CHAPTER 4

Monascus Rice Products

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Contents

Abstract

The fermentation products of *Monascus*, especially those produced by solid-state fermentation of rice, have been used as food and health remedies for over 1000 years in China. *Monascus* rice products (MRPs) are currently being used as health foods in the United States and many Asian countries such as Japan, Taiwan, China, Korea, Thailand, the Philippines, and Indonesia. Many studies have shown that *Monascus* spp. produce commercially viable metabolites, including food colorants, cholesterol-lowering agents, and antibiotics. The most important bioactive compound isolated from *Monascus* is monacolin K, which is identical to the potent cholesterol-lowering, antiatherosclerotic drug lovastatin, a 3-hydroxy-3-methylglutaryl

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Advances in Food and Nutrition Research, Volume 53
ISSN 1043-4526, DOI: 10.1016/S1043-4526(07)53004-4

coenzyme A (HMG-CoA) reductase inhibitor. Several species of the genus *Monascus* also produce citrinin, a mycotoxin harmful to the hepatic and renal systems. Monacolin K and citrinin are polyketide fungal metabolites. The biosynthetic pathways leading to the formation of polyketides, including monacolin K and citrinin, have been elucidated in *Aspergillus* and *Monascus*. The concern for safety is, therefore, high for the development of MRPs as health foods. Other attractive applications for MRPs are likely, as supported by recent studies that indicate that MRPs contain other substances (flavonoids, polyunsaturated fats, phytosterols, pyrrolinic compounds, and others) with a wide variety of biological activities and pharmacological potentials. Their effects in lowering blood sugar and triacylglycerol while raising HDL-C are more pronounced than those of monacolin K alone. Beyond cholesterol lowering, MRP may also be an ideal candidate for the treatment of metabolic syndrome.

I. THE TAXONOMY OF *MONASCUS* SPP.

Monascus spp. have been used as foods and medicines in the Orient for over 1000 years (Wong, 1982). In China and Taiwan, it has been called ''Hong Qu,'' ''Hon-Chi,'' ''Anka,'' or ''Ang-kak'' using the Chinese or Taiwanese phonetic alphabet. The Japanese use the name ''Beni Koji'' or ''red Koji.'' In the United States and Europe, it has been called ''red rice,'' ''red-mold rice,'' or ''red Chinese rice.'' Many publications and commercial products use ''red yeast rice,'' which is not an appropriate name for filamentous fungi.

In taxonomy, the genus *Monascus* belongs to the family Monascaceae and to the order Eurotiales. The so-called yeast *Saccharomyces* belongs to the family Saccharomycetaceae and to the order Endomycetales. The genus was first suggested by van Tieghem (1884) over a century ago, when its species became known to Westerners as contaminants of cereals, starch, silage, and other agricultural products (Iizuka and Lin, 1980; Young, 1930). Some strains of *Monascus* are characterized by their economic importance, being involved in the fermented foods industry in the Orient (Hesseltine, 1965; Lin, 1975). Species of *Monascus* have frequently been found in fermented food, foodstuffs rich in starch, moldy high-moisture fruits, moldy silages, and soil. For example, seven strains of *Monascus* spp. have been isolated from the starters of Kaoliang in Taiwan and Kinmen by Lin (1975). It was found that there were at least six species of *Monascus* in the starters of Kaoliang Brandy, two from Kinmen and four from Taiwan. After the isolation of the first species by van Tieghem in 1884, a long debate followed concerning the nomenclature. Morphological, physiological, and biochemical characteristics, such as the shape of the colony, length of conidial chain, and production of pigment, have been considered suitable keys to the classification of

Monascus. Hawksworth and Pitt (1983) revised the genus to cover three species, namely *M. pilosus* K. Sato, *M. purpureus* Went, and *M. ruber* van Tieghem. *Monascus* sp. is a filamentous fungus producing single-cell conidia for asexual reproduction, or cleistothecium for sexual reproduction (Figure 1). Ascomata, a stalked cleistothecium, arises singly at the tip of stalk-like hyphae scattered on the mycelium. The ascomatal wall is composed of two distinct layers: the inner wall which results from the swelling of the tips of the stalk-like hyphae forming a vesicle-like structure and the outer layer consisting of hyphal branches growing out from

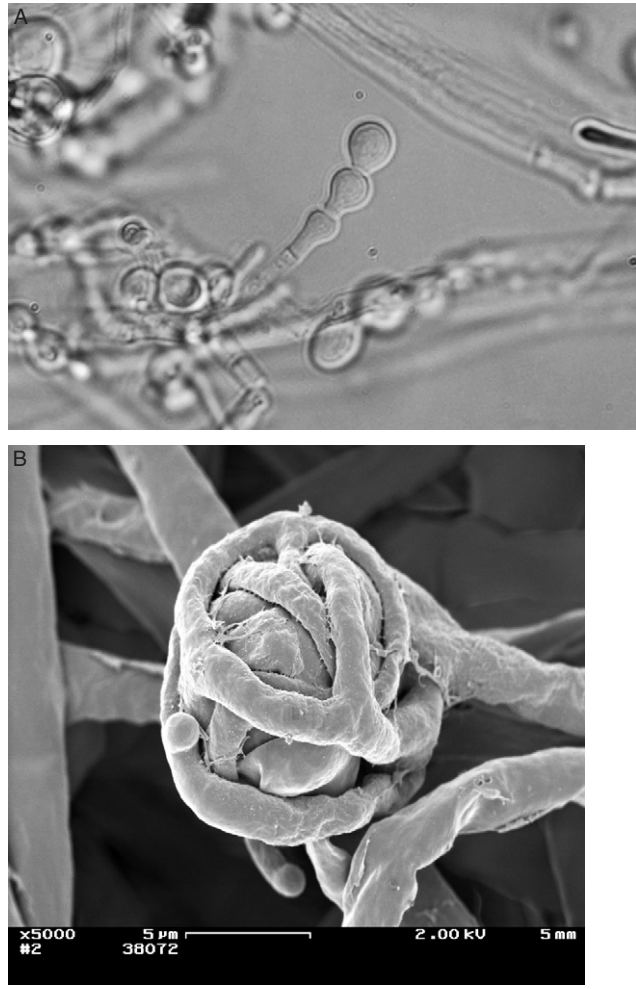


FIGURE 1 The morphology of *Monascus* spp. observed under optical microscope (Panel A) and scanning electron microscopy (SEM) (Panel B).

the base and fusing with the inner vesicle; asci evanescent at an early stage; hyaline, single-celled, ellipsoidal ascospores (Barker, 1903; Carels and Shepherd, 1975).

Based on physiological and morphological characteristics, there are six major species: *M. pilosus*, *M. purpureus*, *M. ruber*, *M. floridanus*, *M. pallens*, and *M. sanguineus*. The phylogenetic relationships of the *Monascus* spp. have been determined by the sequences of the D1/D2 region of the large subunit (LSU) rRNA genes (Park and Jong, 2003). Their results showed *M. ruber* and *M. pilosus* could not be differentiated using these sequences. Park *et al.* (2004) reported the same results using the internal transcribed spacer (ITS) and the partial β-tubulin gene as molecular markers. Although these two species have been recognized as separate species before, recent molecular information has strongly indicated that they are actually the same.

Many species of *Monascus* fungi are readily available to the public from several institutes having culture collections such as the American Type Culture Center (ATCC) in the United States, the National Institute of Technology and Evaluation Biological Resource Center (NBRC) in Japan, and the Food Industry Research and Development Institute in Taiwan assigned with ''CCRC'' prefix.

II. HISTORY OF USING *MONASCUS* RICE PRODUCTS IN ASIA

The *Monascus* rice products (MRPs) that are produced especially by solid-phase fermentation on rice have been used for over 1000 years. The use of MRP in China was first documented in Song Dynasty (sixteenth century), as ''Jiuqu'' to make rice wine (Lin *et al.*, 2005b). Chinese brewers had used ''Jiuqu'' for thousands of years, but did not realize that its innate characteristics were based on microorganisms and enzymes. ''Jiuqu,'' or ''Qu'' in the Chinese phonetic alphabet, are molded cereals that are sources of enzymes necessary for the breakdown of carbohydrates and proteins in the grains. Many strains of *Rhizopus*, *Mucor*, *Aspergillus*, and *Monascus* genera and yeasts with superior hydrolyzing or fermenting power have been isolated from spent grains. MRP (Hong Qu) and its use in alcoholic drinks and in the food industry first appeared in the literature of the Song Dynasty. The traditional techniques of making MRP were recorded in ''Ben Cao Gang Mu'' of Li Shi-zhen (1578) and Song Yin-xing's ''Tian Gong Kai Wu'' (1637).

Traditionally, MRP is cultivated on steamed rice until the mycelium totally covers the whole surface of the grains and the product is used directly (Lotong, 1985; Su and Wang, 1983). Because large quantities of secondary metabolites and hydrolytic enzymes, such as α-amylase, β-amylase, glucoamylase, protease, and lipase, are produced by *Monascus* spp., MRP is widely used as a preservative for meat and fish

and as a colorant or flavor in foods. It is also used for brewing red rice wine and liquor in many Asian countries such as Japan, Taiwan, China, the Philippines, and Indonesia. In China, MRP is legally classified into four types (based on the differences in production methods and appearance of raw materials): ''Ku Qu,'' ''Qing Qu,'' ''Se Qu,'' and ''Wu Yi Hong Qu.'' Among them, ''Ku Qu'' is mainly used for making rice wine. ''Qing Qu'' may be used either for making rice wine or as a colorant for foods. ''Se Qu'' is mainly used as a colorant for foods, and its weight per unit volume is the lightest, due to the longest fermentation time. ''Wu Yi Hong Qu'' is a mixed culture of a *Monascus* sp. with *Aspergillus niger*. ''Wu Yi Hong Qu'' is used in making rice wine with higher amylase activity. The traditional areas of MRP production were centered in South China, in areas such as Fujian Province, Zhejiang Province, Jiangsu Province, Jiangxi Province, and Taiwan. Gutian, a town in Fujian Province, has been one of the most famous red rice production centers. For the use of MRP in Chinese cuisine, ''Hong Cao'' is often the first choice. It is used to stir-fry or steam meat such as roasted red pork ''Hong Cao Pork,'' ''Hong Cao Chicken,'' and ''Hong Cao Fish.'' Another traditional meat product is Chinese-style red sausage ''La Chang.'' The advantages of using *Monascus* spp. to give food color are that they are considered nontoxic and remain stable even when exposed to high temperature. These foods are important mainly because of their color, especially when they are used in the celebration of Chinese New Year as the red color implies ''prosperity.'' In addition to use in meat cuisine, MRP has been used in the fermentation industry for the preparation of red rice wines and foods such as sufu (or ''Dou-Fu-Ru'' in Chinese, a mold-fermented soybean curd product), fish sauce, fish paste, and red soybean curd (a cheese-like product used as a spice). Red rice wine is called ''Hong Qu Jiu'' in Chinese. This kind of rice wine has a long history and the MRP-producing areas associated with production of this rice wine are broad and scattered mainly over the Jiangsu, Jiangxi, Fujian, and Zhejiang provinces in China. This rice wine is brewed from polished glutinous rice with MRP and wheat Qu or rice Qu as saccharifying and fermenting agents. The wine is bright golden-yellow in color, has mellow aromas and elegant flavors, and leaves a relaxing and pleasant aftertaste. Kinetic studies of the headspace components from rice and agar with (experimental) and without (control) inoculation with a *Monascus* sp. have been carried out (Chung *et al*., 2004). The results showed that five alcohols, four esters, two ketones, and one furan, with odor activity values (OAV) > 1 dominated the overall flavor of the product. The application of MRP is not restricted to the above-mentioned products. For example, we have used MRP to invent methods for producing a vegetarian lactic acid beverage similar to yogurt (drinking yogurt) (Wang *et al*., 2003b) and a beer-like, alcohol-free fermented beverage (Lin *et al*., 2006a).

MRP is not only utilized directly for food, but is also used indirectly such as to produce low-cholesterol eggs (Wang and Pan, 2003) and Arbor Acres broiler chickens (Wang *et al*., 2006). In addition to its value in preparing delicious dishes, MRP has also been used in traditional Chinese medicine for centuries to help maintain a healthy heart and circulatory system. A health-promoting effect is mentioned in an ancient Chinese pharmacopoeia of medicinal foods and herbs, ''Ben Cao Gang Mu'' of Li Shi-zhen (1578), where it is proposed to be a mild aid for gastric complication problems (indigestion, diarrhea, and others), blood circulation, and spleen and stomach health. Among the species of *Monascus*, *M. purpureus* has been identified and used in traditional Chinese medicine for the treatment of blood stasis, a disorder related to dyslipidemia and atherosclerosis (Journoud and Jones, 2004). The MRP prepared with the *M. pilosus* IFO 4520 strain produced by Gunze, Ltd., effectively reduces elevated blood pressure and was approved as a food supplement for specified health use in Japan (Himeno, 1997).

III. PRODUCTION METHODS

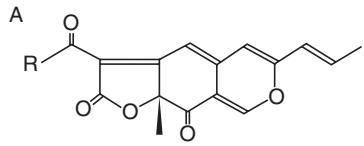
A. Traditional production

MRPs are produced by solid-phase fermentation on rice. A detailed description of making MRPs is found in the ancient Chinese book, ''Tian Gong Kai Wu,'' published in 1637. These traditional methods for manufacturing MRPs are very complicated and time consuming. Since the *Monascus* strain is slow growing, MRPs prepared with this strain are often contaminated with other fast-growing microorganisms during MRP making in the open environment. To prevent contamination, it is necessary to prepare ''seed Koji'' as a starter before making MRPs. The techniques for preparing ''seed Koji'' are proprietary and conducted by ''masters'' with special training. Nonglutinous varieties of rice are most suitable for preparing MRP, since kernels of glutinous varieties tend to stick together and thus reduce the surface to volume ratio of solid material which is critical to the growth of *Monascus*. The best raw material is long-shaped, non-glutinous rice. It is first washed, soaked in water for about 1 day or more, and drained thoroughly. The moist rice is then cooked. On cooling, the steamed rice is mixed with a diluted vinegar solution or a solution of alum to acidify the raw material (because the *Monascus* spp. are acidophilic), then is inoculated with ''seed Koji.'' The inoculated rice is thoroughly mixed and then incubated at an appropriate temperature in the range of 33–42 °C. During the first few days, the rice would have taken on a pink color and been stirred and shaken to redistribute the moisture

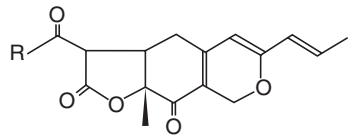
and kernels with respect to depth from the surface of the fermenting mass which should be spread out and piled up in turn. It may have been necessary to add an adequate amount of water to compensate for the moisture lost during incubation. Within about 2 weeks, the rice would take on a deep purplish red color with no observable clumping of the kernels.

B. Production of polyketide metabolites by *Monascus* spp.

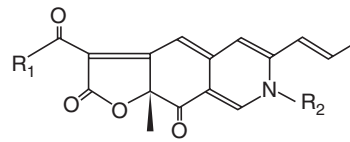
Many secondary metabolites with complex chemical structures, including pigments (Figure 2) and monacolins (Figure 2), are synthesized from the polyketide pathway in *Monascus* spp. (Simpson, 1986). Several effectors controlling the polyketide synthesis of *Monascus* have been reported by using submerged culture systems (Lin, 1991). Considerable research has been conducted on the industrial production of *Monascus* in complex liquid media (Shepherd and Carels, 1983).



	R
rtn Rubropunctatin	n-C_5H_{11}
mbn Monascorubrin	n-C_7H_{15}

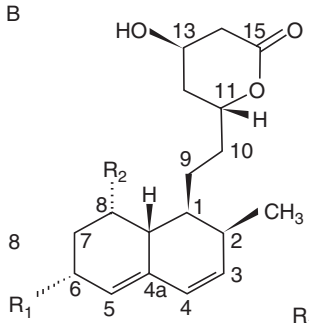


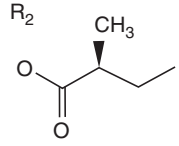
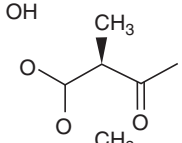
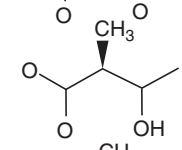
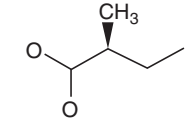
	R
mnc Monascin	n-C_5H_{11}
ank Ankaflavin	n-C_7H_{15}

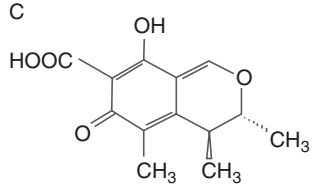


	R_1	R_2
rtm Rubropunctamine	n-C_5H_{11}	H
mbm Monascorubramine	n-C_7H_{15}	H

FIGURE 2 (*continued*)



	R_1	R_2
Mevinolin (monacolin K)	CH_3	
Monacolin L	CH_3	H
Monacolin J	CH_3	
Monacolin X	CH_3	
Monacolin M	CH_3	
Compactin (ML-236B)	H	
ML-236A	H	OH
ML-236C	H	H



Citrinin

FIGURE 2 Chemical structures of selected second metabolites from *Monascus*. (A) *Monascus* pigments, (B) monacolins, (C) citrinin.

Based on the works of Hadfield *et al*. (1967) and Turner (1971), a scheme of the hypothetical routes for the biosynthesis of these pigments was proposed by Hajjaj *et al*. (2000). The condensation of 1 mol of acetyl-CoA with 5 mol of malonyl-CoA leads to the formation of a hexaketide skeleton by the polyketide synthase. Then a medium-chain fatty acid such as octanoic acid, likely produced by the fatty acid biosynthetic pathway, is bound to the hexaketide by a transesterification reaction to generate the orange pigment monascorubrin (or rubropunctatin on transesterification with hexanoic acid). Based on observations from an isotope-labeling experiment, it is probable that the *Monascus* pigments and monacolin K are made by similar polyketide-forming enzymes (Turner and Aldridge, 1983). The biosynthetic pathway of lovastatin in *A. terreus* has been investigated by nuclear magnetic resonance (NMR) and mass spectroscopy (Yoshizawa *et al*., 1994). These studies concluded that lovastatin is composed of two distinct polyketide chains joined through an ester linkage. Proof that these two polyketides are assembled by two discrete polyketide synthases came from the cloning and partial characterization of the lovastatin biosynthetic gene cluster from *A. terreus* (Hendrickson *et al*., 1999; Kennedy *et al*., 1999). Hajjaj *et al*. (1999b) have demonstrated that the biosynthesis of citrinin (Figure 2) by *Monascus* originates from a tetraketide instead of a pentaketide as has been found in *A. terreus* and *Penicillium citrinum*. Since the pigments are produced from a hexaketide, this suggests the existence of a branch point at the tetraketide level, which can account for differential production routes of pigments and citrinin in *M. ruber* (Hajjaj *et al*., 1999a). Several nutrient effectors in controlling polyketide biosynthesis are described separately below.

1. Carbon source

Within the range from 4% to 10%, higher concentrations of glucose support higher production of monacolin K (Buckland *et al*., 1989). Furthermore, addition of glucose during the fermentation increases production to a greater extent than addition of the slowly utilized glycerol. One possible reason for the stimulation of monacolin K production by glucose may be its catabolite repression of nicotinamide adenine dinucleotide phosphate hydrogen (NADPH) generation as well as repression of tricarboxylic acid (TCA) cycle enzymes (Buchanan and Lewis, 1984; Buchanan *et al*., 1985). A decline in TCA cycle flux may lead to the accumulation of acetyl-CoA for the synthesis of polyketides (Demain, 1968). Utilization of carbon sources for growth appears to be strain specific. Yoshimura *et al*. (1975) have reported that 5% ethanol is a very good carbon source for pigment formation but Lin (1982) claimed that ethanol, when its concentration is higher than 2%, inhibits both growth and pigment production. Hajjaj *et al*. (2001) have shown that carbon source starvation is required for lovastatin biosynthesis by *A. terreus*.

Cultured on glucose and glutamate, lovastatin synthesis is initiated when glucose consumption starts to level off.

2. Nitrogen source

Nitrogen source regulation of polyketide biosynthesis is well known, for example, nitrate repression of aflatoxin formation (Bennett *et al*., 1979). However, utilization of different nitrogen sources often causes pH change during fermentation. This independently affects growth and pigment production (Carels and Shepherd, 1977, 1978, 1979; Shepherd and Carels, 1983; Wong *et al*., 1981) and has resulted in confusion with respect to nitrogen control of pigment synthesis. MOPS (3-[*N*-morpholino]propanesulfonic acid) buffer was used to overcome this problem (Lin, 1991). Nitrogen metabolism can lead to the formation of intermediates of the TCA cycle and thus influences the cycle (Berg *et al*., 2006). Bhatnagar *et al*. (1986) have shown that the NAD-requiring glutamate dehydrogenase (catalyzing the conversion of glutamate to α-ketoglutarate) is more active in a medium with ammonium as the sole nitrogen source than in a medium with ammonium plus asparagines as nitrogen sources. The high activity of the NAD-requiring glutamate dehydrogenase during the exponential growth phase results in the accumulation of α-ketoglutarate, which inhibits the TCA cycle, thus minimizing acetyl-CoA oxidation and making it available for increased aflatoxin synthesis.

3. Other Factors

Other factors reported to have significant effects on the synthesis of polyketide mycotoxins and *Monascus* pigments include metals, oxygen, and temperature (Demain, 1986). Metals have important effects on secondary metabolism (Weinberg, 1989). The growth of *M. purpureus* is inhibited, but the production of certain pigments is promoted, by the addition of zinc sulfate (Johnson and McHan, 1975; Wong, 1982; Wong and Bau, 1977). In the case of the production of *Monascus* pigments, Su (1978) found that the agitation of fermentation broths at 500 and 700 rpm yielded the same mycelial form, but pigment formation was higher at 500 rpm than at 700 rpm. The lower production of *Monascus* pigments at the higher agitation rate may be caused by the inhibition of pigment formation by oxygen and/or shear stress on the mycelia (Yoshimura *et al*., 1975).

An additional problem is that polyketide formation requires acetyl-CoA, malonyl-CoA, and NADPH generated by primary metabolic pathways. These precursors and the cofactor are also used for fatty acid biosynthesis. An inverse relationship between the synthesis of fatty acids and polyketide compounds has been found in the mevinolin (lovastatin)-producing species of *Aspergillus* (Dutton, 1988; Greenspan and Yudkovitz, 1985). Thus, any regulatory factor that substantially alters the rate or extent of formation of these precursors and cofactor may affect polyketide formation.

C. Pigments production

There are six well-known *Monascus* pigments (azaphilones) that are produced and are divided into three pairs. Rubropunctatin ($C_{21}H_{22}O_5$) and monascorubrin ($C_{23}H_{26}O_5$) are orange pigments with different aliphatic side chains on the β-ozo-lactone ring. The two corresponding red pigments, rubropunctamine ($C_{21}H_{23}NO_4$) and monascorubramine ($C_{23}H_{27}NO_4$), are nitrogen analogues of the orange pigments. The yellow pigments, monascin ($C_{21}H_{26}O_5$) (syn. monascoflavin) and ankaflavin ($C_{23}H_{30}O_5$), are the reduced forms of the orange pigments (Kurono *et al*. 1963; Sweeny *et al*., 1981). Carels and Shepherd (1977) have proposed that the orange pigments are initially synthesized and the yellow and red pigments are derived from the orange counterparts. *Monascus* pigments are stable in the pH range of 2–10. They are also heat stable and can be autoclaved (Francis, 1987). Mutation is a useful technique for the enhancement of pigment production. For example, Lin and Iizuka (1982) have derived a hyperpigment-producing mutant, *M. kaoliang* R-10847, through a series of mutagenesis steps. This mutant, derived from an intracellular parent, produces extracellular pigment. The productivity of pigment is about 100-fold greater than that of the wild type. Pigment production can also be improved by optimizing the culture condition of *Monascus*. Shin *et al*. (1998) showed that when a *Monascus* isolate was cocultured with either *Saccharomyces cerevisiae* or *A. oryzae* in a solid sucrose medium, there were significant morphological changes in the *Monascus* culture. Cocultures exhibited cell mass increases of 2 times and pigment yield increases of 30–40 times compared to monocultures of *Monascus*. The hydrolytic enzymes produced by *S. cerevisiae*, such as amylase and chitinase, are thought to be the effectors. Using transformation systems, Campoy *et al*. (2003) were able to manipulate the natural pigment producers, *M. purpureus* and *M. ruber*. The high-level pigment-producing *Monascus* strain IBCC1 was characterized by random amplification of polymorphic DNA as *M. purpureus*. Their results showed that 60% of the *Monascus* transformants were found to be stable in mitosis and retained the plasmid inserted in the chromosome after repeated sporulation cycles. The transformants obtained by *Agrobacterium*-mediated DNA transfer remained fully stable (98%) after four sporulation rounds and showed bands of hybridization corresponding to integration of the plasmid in different sites of the genome. A characterization of a non-pigment-producing mutant, *M. purpureus* M12, compared with its parental strain, *M. purpureus* Went CBS 109.07, has been performed aiming to investigate the relation between pigment biosynthesis and other characteristics (Rasheva *et al*., 2003). In a selected albino mutant, the growth rate, metabolic activity, and capacity for polyketide production typical for *Monascus* secondary metabolites were reduced considerably. The mutant strain produced C17, C20, and C22 fatty acids but did not produce citrinin. By immobilized repeated-batch

processes, extracellular production of *M. purpureus* C322 pigment was studied by Fenice *et al*. (2000). Using Ca-alginate as an immobilizing carrier, pigment production reached a plateau while the cell leakage was negligible and mechanical stability of the Ca-alginate bead was good. By the addition of 20 amino acids, the red derivative pigments were produced (Jung *et al*., 2003). Liquid chromatography-mass spectroscopy (LC-MS) and ^{1}H and ^{13}C NMR structural analyses confirmed that the derivative pigments contained the moieties of the added amino acids. These pigments showed enhanced photostability (Jung *et al*., 2005). Under sunlight, the half-life of derivatives was increased to 1.45–5.58 hours, corresponding to a 6- to –25-fold improvement over a control red pigment (0.22 hours).

MRP has been cultured traditionally on rice and other cereals by solid-state fermentation. However, for large-scale cultivation, solid-state cultures were associated with some problems such as contamination and scale-up requirements. There have been reports that *Monascus* could be cultured in submerged culture systems; however, the pigment production in submerged culture was reduced to one-tenth of that achievable in the solid-state fermentation (Lin, 1973). For the submerged culture studies with rice particles, a stirred-tank fermentor was not suitable as the impeller tended to break the particles into small pieces. A conventional bubble column was also unsuitable since its mixing capability was poor. Wu *et al*. (2000) developed a modified bubble column with wire-mesh draft tubes for the cultivation of *M. purpureus*. The production of pigments using the proposed column was 80% higher than that achieved using the conventional bubble column. A process combining solid-state and submerged cultivations, intermittently rinsing the rice with monosodium glutamate (MSG) solutions every 12 h, shows advantage. Following an adsorptive extraction of the red pigment dissolved in the rinsing solution, the *Monascus* red pigment production was found to increase by 24% as compared with that realized by the plain fixed-bed cultivation (Hsu *et al*., 2002). Considerable research has been conducted on the industrial production of *Monascus* pigments in complex liquid media (Campoy *et al*., 2005; Krairak *et al*., 2000). In general, high broth viscosity is a key factor to be considered in a submerged fermentation of filamentous fungi. The resultant high viscosity induced heterogeneity inside the fermentor, poor oxygen transfer, and low pigment yield. However, these problems could be overcome by reducing fungal growth rate by culturing at low temperature (25 °C). As a result, the pigment yield at 25 °C was 10 times greater than at 30 °C (Ahn *et al*., 2006). Light is another factor. In nature, light is one of the most crucial environmental signals for developmental and physiological processes. Miyake *et al*. (2005) have found that both red and blue lights affect development in *Monascus*, influencing the processes of mycelium and spore formation, and the production of secondary metabolites such as γ-aminobutyric acid (GABA), red pigments, monacolin K, and citrinin.

Although toxicity studies show that *Monascus* pigments are safe for human consumption (Su and Wang, 1983), low solubility in water and the sensitivity to decoloration by sunlight restricted the wide use of the *Monascus* pigments in the beverage and confectionary industries (Sweeny *et al*., 1981). Thus, many patents have focused on the improvement of extraction and solubilization of *Monascus* pigments (Wong, 1982). Several chemical processes have been patented for semisynthetic water-soluble red pigments (Moll and Farr, 1976). Direct production of water-soluble pigments by fermentation offers a more acceptable alternative to the total and semisynthetic processes, since it avoids the use of ''chemical additives'' in foods (Spears, 1988). We have developed a chemically defined medium and a resting-cell system, and discovered new water-soluble red pigments (Lin, 1991). These were isolated and characterized as glutamate derivatives of the orange pigments (rubropunctatin and monascorubrin). These new pigments showed superior properties such as higher water solubility, higher absorption coefficient (ε value), and greater resistance to decoloration by light.

D. Monacolin K production

Initially identified from *Monascus* spp., monacolin K ($C_{24}H_{36}O_5$) is a polyketide, which is structurally identical to lovastatin (Endo, 1979, 1980). In addition to monacolin K, there are several other minor monacolins (Ma *et al*., 2000). At least six structurally related monacolins have been identified from the genus *Monascus*, namely monacolin J, K, L, and X, dihydromonacolin K, and dihydromonacolin L (Endo *et al*., 1985a,b).

Many analytical procedures based on high-performance liquid chromatography (HPLC) and LC-MS have been developed for the determination of lovastatin and other statins in biological samples. Li *et al*. (2004) developed a chemical fingerprint-profiling method using HPLC with a photodiode array (PDA) detector and tandem mass spectrometry (MS/MS). A fingerprint profile containing 14 monacolin compounds, including monacolin K (mevinolin), J, L, M, and X, and their hydroxyl acid forms, as well as dehydromonacolin K, dihydromonacolin L, compactin, and α-hydroxy-3, 5-dihydromonacolin L in *Monascus* spp., can be obtained using that method. Another detection method for the assay of the monacolin series compounds was established by Li *et al*. (2005c). By using reversed phase HPLC (RP-HPLC) with PDA, well-resolved peaks of seven main compounds of the monacolin family were profiled. The method was established with 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. Li *et al*. (2005c) used this method to study the stability of monacolin K under different storage conditions. The results showed that the monacolins in MRP powder are light sensitive and thermal sensitive. Monacolins

decomposed 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 dehydrated and converted 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).

Only a few species of *Monascus* can produce monacolin K (Table 1). Since *Monascus* pigments and monacolin K are made by the same or similar polyketide-forming enzymes (Turner and Aldridge, 1983), the ability of various species of *Monascus* to produce monacolin K may be predicted based on its mycelia color.

Response surface methodology (RSM) was employed by Chang *et al.* (2002) to study the effect of the composition of the rice–glycerol complex medium on the production of monacolin K by *M. ruber* in mixed solid–liquid (or submerged) cultures at 25 °C. The best composition (in g/liter) derived from RSM regression was rice powder 34.4 g, peptone 10.8 g, glucose 129 g, KNO_3 8.0 g, $MgSO_4 \cdot 7H_2O$ 4.0 g, and glycerol 36.4 ml per liter. With this composition, the monacolin K production was 157 mg/liter after 10 days of cultivation. In a study by Miyake *et al.* (2006), it was found that *M. pilosus* required a suitable concentration of organic peptone for high monacolin K production. They had developed a glucose-glycerol-peptone (GGP) medium which contained 3% glucose, 7% glycerol, 3.8% peptone, 0.1% $MgSO_4 \cdot 7H_2O$, and 0.2% $NaNO_3$; and with this medium, *M. pilosus* MK-1 produced the highest level of monacolin K.

For the production of monacolin K-containing MRP on an industrial scale especially, we have successfully developed a fermentation process by cultivating a low-citrinin, high-monacolin K-producing strain of *M. purpureus* on rice in an aseptic rotary vessel to minimize microbial contamination due to the slow growth of *Monascus*. In brief, steamed rice was inoculated with *M. purpureus* with an inoculum of 100 spores/kg raw rice. The inoculated rice was cultivated at 30 °C for 5 days until the steamed rice turned a deep red color. The colored rice was used as ''seed Koji.'' The ''seed Koji'' was uniformly mixed with steamed rice at a ratio of 3% based

TABLE 1 The species of *Monascus* that produce monacolin K

Species	Monacolin K production	References
M. ruber	17.4 mg/liter	Endo, 1979
M. purpureus NTU 601	0.53 mg/g	Wang *et al.*, 2003
M. ruber	2.5–3.0 mg/g	Wei *et al.*, 2004
M. pilosus	2.52 mg/g	Chen and Hu, 2005
M. purpureus NTU 301	2.584 mg/g	Lee *et al.*, 2006
M. pilosus MK-1	725 mg/liter	Miyake *et al.*, 2006

on the weight of steamed rice. The resulting mixture was soaked in water twice and cultivated for 6 days at a temperature of 35 °C and a relative humidity below 95% to produce *Monascus* rice with a bright red color.

Since the formation of the secondary metabolites of the *Monascus* spp. is affected by cultivation conditions, Lee *et al.* (2006) used sweet potato (*Ipomoea batatas*), potato (*Solanum tuberosum*), cassava (*Manihot esculenta*), and dioscorea (*Dioscorea batatas*) as the substrates to identify the best choice for monacolin K production. The results showed that *M. purpureus* NTU 301, with dioscorea as the substrate, could produce monacolin K at 2584 mg/kg, which is 5.37 times more than that resulted when rice is used as the substrate.

For storage, monacolins in MRP powder decreased significantly under the conditions of high humidity at high temperature (75% RH, 60 °C) and sunlight. Therefore it has been suggested that the preparations containing monacolins be stored in a cool and lightproof place (Li *et al.*, 2005c).

E. GABA production

GABA is produced by the decarboxylation of glutamic acid by glutamate decarboxylase. In the process of making ''Jiuqu'' (mold-containing MRP for making rice wine), glutamic acid is produced from steamed rice by an acid protease and an acid carboxypeptidase that are secreted by the mold. GABA has several physiological functions, including neurotransmitting, hypotensive, and diuretic effects (Keisuke *et al.*, 1992; Matheson *et al.*, 1986). The changes in GABA content during the preparation of MRP have been examined by Kono and Himeno (2000). When prepared with *M. pilosus* IFO 4520, the production of GABA in MRP peaked on the fifth day and thereafter declined. Su *et al.* (2003) showed that the amounts of GABA produced by different strains varied greatly. In their study, solid-state cultivation always produced more GABA than submerged cultivation did. The addition of sodium nitrate during the solid-state fermentation of *M. purpureus* improved the productivity of GABA to 1267.6 mg/kg. GABA productivity increased further to 1493.6 mg/kg when dipotassium hydrophosphate was added to the medium. Wang *et al.* (2003a) showed that addition of 0.5% ethanol increased production of GABA from 1060 to 7453 mg/kg.

IV. EVIDENCE FOR HEALTH BENEFITS

A. Cholesterol-lowering effect

1. The inhibitor of HMG-CoA reductase

Hypercholesterolemia, especially elevated plasma low-density lipoprotein cholesterol (LDL-C), is a key risk factor leading to the pathogenesis of atherosclerosis (Steinberg, 2002). The identification of compactin

(ML-236B) from *P. citrinum* (Endo *et al.*, 1976) and *P. brevicompactum* (Brown *et al.*, 1976) and lovastatin (mevinolin) from *A. terreus* (Alberts *et al.*, 1980) and *M. purpureus* as the inhibitors of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase has greatly advanced the development of cholesterol-lowering drugs. Inhibition of hepatic HMG-CoA reductase, the rate-limiting enzyme in the cholesterol biosynthetic pathway, stimulates the expression of LDL receptor (also called ApoB/E receptor) (Brown and Goldstein, 1986). Increased uptake of LDL through a receptor-mediated pathway reduces plasma LDL-C. Many clinical studies have shown that lovastatin and other statin drugs reduce plasma total cholesterol (TC) and LDL-C. Treatment with lovastatin also reduces plasma triacylglycerols (TG) and increases high-density lipoprotein cholesterol (HDL-C) to an extent less than the magnitude of TC and LDL-C lowering. MRP is used for dietary supplements with the health claim that the product will lower plasma lipids, especially plasma TC and LDL-C. The MRP supplements contain a family of naturally occurring statins (monacolins) including monacolin K. There are many commercial products of MRP worldwide. Among them ''Cholestin'' (2.4 g/day contained 9.6 mg total monacolins, Pharmanex, Inc., Simi Valley, CA), ''Xuezhikang'' (1.2 g/day contained 13.5 mg total monacolins, WBL Peking University Biotech Co., Ltd., China), and ''Unchole'' (1.0 g/day contained 8.0 mg total monacolins, Taiwan Tobacco & Liquor Corp., Taiwan, ROC) have demonstrated a cholesterol-inhibiting effect similar to statins in animal studies and clinical trials.

2. Animal studies

The effects of ''Xuezhikang'' on plasma cholesterol and functions of endothelial cells in cholesterol-fed rabbits have been studied by Wu *et al.* (2003). After a 12-week feeding experiment, serum TC, LDL-C, TG, and plasma endothelin-1(ET-1) decreased and serum NO level increased in the ''Xuezhikang'' group as compared with those of the hypercholesterol-fed control group ($p < 0.05$). The areas of lipid deposition on the intimal surface of the aorta and coronary arteries were reduced and the ultrastructural injuries of endothelial cells were milder in the ''Xuezhikang'' group. Apolipoprotein E-deficient [ApoE (−/−)] mice are the common animal model and display high similarity to human atherosclerosis. Using ApoE (−/−) mice, Zheng *et al.* (2003) have shown that ''Xuezhikang'' lowers the serum TC, TG, and LDL-C and reduces the atherosclerotic lesions after a 14-week feeding. For animal studies, hamsters are considered to be one of the most suitable animal models for human lipid and lipoprotein metabolism (Harris, 1997; Ntanios and Jones, 1999). Using this animal model fed with products from a *Monascus* strain, *M. purpureus* NTU568, similar results with decreases in TC, TG, and LDL-C levels were shown by Lee *et al.* (2005).

Long-term feeding effects of *M. purpureus*-fermented rice (Cholestin) on serum lipids and the severity of atherosclerosis were examined in

rabbits fed for 200 days on a semipurified diet containing 0.25% cholesterol (Wei *et al.*, 2003). Total serum cholesterol was 25% and 40% lower, respectively, in rabbits fed 0.4 or 1.35 g/kg/day of MRP compared to those of controls. This treatment also lowered serum LDL-C, serum TG, and the atherosclerotic index (ratio of non-HDL-C to HDL-C). Although similar reductions of TC, LDL-C, and TG were observed, a parallel group of rabbits fed with lovastatin (0.0024 g/kg/day) did not have a significantly reduced atherosclerotic index.

We have used hamsters as an animal model to elucidate the effects of a monacolin K-containing *M. purpureus* rice product (''Unchole,'' 1.0 g/day containing 8.0 mg total monacolins, Taiwan Tobacco & Liquor Corp.) on serum lipid and lipoproteins (Lin *et al.*, 2005b; Table 2). Results showed that MRP treatment lowered total serum cholesterol and LDL-C. The MRP treatment also increased the secretion of fecal cholesterol, which was not found in the lovastatin-treated group (Lin *et al.*, 2005b; Table 3). Treatment with ''Unchole'' also lowered serum TG, although its TG-lowering mechanism is unclear.

TABLE 2 Lipid and glucose concentrations of hyperlipidemic hamster after 31 days feeding study

	Basline ($n = 8$)	Control ($n = 8$)	"Lovastatin" ($n = 8$)	"Unchole" ($n = 8$)
	(mg/dl)			
TC	98.5 ± 0.7	277.0 ± 33.9	235.0 ± 33.9	184.5 ± 24.7
TG	239 ± 46	274 ± 94	268 ± 76	156 ± 64
Glucose	192 ± 42	222 ± 34	174 ± 27	114 ± 32
VLDL-C	17.0 ± 8.5	79.5 ± 26.2	49.5 ± 2.1	14.5 ± 3.5
LDL-C	31.0 ± 7.1	135.5 ± 6.4	106.0 ± 29.7	88.0 ± 12.7
HDL-C	50.5 ± 16.3	62.0 ± 1.4	79.5 ± 2.1	82.0 ± 8.5

Baseline, chow diet (Purina 5001); Control, chow diet plus 0.25% cholesterol (w/w) and 5% soybean oil; ''Lovastatin,'' Control diet plus Lovastatin (50 mg/kg); ''Unchole,'' Control diet plus ''Unchole'' (25 g/kg).
HDL-C was determined by the following formula: (HDL-C) = TC − (LDL-C) − (VLDL-C).
Results were shown as mean ± S.D. Data with same superscript in the same row were not significantly different ($p > 0.05$).

TABLE 3 Fecal cholesterol

	Basline ($n = 8$)	Control ($n = 8$)	"Lovastatin" ($n = 8$)	"Unchole" ($n = 8$)
Cholesterol (mg/g dry feces)	5.8 ± 0.4	11.6 ± 0.2	13.4 ± 0.6	19.4 ± 0.1

Fecal cholesterol was recovered by saponification and extraction with n-hexane.
The recovered cholesterol was determination by a modified Liebermann-Burchard method.

The most plausible explanation of monacolin K-containing MRP reducing plasma cholesterol and LDL-C, beyond the contribution of its monacolin K content, may be due to the additive and/or synergistic effects of monacolin K with other monacolins and substances (such as phytosterols) in MRP. In addition to the phytosterols (β-sitosterol, campesterol, stigmasterol, and sapogenin), MRP has been found to contain isoflavones and isoflavone glycosides, and monounsaturated fatty acids (Heber *et al.*, 1999). Based on the observation that the solid-phase fermentation is a prolonged and extensive process (which is a slow process of over 10 days), we propose that the resulting *Monascus* preparation is a product of extensive utilization of starch and significant enrichment of phytosterols. Many studies have demonstrated the cholesterol-lowering effects of plant sterols (Ostlund, 2002). Plant sterols may competitively inhibit the intestinal absorption of cholesterol and therefore lower its level in the plasma.

3. Clinical studies

Several pilot studies (Cicero *et al.*, 2005; Liu *et al.*, 2003; Sumioka *et al.*, 2006) and clinical trials (Table 4) have demonstrated that the intake of MRP significantly decreases TC, LDL-C, and TG in subjects, without causing clinically adverse effects in the liver and muscle tissue. Since double-blind, randomized, placebo-controlled prospective studies have been performed on subjects with ''Xuezhikang,'' ''Cholestin,'' and other MRPs, it suggests that MRP can be a safe and efficacious agent for subjects at risk for cardiovascular diseases. Although one proprietary strain of MRP has been demonstrated to lower cholesterol levels significantly in clinical trials, not all strains being sold as dietary supplements have undergone similar evaluation. In order to determine whether the results of a clinical trial conducted with one strain of MRP could be extended to other preparations of MRP, nine different commercially available dietary supplements were purchased and tested for chemical constituents by Heber *et al.* (2001). They concluded that standardized manufacturing practices should be established for MRP as a dietary supplement. It can ensure equivalence of content of active ingredients in preparations being sold to the public and to limit the production of unwanted by-products of fermentation such as citrinin. There is still a need for further study on the long-term safety and efficacy of MRP as a dietary supplement in a larger population. The effects in lowering TG and increasing HDL-C also need to be elucidated in future studies.

B. Other effects

Besides monacolin K (lovastatin, once the world's largest selling class of cholesterol-lowering drugs), the *Monascus* products also contain many other substances (flavonoids, polyunsaturated fats, pyrrolinic compounds, and so on) with a wide variety of actions. Their effects may be

TABLE 4 Preliminary clinical data of MRP in colesterol-lowering effect

References	Design	Dosage	Patients(n)	Results
Kou *et al.*, 1997	R comparative study of Xuezhikang and Simvastatin (Zocor)	Xuezhikang (1.2 g/day, ~13.5 mg total monacolins) Zocor (10 mg/day) 8 weeks	108 patients with primary hyperlipidemia	Xuezhikang TC: −23% ($p < 0.001$) TG: −28.1% ($p < 0.001$) LDL-C: −28% ($p < 0.001$) HDL-C: +5% ($p > 0.05$) Zocor TC: −23.3% ($p < 0.001$) TG: −29.5% ($p < 0.001$) LDL-C: −29.5% ($p < 0.001$) HDL-C: +14.3% ($p < 0.01$)
Jing *et al.*, 1999	R comparative study of Xuezhikang and Gemfibrozil	Xuezhikang (1.2 g/day) Gemfibrozil (1.2 g/day) 8 weeks	91 patients with hyperlipidemia	Xuezhikang TC: −21.6% ($p < 0.01$) TG: −23.3% ($p < 0.01$) LDL-C: −33.3% ($p < 0.01$) HDL-C: +33.7% ($p < 0.01$) LP(a): −28.2% ($p < 0.01$) TXB_2: −34.2% ($p < 0.01$) 6-keto-$PGF_{1\alpha}$: +65.4% ($p < 0.01$) Gemfibrozil TC: −20.4% ($p < 0.01$) TG: −40.3% ($p < 0.01$) LDL-C: −24.8% ($p < 0.01$) HDL-C: +26.9% ($p < 0.01$) LP(a): −4.9% ($p < 0.01$) TXB_2: −8.4% ($p < 0.01$) 6-keto-$PGF_{1\alpha}$: +11.7% ($p < 0.01$)

(*continued*)

TABLE 4 (*continued*)

References	Design	Dosage	Patients(n)	Results
Heber *et al.*, 1999	PC; R; DB	Cholestin (2.4 g/day, ~9.6 mg total monacolins) 8 and 12 weeks	83 healthy sunjects with hyperlipidemia	8 weeks TC: −16.8% ($p < 0.05$) TG: −11.3% ($p < 0.05$) LDL-C: −22.3% ($p < 0.05$) HDL-C: 0% 12 weeks TC: −16.1% ($p < 0.05$) TG: −6.7% ($p < 0.05$) LDL-C: −21.9% ($p < 0.05$) HDL-C: 0%
Lin *et al.*, 2003	PC; R	Xuezhikang (1.2 g/day) 8 weeks	60 CHD patients	TC: −21% ($p < 0.05$) TG: −25% ($p < 0.05$) LDL-C: −30% ($p < 0.05$) HDL-C: +16% ($p < 0.05$) LP(a): −23% ($p < 0.05$)
Zhao *et al.*, 2004	PC; R	Xuezhikang (1.2 g/day) 6 weeks	50 CHD patients	TC: −18.8% ($p < 0.001$) TG: −31.1% ($p < 0.001$) LDL-C: −28.3% ($p < 0.001$) HDL-C: +17.4% ($p < 0.001$) Hs-CRP: −50% ($p < 0.001$)

Li *et al.*, 2005b	R	Xuezhikang (1.2 or 2.4 g/day) 14 days	48 patients with stable angina	1.2 g/day, median CRP: −28.6%; TC: −13%; LDL-C: −23%, ($p < 0.05$) 2.4 g/day, median CRP: −30.4%; TC: −22%; LDL-C: −32%, ($p < 0.01$)
Lin *et al.*, 2005a	PC; R; DB	*M. purpureus* Went rice (600 mg) 8 weeks	79 patients with a mean baseline LDL-C level of 203.9 mg/dl	LDL-C: −27.7% TC: −21.5% TG: −15.8% ApoB: −26.0%
Lu and Fu, 2005	PC; R; DB	Xuezhikang (0.6 g, bid) + conventional therapy 4 years	4870 CHD patients	Incidence of nonfatal MI was reduced by 60.8% ($p < 0.0000$) Incidence of death from CHD reduced by 31.0% ($p < 0.0048$) Total mortality was lowered by 33.0% ($p = 0.0003$)
Lu *et al.*, 2005	PC; R; DB	Xuezhikang (0.6 g, Bid) + conventional therapy 4 years	591 CHD patients with diabetes	Incidence of CHD events reduced by 50.8% ($p = 0.0008$) Incidence of death from CHD reduced by 44.1%($p = 0.0246$) Incidence of nonfatal MI was reduced by 63.8% ($p = 0.0151$) Total mortality was lowered by 44.1% ($p = 0.0097$)

(*continued*)

TABLE 4 (*continued*)

References	Design	Dosage	Patients(n)	Results
Du *et al.*, 2006	PC	Xuezhikang (1.2 g/day)	2135 patients with MI history of 28 days to 3 months (group A)	Group A: reduced the risk of CHD events by 56.7% ($p < 0.0001$)
		8 weeks	2735 patients with MI history of 3–60 months (group B)	Group B: decreased the risk of CHD events by 5.3% ($p = 0.0008$)

PC = Placebo-controlled; R = randomized; DB = double-blind; ACS = acute coronary syndrome; Hs-CRP = high sensitivity-C reactive protein; MMP-9 = matrix metalloproteinase-9; CHD = coronary heart disease; MI = myocardial infarction; LP(a) = lipoprotein a; ApoB = apolipoprotein B; TXB_2 = thromboxane B_2; $PGF_{1\alpha}$ = prostaglandins $F_{1\alpha}$.

more extensive and complex than those of statins alone. It also makes MRP an ideal candidate for the treatment of the metabolic syndrome (Bianchi, 2005). The most recent studies are described briefly as follows and are summarized in Table 5.

1. Antihypertensive effect

The vasodilatory effects of an aqueous extract of MRP fermented with *M. ruber* IFO 32318 were examined on the isolated rat aorta by Rhyu *et al.* (2000). The results showed that MRP-induced aortic relaxation involved the release of NO from endothelium. It seems that an unknown factor(s), other than acetylcholine (Ach) and GABA, in the aqueous extract of MRP, may stimulate vascular endothelial cells to produce and/or release NO.

Hsieh and Tai (2003) showed that the intragastric loading of fructose-fed rats with *M. purpureus* M9011 containing GABA(1 mg/kg/day) prevented the development of fructose-induced hypertension. Additionally, they tested the reverse effect. After fructose-induced hypertension had been established, intragastric loading of *M. purpureus* M9011 reversed the elevated blood pressure to a normal level. However, administration of pure GABA at the same dose as that contained in *M. purpureus* M9011 failed to prevent or reverse hypertension due to high fructose consumption. Prolonged *M. purpureus* M9011 treatment significantly suppressed the fructose-induced elevation in plasma TC and improved the HDL-C:TC ratio.

2. Antioxidant effect

The antioxidant and hepatoprotective actions of *M. anka* against acetaminophen (AAP)-induced liver toxicity have been investigated (Aniya *et al.*, 1998). Their results show that *M. anka* prevents AAP-induced liver toxicity by both antioxidant action and the inhibition of AAP metabolism. Further antioxidant action of *M. anka* was studied *in vitro* and *in vivo*

TABLE 5 Summary of the biological activities and pharmacological potentials (see text in detail) reported to MRP

1. Cholesterol-lowering effect
2. Antihypertensive effect
3. Antioxidant effect
4. Antihyperglycemic activity
5. Antiproliferate effect
6. Suppression of adipogenesis
7. Antimicrobial activity
8. Macrophage-stimulating activity

(Aniya *et al.*, 1999). Antioxidant activity was evaluated by scavenging stable free radical 1,1-diphenyl-2-picrylhydrazyl (DPPH) and lipid peroxidation of rat liver microsomes. *M. anka* was shown to have the strongest action. When galactosamine (GalN, 400 mg/kg) or GalN plus lipopolysaccharide (LPS, 0.5 μg/kg) was given intraperitoneally to Sprague-Dawley rats, aspartate aminotransferase (AST) and glutathione (GSH) *S*-transferase (GST) activities in serum were significantly increased. Hepatotoxicity marked by an increase in serum enzyme levels was reduced when the extract prepared from *M. anka* was given 1 and 15 hours before the toxic insult. In further studies, Aniya *et al.* (2000) isolated and identified the antioxidant component of *M. anka* as dimerumic acid. When the dimerumic acid (12 mg/kg) was given to mice prior to a carbon tetrachloride (CCl_4, 20 μl/kg, ip) treatment known to elicit liver toxicity in mice, the elevation of serum AST and alanine aminotransferase (ALT) activities was decreased, suggesting a hepatoprotective action of dimerumic acid. The antioxidant mechanism of dimerumic acid is due to one electron donation of the hydroxamic acid group in the dimerumic acid molecule toward oxidants, resulting in formation of nitroxide radical (Taira *et al.*, 2002).

3. Antihyperglycemic activity

MRPs produced by fermentation with *M. pilosus* and *M. purpureus* were used for antihyperglycemic activity screening in streptozotocin-induced diabetic rats (STZ-diabetic rats) (Chang *et al.*, 2006). Single oral administration of MRP decreased plasma glucose in STZ-diabetic rats in a dose-dependent manner from 50 to 350 mg/kg. Moreover, mRNA levels of phosphoenolpyruvate carboxykinase (PEPCK) in liver from STZ-diabetic rats were reversed in a dose-dependent manner by the repeated oral treatment of MRP 3 times daily for 2 weeks. These results suggest that oral administration of MRP could decrease hepatic gluconeogenesis to lower plasma glucose in diabetic rats with insulin deficiency. The hypoglycemic effect of MRP was also studied by another group, Chen and Liu (2006). Oral administration of MRP, fermented with *M. pilosus* and *M. purpureus* for 90 min to fasting Wistar rats resulted in a decrease in plasma glucose in a dose-dependent manner. In parallel to the reduction of plasma glucose, an increase in the plasma level of insulin and C-peptide was also observed. The study also suggests that MRP has an ability to stimulate the release of acetylcholine from nerve synapses, which in turn stimulates muscarinic M(3) receptors in pancreatic cells and augments insulin release to result in a plasma glucose-lowering action.

4. Antiproliferative effect

Using a cell-based cytotoxicity assay, a cytotoxic compound was found in *Monascus* by Su *et al.* (2005). Ankaflavin, but not monascin, was found to be toxic to human cancer cell lines (Hep G2 and A549) with a similar IC50

value of 15 μg/ml, but posed no significant toxicity to normal cells (MRC-5 and WI-38) at the same concentration. From a morphological observation of the chromatin, Su *et al*. proposed that apoptosis should be the possible mechanism. To elucidate the molecular mechanisms responsible for the antiproliferative effect of monacolin K in cancer cells, Lin *et al*. (2006b) have used proteomic analysis by two-dimensional gel electrophoresis, matrix-assisted laser desorption ionization time-of-flight/time-of-flight mass spectrometry (MALDI-TOF/TOF MS), MS/MS, and database interrogation to separate and identify the proteins of Caco-2 cells treated with monacolin K. Their results showed that monacolin K inhibited the proliferation of Caco-2 cells in a dose-dependent manner. The proteins identified in the proteomic analysis included antioxidation enzymes related to reactive oxygen species stress, cytoskeleton proteins, glycolytic enzymes, and enzymes involved in mediating protein interactions.

5. Suppression of adipogenesis

Jeon *et al*. (2004) demonstrated that MRP extracts, which were extracted from embryonic rice fermented with *M. ruber*, significantly decreased glycerol-3-phosphate dehydrogenase (GPDH) activity and lipid accumulation, a marker of adipogenesis, in a dose-dependent manner. Moreover, MRP extracts significantly decreased gene expression of adipocyte fatty acid binding protein (aP2) and leptin, two adipogenic marker proteins and C/EBPα and PPARγ target genes. Their results suggested that the inhibitory effect of MRP extracts on adipocyte differentiation might be mediated through the downregulated expression of adipogenic transcription factors and other specific genes.

6. Antimicrobial activity

MRP has been used as a food preservative from old times. The antibacterial activity of *M. purpureus* was demonstrated by Wong and Bau (1977). The active compound(s) was named monascidin, and is a potent but not a broad-spectrum antimicrobial agent. It is effective against *Bacillus* spp. tested, especially *B. megaterium*. In Japan, several species of *Monascus*, such as *M. paxii* (Matsumoto *et al*., 1989), *Monascus* sp. ATCC 16775 (Araki *et al*., 1998), and *M. pilosus* IFO 4520 (Kono and Himeno, 1999), are also reported to have demonstrated antimicrobial activity. The pigments from the mycelium of *M. purpureus* display significant antimicrobial activity against *B. subtilis* and *Candida pseudotropicalis* (Martinkova *et al*., 1999). The antimicrobial compounds have been identified as rubropunctatin and monascorubrin.

7. Macrophage-stimulating activity

The pigments, monascin and ankaflavin, from the mycelium of *M. purpureus* have immunosuppressive activity on mouse T-splenocytes (Martinkova *et al*., 1999). Yu *et al*. (2005) developed a liquid medium for the production of an

exobiopolymer with macrophage-stimulating activity in a submerged culture of *M. pilosus*. The highest amount of the exobiopolymer (20.1 mg/ml) was obtained on the fourth day of cultivation in the optimal medium having the following composition (g/liter): 20 g of rice bran, 5 g of peptone, and 1 g of KH_2PO_4. The optimal culture pH and temperature for mycelial growth and exobiopolymer production was pH 5.0 and 25 °C, respectively. The exobiopolymer, a crude polysaccharide fraction, mainly contained neutral sugar (81.8%) with a considerable amount of uronic acid (18.2%).

V. SAFETY

Although MRP is now used as a natural colorant and a dietary supplement all over the world, the discovery of citrinin in MRP has led to a controversy about the safety. Citrinin is a fungal metabolite known since 1931, when it was isolated from *P. citrinum* by Hetherington and Raistrick (1931). Citrinin has been associated with yellow rice disease in Japan (Saito *et al*., 1971). It has also been implicated as a contributor to porcine nephropathy. Citrinin acts as a nephrotoxin in all animal species tested, but its acute toxicity varies in different species (Carlton and Tuite, 1977). Citrinin was characterized as an antibacterial compound (Betina, 1984). Citrinin was tested for activities against bacteriophages, sarcomas, protozoa, animal cells, and plant cells (Betina, 1984). Citrinin was identified in over a dozen species of *Penicillium* (e.g., *P. camemberti*), several species of *Aspergillus* (e.g., *A. terreus*, *A. niveus*, and *A. oryzae*), and *Monascus* spp. (Bennett and Klich, 2003). Blanc *et al*. (1995) characterized the antimicrobial compound, monascidin A, from *Monascus* as citrinin using qualitative methods, mass spectra, and NMR from *M. purpureus* and *M. ruber*. With an acidic character, citrinin is practically insoluble in water. It is soluble in hot alcohol, dioxane, and other nonpolar solvents. Due to its conjugated double bonds, citrinin absorbs light in the visible wavelength range. Its color varies from lemon yellow at pH 4.6 to cherry red at pH 9.9. Its absorption maxima are in the UV range: 250–331 nm. It has a melting point of 175 °C and molecular mass 250.25 g/mol. The 50% lethal dose (LC_{50}) is 57 mg/kg (body weight) for ducks, 95 mg/kg for chickens, and 134 mg/kg for rabbits (Hanika and Carlton, 1994). Citrinin can act synergistically with ochratoxin A to suppress RNA synthesis in murine kidney (Sansing *et al*., 1976). The cytotoxic effects of citrinin have been extensively studied by Liu *et al*. (2005). Using human embryonic kidney cells (HEK293) as a cellular model, the concentrations causing 50% cell death by the lipophilic extracts of *Monascus* were in the range of 1.8–4.7 mg/ml. The aqueous extract showed a lower cytotoxicity. Incubation of HEK293 cells with 60-μM pure citrinin for 72 hours caused cell viability to fall to 50% of control levels. In addition, coadministration of pure citrinin and

the lipid extracts from *Monascus* samples significantly enhanced citrinin cytotoxicity for HEK293 cells using the MTT assay.

For analysis of citrinin in *Monascus*, several methods were developed including thin layer chromatography (TLC), RP-HPLC, and immunoassays (Chu, 1991). A highly sensitive determination (~1 ppb) of citrinin in *Monascus* by GC-selected ion-monitoring mass spectrometry was developed by Shu and Lin (2002). Pisareva *et al.* (2005) chose 16 *Monascus* strains to monitor the biosynthesis of citrinin and pigments quantitatively. The results showed that the formation of citrinin appeared to be strain-specific and did not correlate with the pigment formation. In China, researchers from the Institute for Nutrition and Food Safety, Chinese Center for Disease Control and Prevention, have also screened 35 *Monascus* strains used in the food industry to investigate the effect of cultivation conditions and the medium composition on citrinin production (Li *et al.*, 2003). The results indicated that all strains produced citrinin during fermentation on rice with the levels ranging from 0.28 to 2458.80 mg/kg (201.60 mg/kg for the average and 61.99 mg/kg for the median, respectively). The citrinin level resulting from fermentation on rice was higher than in a liquid medium. The survey reported by Li *et al.* (2005a) showed that 68 of 114 (59.65%) samples from either solid or liquid phases and collected from either markets or food processing facilities were positive for citrinin with the levels between 0.18 and 1739.23 mg/kg. In Taiwan, HPLC was used to analyze citrinin levels in commercialized MRPs including capsule, colorant, and daily MRP food products (Hsieh and Pan, 2002). The results showed that the amount of citrinin ranges from undetectable to 122.09 mg/kg in the MRPs. However, citrinin is very low or undetectable in the daily food products with *Monascus* additives, such as bread, salad, *Monascus* sauce, fermented glutinous rice, Chinese cheese, and wines. Liu *et al.* (2005) showed that citrinin was detected in lipid extracts of all examined commercialized *Monascus* products at concentrations ranging between 0.28 and 6.29 mg/kg, but was not found in aqueous extracts. Thus, domestic MRPs may be contaminated by citrinin, which would result in consumer exposure to this mycotoxin.

Since citrinin is a mycotoxin and possesses nephrotoxic and hepatotoxic effects, it has a negative impact on the acceptance of red mold rice by consumers. Studies on MRP with a high concentration of monacolin K and a low concentration of citrinin have been conducted by several laboratories (Chen and Hu, 2005; Wang *et al.*, 2004). Since citrinin possesses antibacterial activity for *B. subtilis*, a simple and quick selection method for mutant strains with low citrinin production was developed based on the formation of an inhibition zone around the colony of the *Monascus* strain (Wang *et al.*, 2004). Their results showed that mutant strain, *M. purpureus* N 301, only produced 0.23 mg/kg citrinin, which was 50% less than that of the parent strain. Chen and Hu (2005) obtained

a mutant strain of *M. pilosus* by treating a wild strain collected in China with mutagenic agents. At the optimum conditions, the concentrations of monacolin K and citrinin were 2.52 mg/g and 0.13 ng/g, respectively. In exploring the effects of a nanoparticulate dispersion of MRP after wet-milling technology treatment, Yu *et al*. (2006) have shown that monacolin K was reduced to 50–92% of its base level and citrinin was reduced to 48–74% of its base level. Further experimentation will be needed to evaluate the safety and verify the functionality of this nanoparticulate dispersion.

The polyketide pathway is a major route for the formation of secondary metabolites including various mycotoxins (including citrinin) in filamentous fungi (e.g., *Monascus*) (Chandle *et al*., 1992). The incorporation of ^{13}C isotope into citrinin from *M. ruber* incubated with ^{13}C acetate revealed that citrinin is biogenetically originated from a tetraketide, instead of a pentaketide, as has been shown for *Penicillium* and *Aspergillus* spp. The production of polyketide red pigments and citrinin by *M. ruber* may therefore be regulated at the level of the tetraketide branch point (Hajjaj *et al*., 1999a). Hajjaj *et al*. (2000) investigated the effects of medium- and long-chain fatty acids on pigment and citrinin production. Their results show that the synthesis of pigments is barely affected whereas the production of citrinin is strongly inhibited, likely by a hydrogen peroxide-mediated degradation of the toxin due to fatty acid-induced peroxisome proliferation.

A full-length polyketide synthase (PKS) gene (*pksCT*) of 7838 bp from *M. purpureus* has been cloned. It encodes a 2593-amino acid protein that contains putative domains for ketosynthase, acyltransferase, acyl carrier protein (ACP), and a rare methyltransferase (Shimizu *et al*., 2005). Using a truncated disruption construct resulted in a *pksCT*-disrupted strain of *M. purpureus*. Shimizu *et al*. (2005) have shown that the disruptant does not produce citrinin, but a *pksCT* revertant generated by successive endogenous recombination events in the *pksCT* disruptant restores citrinin production. These observations indicate that *pksCT* encoding the PKS is responsible for citrinin biosynthesis in *M. purpureus*. Subsequently, these investigators used the gateway system to facilitate the introduction of 7.8 kbp DNA fragments into *M. purpureus* (Shimizu *et al*., 2006). The transformants showed 1.5-fold higher production of citrinin than the wild-type strain.

Wild *et al*. (2003) detected two compounds with identical UV absorption at 306–307 nm wavelength. They were purified by HPLC and structurally elucidated by mass spectrometry and NMR spectroscopy. Among them, monascopyridine A contains a γ-lactone, propenyl group, hexanoyl side chain, and a pyridine ring. The more lipophilic monascopyridine B is a higher homologue of monascopyridine A with a more lipophilic octanoyl instead of a hexanoyl side chain. The toxicological properties

of monascopyridine A and B, which were the dehydrogenated derivatives of the corresponding red pigments rubropunctamine and monascorubramine, were studied using immortalized human kidney epithelial cells (Knecht and Humpf, 2006; Knecht *et al*., 2006). The results show that these two compounds are cytotoxic in the micromolar range with median effective concentration values between 20.7 and 43.2 μM. These effects indicate an aneuploidic potential and that monascopyridines may contribute to tumor formation.

Several cases of anaphylaxis or asthma may have been caused by MRP (Hipler *et al*., 2000; Vandenplas *et al*., 2000; Wigger-Alberti *et al*., 1999). Hipler *et al*. (2002) reported the first case of *M. purpureus* as a possible allergic agent by means of a prick-to-prick test, ''cellular antigen stimulation test'' (CAST), and different immunoblots. In their study, a 26-year-old butcher experienced a severe anaphylactic reaction with sneezing, rhinitis, conjunctivitis, generalized pruritus, followed by widespread urticaria, Quincke's edema, and dyspnea after starting to prepare sausages containing MRP.

ACKNOWLEDGMENTS

Research in our laboratory was supported by Taiwan Tobacco & Liquor Corp., Taipei, Taiwan, Republic of China. The animal study of the product, Unchole, was performed under the supervision of Professor Ming-Shi Shiao, Department of Life Science, Chang Gung University, Taiwan. We also thank Professor. Ming-Shi Shiao for reviewing the chapter.

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