin K (acid form, MW=422; lactone form, MW=404) in methanol were eluted separately at 10.5–11 and 13.5–14 min. Monacolin K production was increased by red light compared to dark, and monacolin K production under all conditions reached approximately the same level after 14 days of culture.

Chang *et al.* (2002) had employed response surface methodology (RSM) to study the effect of culture medium on the production of lovastatin (monacolin K) in mixed solid-liquid state (or submerged) cultures by *Monascus ruber* CCRC 31535. Four medium ingredients (rice powder, peptone, glycerin, and glucose) were chosen for optimization through RSM. The HPLC system for analysis of lovastatin concentration was composed of a Hitachi L-6200 solvent delivery controller, a Hitachi 4250 UV-VIS detector, a Hitachi-D-2500 Chromato-Integrator, and a hyperbond C18 column (ThermoQuest Hypersil, UK, 300 x3.9 mm, 10 μ). The injection volume was 20 μL. The sample was eluted with a mobile phase comprising 65% acetonitrile at a flow rate of 1.0 ml/min. The chromatogram was monitored at 240 nm. The maximal lovastatin yield (131 mg/L) appeared at the region where the respective concentrations of rice powder, peptone, glycerin, and glucose were around 34.4 g/L, 10.8 g/L, 26.4 ml/L, and 129.2 g/L, respectively. The optimized medium resulted in a significant increase of lovastatin yield, as compared with that obtained by the fermentation of many other *M. ruber* species.

Chung *et al.* (2007) had applied the Taguchi method to determine the optimum conditions for producing a high yield of monacolin K from *Monascus* spp. in

submerged culture, using a rotary aerobic liquid culture in an incubator. The control factors of liquid medium included carbon, nitrogen, oil, and salt sources and pH values. The results revealed that in the growth phase the optimum culture conditions are 1% whole wheat flour, 1% peptone, 0.01% olive oil and 0.01% potassium phosphate with a pH of 5.0. In the metabolic phase the optimal culture conditions are 1%whole wheat flour, 1% peptone, 0.01% soybean oil, 0.01% potassium phosphate with a pH of 3.0. The HPLC system used for analysis of monacolin K concentration was a hyperbond C18 column (LiChroCART 250-4, 250x4.6 mm, 5 μm, Merck Biosciences Ltd., Darmstadt, Germany). The injection sample volume was 20 μL. A mobile phase was comprised of 65% acetonitrile and 35% methanol. A flow rate was 1.0 mL/ min, and the chromatogram was monitored at 238 nm. By using these optimal culture conditions, the yield of monacolin K in the fermentation process was 151.06 ppm.

The ultrasonic effect on biological characteristics of *Monascus* sp. was reported by Yang *et al.* (2005). The selected *Monascus* sp. was used as the starting strain by the mutagenic treatment of ultrasonic wave. During the fermentation, the culture medium was subject to intermitted ultrasonic wave treatment. The culture medium was treated once every 6 hours. The time periods of treatment were 20 min, 10 min, 5 min, 2 min, 1 min, 30 s, 10 s, and 5 s based on requirement. The culture then was incubated at 28°C for 7 days, and analyzed for monacolin K content. A HPLC with an Econosphere C18 column (250×4.6 mm; inner diameter, 5μm) was used for detection. The mobile phase was comprised of acetonitrile/H_4PO_3 (65:35), flow rate was controlled at 1.0 mL/min. Monacolin K was detected by UV absorbance at 238 nm. The results indicated that content of the *Monascus* pigment and monacolin K

produced by the mutant increased 29.74% and 39.96%, respectively, compared with those from the starting strain.

Sayyad *et al.* (2007) had optimized the nutrients for lovastatin (monacolin K) production by *M. purpureus* MTCC 369 using shake flask method and response surface methodology. Five nutritional parameters screened by Plackett–Burman experimental design were optimized by Box–Behnken factorial design of response surface methodology for lovastatin production in submerged fermentation. Lovastatin was estimated by HPLC (SHIMADZU, Japan) with Lichrosper® 100 C18 column, 5 μm particle size (250×4.6 mm ID). Acetonitrile to water acidified (0.1%) with ortho-phosphoric acid (65:35 v/v), was used as mobile phase. Flow rate of mobile phase was maintained at 1.5 ml/min and UV detector was set at 235 nm. Maximum lovastatin production of 351 mg/L were predicted in medium containing 29.59g/Ldextrose, 3.86g/LNH4Cl, 1.73 g/L KH_2PO_4, 0.86 g/L $MgSO_4 \cdot 7H_2O$, and 0.19 g/L $MnSO_4 \cdot H_2O$ using response surface plots and point prediction tool of DESIGN EXPERT 7.0 (Statease, USA) software.

Miyake *et al.*(2006) examined the effects of several carbon and nitrogen sources on lovastatin (monacolin K) production to develop a define medium for the higher production . After cultures of *M.pilosus* IFO4520 were grown at 25°C for 21 days in GGP medium, lovastatin production was determined by HPLC (Agilent 1100 system; Agilent Technologies, Palo Alto, Calif.) connected to TOFMS (JMS-T100LC ''AccuTOF''; JEOL, Tokyo, Japan) under established conditions [HPLC column, COSMOSIL 5C18-MS (4.6x150 mm; Nakarai Tesque, Kyoto, Japan); linear gradient, acetonitrile-water containing 0.1% formic acid (60:40, v/v) to acetonitrile-water containing 0.1% formic acid (100:0, v/v) in 20 min; flow rate, 0.5 mL/min; oven

temperature, 40°C; injection volume, 10 μL]. Standards of lovastatin (acid form, MW=422; lactone form, MW=404) in methanol were eluted separately at 10.5–11 and 13.5–14 min. The results revealed that the type and dose of carbon and nitrogen sources strongly influenced lovastatin production by *M.pilosus* and *Monascus pilosus* requires a medium with a suitable C/N ratio for high lovastatin production and growth. It appeared that 3.8% peptone in GGP medium resulted in high lovastatin production which was approximately 130mg/L. More interestingly, a combination between carbon sources, 1% maltose and 7% glycerol, resulted in enhancing lovastatin production by 3 folds, up to 444mg/L.

Wang et al. (2004) had sought for mutant strains of *Monascus purpureus* with low citrinin and high monacolin K production. *M. purpureus* NTU 601 was used as the parental strain and used *N*-methyl-*N*'-nitro-*N*-nitrosoguanidine (NTG), ethylmethanesulfonate (EMS), and UV radiation to induce 11 mutant strains with low citrinin production (citrinin production was in the range of 0.16-0.73 ppm). From 11 strains, *M. purpureus* N 311 produced the least amount of citrinin in PDA, but with low monacolin K as well. The results also showed that concentration of monacolin K produced by the 11 strains had no direct relationship with the concentration of citrinin. Examining the concentrations of monacolin K and citrinin of the 11 strains in the PDA medium, *M. purpureus* N 301 and *M. purpureus* N 310 were found to produce a significant amount of monacolin K, and the concentrations of citrinin were reduced by 47-26% as compared to that of the parental 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 which 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.

2.3 Clinical Studies

Li *et al.* (1998) studied the effects of *Monascus purpureus* (red yeast rice) on blood lipid and lipoprotein concentrations in three animal models. The results showed that rabbits fed a diet of 25% casein, a model of endogenous hyperlipidemia, which subsequently treated with *Monascus purpureus* (red yeast rice) for 30 days while maintaining the casein diet, total serum cholesterol (TC) and LDL cholesterol (LDL-c) were reduced in a dose-dependent fashion. At the highest dose of 800 mg/kg/day, *M. purpureus* reduced TC and LDL-c levels by 59 and 44% respectively. In a second rabbit model, exogenous hyperlipidemia was induced with a diet containing lard, cholesterol and yolk powder. Treatment with red yeast rice 0.8 g/kg/day for 40 days prevented increases of serum total cholesterol (TC), triglyceride (TG) concentration and TC:HDL-c ratio. In quail where hyperlipidemia was induced exogenously by an atherogenic diet included 1% cholesterol, 14% lard, 6% Soya-bean oil, oral red yeast rice (0.1, 0.2, and 0.4 g/kg/day for 2 weeks) largely prevented increases of serum TC and TG concentrations.

Microorganism *M. purpureus* NTU568 which was mutated from wild strain *M. purpureus* HM105 was selected by Lee *et al.* (2005) to prepare red mold rice in order to investigate on the effects of oral administration of a small amount of *Monascus* powder fermented by *M. purpureus* NTU568 in hyperlipidemia hamsters. The results showed that the oral administration of *Monascus* powder in hyperlipidemia hamsters

was proven to decrease TC, TG, and LDL-C levels. Plasma TC levels in hamster fed with *Monascus* powder at one fold dosage [10.78 mg (day 100 g bw)$^{-1}$] for 4 and 8 weeks were significantly lower (31.2 and 22.0%, respectively) than that in hyperlipidemia hamsters. Plasma TG (30.1 and 17.9%) and LDL-C levels (36.0 and 20.7%) were also significantly lowered by feeding with *Monascus* powder at one fold dosage for 4 and 8 weeks compared to hyperlipidemia hamsters. In addition, the plasma glutamyl oxaloacetic transaminase (GOT) and glutamyl pyruvic transaminase (GPT) levels were not significantly increased by feeding with *Monascus* powder.

Wang *et al.* (1997) had established a multicenter, single-masked clinical trial to examine the ability of a natural product *Monascus purpureus* (red yeast) rice preparation in regulating serum lipid. A group of 324 hyperlipidemia patients received an alcoholic extraction of *M. purpureus* (red yeast) rice 0.6 g twice a day, and a positive control group of 122 hyperlipidemia patients received Chinese herbal medicine, Jiaogulan *(Gynostemma pentaphllla)* three tablets a day (1.2 g/day). After 8 weeks, total serum cholesterol decreased significantly by 22.7% and low-density lipoprotein cholesterol decreased by 30.9% in the group treated with *M. purpureus* rice preparation, and the positive control group showed 7.0% and 8.33% reduction in total cholesterol and low-density lipoprotein cholesterol respectively. In conclusion, when the overall therapeutic effects of *M. purpureus* rice were scored according to criteria established by the Ministry of Public Health of China, 93.2% of patients in the treatment group benefited from *M. purpureus.*

Cicero *et al.* (2005) had evaluated the efficacy and safety as an antihypercholesterolemic agent of a brand dietary supplement. The tested product is made of a patented dietary supplement association (*DIF1STAT*®) of *M. purpureus*

(1.5% monacolin K), linear aliphatic alcohols (60% octacosanol) and niacine. Each capsule (daily dose) contains *M. purpureus* 340 mg (the maximal amount that can be used in a dietary supplement following Italian law), octacosanol 10 mg , and Niacin 27 mg (Difass S.r.l., San Marino Republic). By carring the study on 111 Caucasian patients with low cardiovascular disease risk, the tested dietary supplement determined a significant decrease of Total Cholesterol (TC), Low Density Lipoprotein Cholesterol (LDL-C), and Triglycerides (TG) in moderately hypercholesterolemic subjects without clinically relevant change in liver and muscular toxicity markers. The reduction of LDL-C reached the 20%, which is similar to that obtained with a well-known effective statin, Pravastatin.

In the study of 72 patients with idiopathic persistent Nephrotic dyslipidemia with secondary dyslipidemia, three groups were formed. The first group received *M. purpureus* Went rice (600 mg twice a day for one month then once daily). The second group received oral fluvastatin in a daily dose of 20 mg. The third group received no additional therapy and ranked as a control. The results showed that both fluvastatin and *M. purpureus* Went rice were well-tolerated with no evidence of significant side effects, including no neuromuscular dysfunction. Also, a significant reduction of serum cholesterol was clearly noticed by 28.8 and 30.2% at six months and at one year of *M. purpureus* Went rice or fluvastatin therapy, respectively. In contrast, there were no significant changes in serum cholesterol in the control group. To sum up, *Monascus purpureus* Went rice is a safe, effective and economic treatment strategy for nephritic dyslipidemia (Gheith *et al.*, 2008).

In the United States, an important study has been carried out on a preparation standardized to 0.4 % in monacolin K sold as a dietary supplement for the prevention

of hyperlipoproteinemia, Cholestin®. This study was carried out on 83 patients with hyperlipidemia (46 males and 37 females) with TC: 5.28 -8.74 mmol/L; LDL-C: 3.31 - 7.16 mmol/L; TG: 0.62 - 2.78 mmol/L and HDL-C: 0.78 -2.46 mmol/L. Patients were given 2.4 g/day of extract of *Monascus purpureus* (4 capsules of 600 mg) while the control group was given a placebo substance. The level of the lipids in the plasma was measured at weeks 8, 9, 11 and 12. The results of this study showed a significant decrease in the treated group from week 8 regarding TC, LDL-C and TG (Heber *et al.*, 1999).

2.4 Effects of Monacolin K on Cholesterol Metabolism

Cholesterol is a 27-carbon steroid that is an essential component of the cell membrane, the immediate precursor of steroid hormones, the substrate for the formation of bile acids, and is required for the assembly of very low density lipoprotein in the liver. Since as much as two-third of total body cholesterol is of endogenous origin, and only approximately one-third is from diet, a potentially effective way to lower plasma cholesterol level is to control its biosynthesis which involves more than 25 separate enzymatic reactions. The critical reaction in the pathway of cholesterol biosynthesis is the formation of mevalonic acid from 3-hydroxy-3-methyglutaryl coenzyme A (HMG CoA) by HMG CoA reductase (Alberts, 1988). Monacolin K (also known as Lovastatin, Mevinolin or Mevacor®) is structurally similar to HMG CoA and plays a role as a competitive inhibitor which competes with HMG CoA and reduces the biosynthesis of cholesterol as shown in Fig 2.6 (Endo, 1980 and 1985; Manzoni and Rollini, 2002). Inhibition of this enzyme results in an inability of hepatocytes to increase cholesterol biosynthesis and decrease

hepatocyte cholesterol concentration. Decreased cellular cholesterol activates a cellular signaling cascade culminating in the activation of sterol regulatory element binding proteins (SREBPs), which are transcription factors that activate transcription of the gene encoding LDL-R. This leads to increased hepatocyte LDL-R expression, which mediates increased plasma LDL clearance and results in reduced circulating LDL levels (Golen *et al.*, 2005).

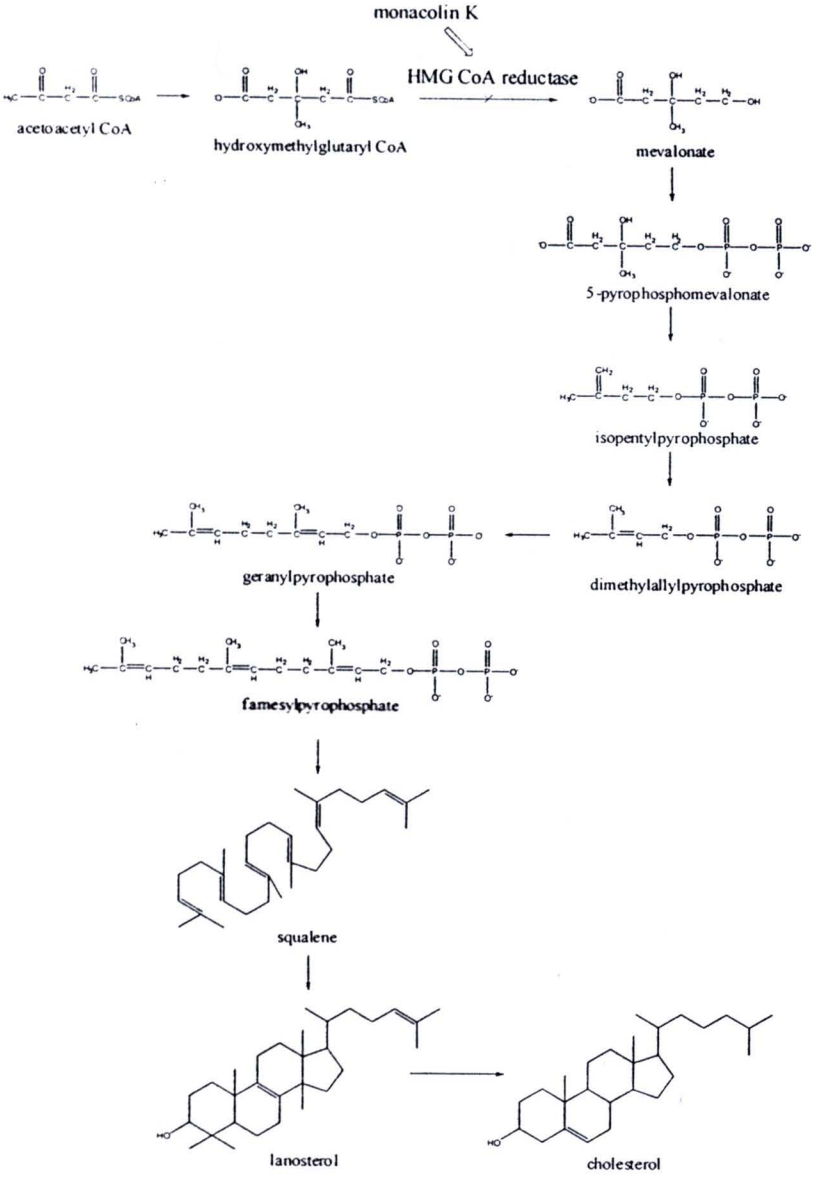


Fig 2.6 Inhibition of cholesterol biosynthesis by monacolin K (source: Manzoni and Rollini, 2002

2.5 Citrinin

Red Yeast rice has been used in food coloring and meat preservation in Asia for centuries and has also been used as dietary supplements all over the world because of their cholesterol-lowering ability. However, the discovery of citrinin in RFR has led to a controversy about the safety of RFR. In 1999 a study in the Netherlands detected the mycotoxin citrinin in all the commercial *Monascus* samples at concentrations varying between 0.2 and 17.1 mg/kg (Sabater-Vilar *et al.* 1999). In the USA, nine different commercially available Red Mold Rice dietary supplements were tested and citrinin was found in seven of them (Heber *et al.* 2001). In Taiwan, all *Monascus* products were found to contain citrinin at concentrations of 0.28–6.29mg/kg (Liu *et al.* 2005). A mutant strain, *Monascus* sp. M12-69, was acquired by treatment with mutagenic agents of a wild strain M12 of *Monascus* screened from Red Mold Rice samples gathered in China (Chen and Hu 2005). This strain can produce RMR with a high concentration of monacolin K and low concentrations of CIT under optimum conditions.

Citrinin [$C_{13}H_{14}O_5$, IUPAC: (3R,4S)-4,6-dihydro-8-hydroxy-3, 4, 5-trimethyl-6-oxo-3H-2-benzopyran-7-carboxylic acid; CAS No.: 518-75-2], is an acidic lemon-yellow crystal with maximal UV absorption at 250 nm and 333 nm (in methanol), melting at 172 ^{O}C. It is sparingly soluble in water but soluble in dilute sodium hydroxide, sodium carbonate, or sodium acetate; in methanol, acetonitrile, ethanol, and most of other polar organic solvents. Citrinin crystallizes in a disordered structure, with the p-quinone and o-quinone two tautomeric forms (as shown in Fig 2.7) in a dynamic equilibrium in the solid state. (Xu *et al.*, 2006)

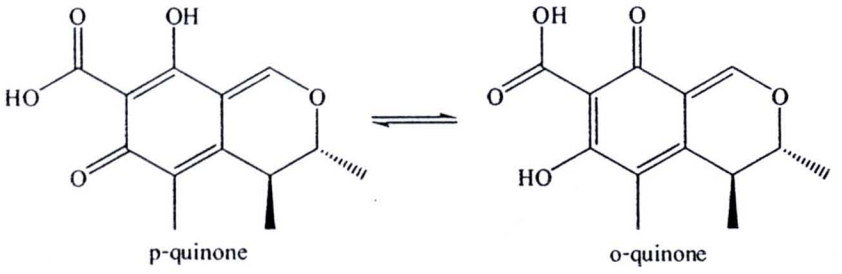


Fig 2.7 Structural formula of citrinin isomers (source: Xu *et al.*, 2006)

Wang *et al.* (2005) investigated citrinin variability in type cultures of *Monascus* species by HPLC to test citrinin producing ability. Twenty-three *Monascus* type cultures representing eight species (*M.purpureus, M. ruber, M. lunisporas, M. pallens, M. pilosus, M. sanguineus, M. aurantiacus, M. floridanus*) were studied. All these strains were found to produce citrinin in yeast extract sucrose (YES) medium in quantities varying from 65 to 480 mg/l. The highest yield (480 mg/l) was produced from *Monascus pallens* IMI 356820, while the lowest (65 mg/l) was from *Monascus ruber* IFO 8201. The results of this study also revealed that the production of citrinin is independent of pigment production by *Monascus* species. It is suggested that safety measures should be taken when the pigments or other products from *Monascus* are utilized.

HPLC was used to analyze citrinin levels in lipid and aqueous extracts of commercialized *Monascus* products by Liu *et al.* (2005). A Lichrospher C_{18} reverse-phase column (5 μm particle size, 4.0 mm x 250 mm; Merck) was equilibrated with a mobile phase of methanol/0.25 M orthophosphoric acid, pH 2.5 (70:30, v/v) at a flow rate of 0.6 mL/min. Chromatograms were monitored using the fluorescence detector set at an excitation wavelength of 330 nm and an emission wavelength of 500 nm. Citrinin was detected in lipid extracts of all examined samples at concentrations varying between 0.28 and 6.29 μg/g, but was not found in aqueous extracts. When

human embryonic kidney cells (HEK293) were incubated for 72 h with *Monascus* extracts, the concentrations causing 50% cell death by all lipid extracts were in the range of 1.8-4.7 mg/mL, while aqueous extracts showed a lower cytotoxicity. Incubation of HEK293 cells with 60 μM pure CTN for 72 h caused cell viability to fall to 50% of control levels.

Hajjaj *et al.* (1999) have investigated the biosynthetic pathway of citrinin in *M. ruber* by ^{13}C nuclear magnetic resonance (NMR) analysis of carbon isotope distribution of ^{13}C-enriched citrinin after feeding the culture with ^{13}C-labeled acetate, and they found that this pathway is different from the one previously identified in *Aspergillus* or *Penicillium* (Fig 2.8) .

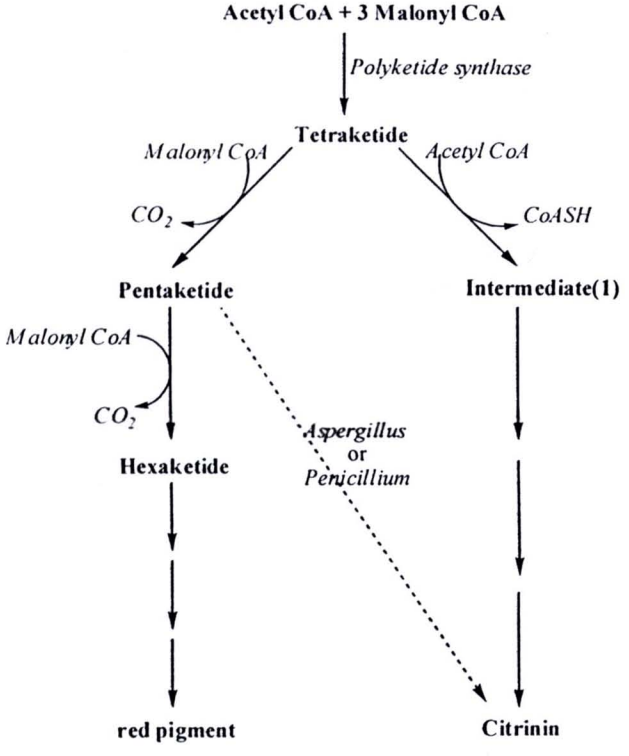


Fig 2.8 Biosynthesis of citrinin and red pigment in *M.ruber*. The toxin pathway in *Aspergillus* and *Penicillium* is indicated by the dash arrow. (source: Hajjaj *et al.*,1999)

The precursor for citrinin is a tetraketide arising from the condensation of one acetyl CoA molecule with three malonyl CoA molecules instead of a pentaketide (one acetyl CoA molecule with four malonyl CoA molecules). Then, an additional acetyl CoA molecule is added to the tetraketide to form intermediate 1. Subsequent reactions include O alkylation and the cleavage of the single bond between C-1 and C-9. This cleavage also agrees with the proximity of C-3 and C-9 as described in Fig 2.9. (Hajjaj *et al.*,1999)

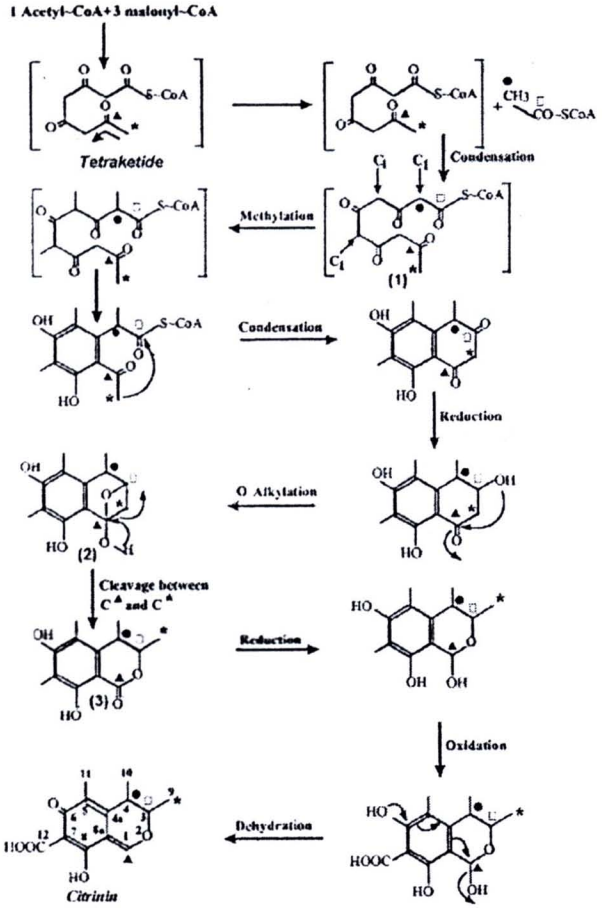


Fig 2.9 Scheme of the biosynthesis of citrinin by *M. ruber*. The start of the condensing reaction is indicated by the bent arrow in the upper left panel. Intermediates are numbered. Enrichment of C-1 (▲), C-3 (□), C-9 (*), and C-4 (●) is indicated. (source: Hajjaj *et al.*,1999)

2.6 Toxicology

Since there have been some controversy about using red yeast rice, Journoud and Jones (2004) had summarized the evidence of adverse effects and food/drug interactions encountered with the use of red yeast rice during human trials and provided a comparison with available marketed HMG-CoA reductase inhibitors as shown in table 2.1.

Table 2.1 comparison of adverse effects and food/drugs interactions

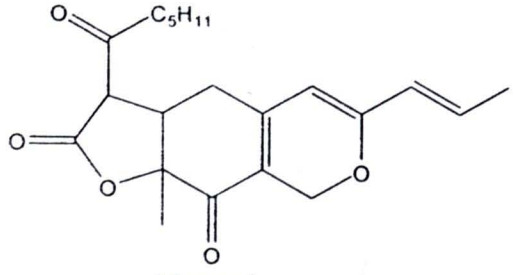
Drug	Adverse effects	Food interaction	Drug interaction
Red yeast rice	Allergy, heart burn, abdominal discomfort, flatulence, and dizziness	None reported. In theory, same as lovastatin	None reported. In theory, same as lovastatin
Atorvastatin (Lipitor™)	Nausea, dyspepsia, abdominal and muscle pain, constipation, flatulence, rash, oedema, dizziness, chest pain, insomnia	Grapefruit juice, Alcohol	With high dose of niacin, myopathy
Fluvastatin (Lescol™)	Dyspepsia, nausea, abdominal cramps, headache, insomnia,	None reported	With high dose of niacin, myopath
Lovastatin (Mevacor™)	Nausea, dyspepsia, abdominal pain, constipation, flatulence, headache, rash, blurred vision, dizziness, muscle pain, insomnia, rare rhabdomyolysis	Grapefruit juice, alcohol, fibre, pectin, and oat bran	With high dose of niacin, myopathy
Pravastatin (Pravachol™)	Nausea, vomiting, diarrhoea. headache, muscle pain, rash	Alcohol	With high dose of niacin, myopathy
Simvastatin (Zocor™)	Dyspepsia, constipation muscle pain, insomnia, rare rhabdomyolysis	Grapefruit juice alcohol	With high dose of niacin, myopathy

Blanc *et al.* (1995) isolated and identified Monascidin A from various species of *Monascus*, and used mass spectroscopy to indicate that its structure was identical to citrinin. Citrinin, one of mycotoxins, possesses antibiotic, bacteriostatic, antifungal and antiprotozoal properties, while it is also known as a hepato-nephrotoxin which is produced from a wide range of *Monascus* spp. (Xu *et al.*, 2006) Therefore, it can limit the acceptability of the red yeast rice product. It had also been reported to have nephrotoxic and hepatotoxic properties in animals with LD_{50} values of 35 mg/kg in mouse and 67 mg/kg in rat. Citrinin can be found in both solid and submerged cultures in the 100–400 mg/L range. (Monica *et al.*, 1999; reviewed in Wang *et al.*, 2003)

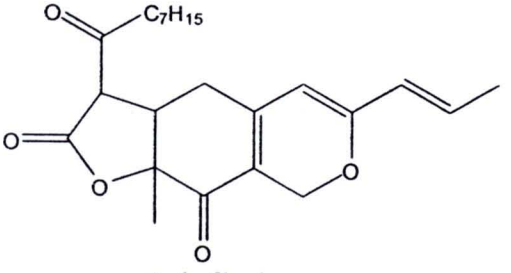
2.7 Pigments

Monascus species produce a mixture of six major pigments from polyketide pathway. These pigments are: 1) yellow pigments : monascin and ankaflavin; 2) Orange pigments : rubropunctatin and monascorubrin; 3) Red pigments : rubropunctatamine and monascorubramine (Fig 2.10). (Teng *et al.*, 1998) The orange pigments, monascorubrin and rubropunctatin, are synthesized in the cytosol from acetyl coenzyme A (Fig 2.11) by the multienzyme complex of polyketide synthase I. 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 (Fig 2.12). The mechanism of formation of the yellow pigments, ankaflavin and monascin, has not yet been elucidated. It is supposed that these compounds originated from chemical oxidation of monascorubrin and rubropunctatin. However, their

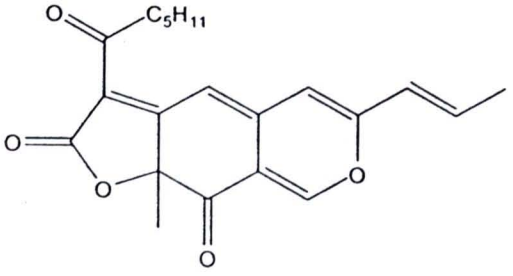
structures strongly suggest that the yellow pigments are reduced derivatives of the orange ones. (Juzlova *et al.*, 1996)



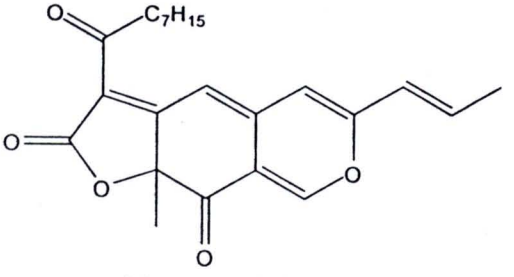
Monascin



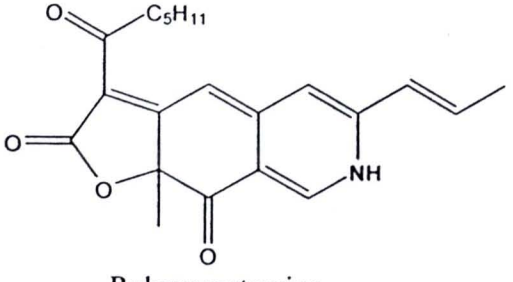
Ankaflavin



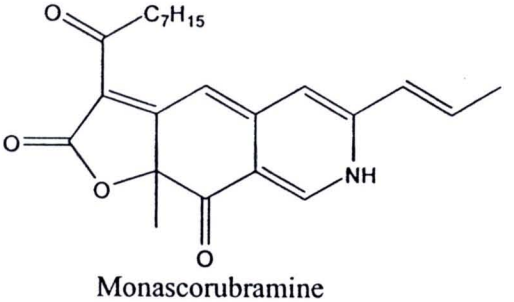
Rubropunctatin



Monascorubrin



Rubropunctamine



Monascorubramine

Fig 2.10 Pigments produced by the genus *Monascus* (source : Teng and Feldheim, 1998)

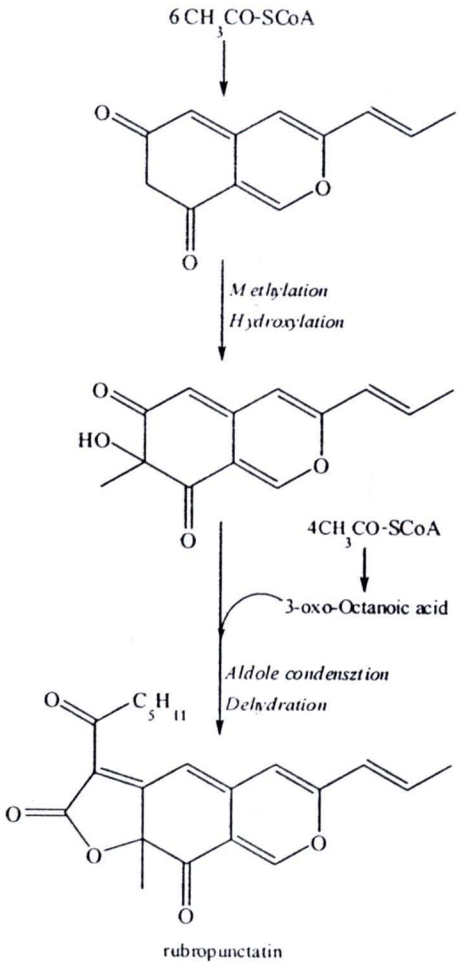


Fig 2.11 Probable mechanisms of the biosynthesis of rubropunctatin (source: Juzlova *et al.*, 1996)

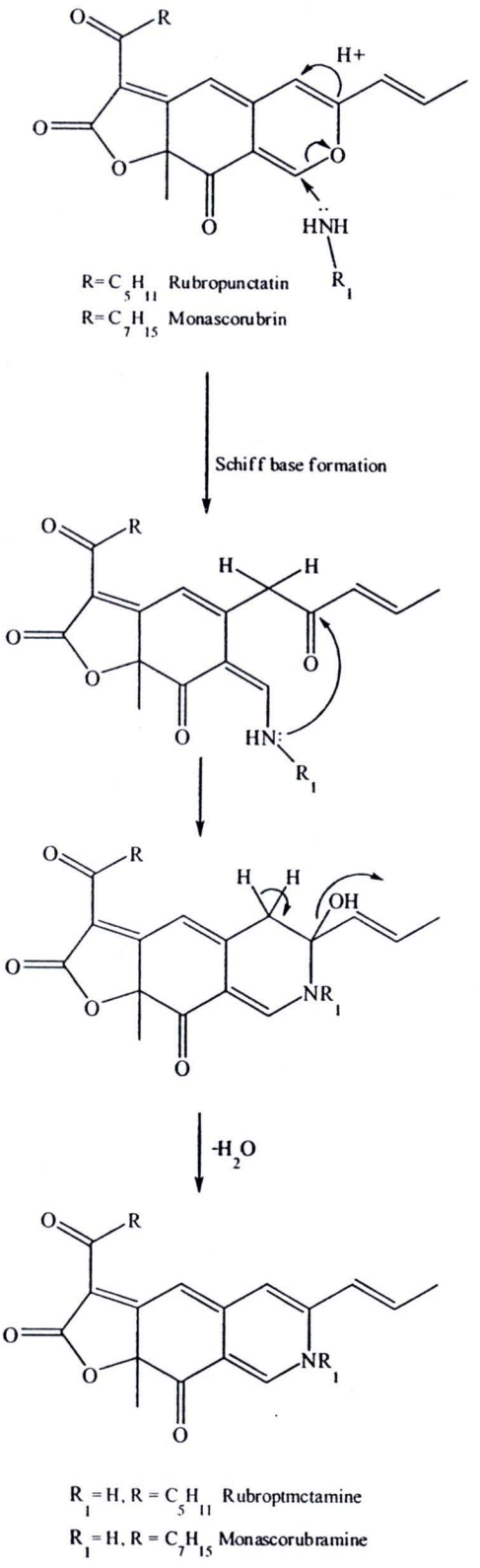


Fig 2.12 Formation of red pigments (source: Juzlova *et al.*, 1996)

Carvalho *et al.*, 2006 established relation between growth, respirometric analysis and biopigments production from *Monascus* sp by solid-state fermentation in columns and in a drum-type bioreactor with forced air. In these reactors, the best aeration rate for biopigment production was 1 ml of air, per gram of wet substrate, per minute. Under ideal conditions in column fermentation, a maximum specific growth velocity of 0.039/h and a specific pigment production velocity of 27.5AU/g biomass h were obtained, at 140 h, with 500AU/g dry weight after 12 days. The specific product formation velocity in the bioreactor was 4.7AU/g h, at 240 h, and the total pigment production was 108.7AU/g dry weight after 15 days. The results indicated that aeration played a critical influence in pigment formation and on the metabolism. On the other hand, even though *Monascus* pigments are secondary metabolites (not associated to growth) the amount of pigments produced was directly related to the biomass produced and cultivation conditions. Therefore, it is possibly that the aeration in different fermentation phases could work as a tool to increase productivity of *Monascus* pigments.

Ahn *et al.*, 2006 suggested that high broth viscosity is a key factor to be considered in a submerged fermentation of filamentous fungi. To improve the productivity of *Monascus* pigment in a submerged fermentation of *Monascus* sp. J101, it was also necessary to reduce broth viscosity. In a batch culture of *Monascus* sp. J101 at 30 °C, most cell growth was accomplished within 48 h, which induced viscosity and heterogeneity inside the fermentor. Consequentlty, it resulted in poor oxygen transfer, and low pigment yield. However, at low temperature (25 °C), cell growth was moderate and continued for 120 h, and low viscosity was maintained. The

DO levels remained at 50% or higher with good mixing. As a result, the pigment yield at 25 °C was 10 times greater than that at 30 °C.