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Review

Factors and advances on fermentation of *Monascus* sp. for pigments and monacolin K production: a review

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Abstract

A monascal product or angkak is a kind of fermented product obtained from the fermentation of polished rice (*Oryza sativa*). or other kind of high carbohydrate raw materials with *Monascus* sp. It has been utilised as a natural food colorant and a folk medicine in many countries for a long time. Monascal rice is well known for lowering blood cholesterol levels and antioxidant activities because of its significantly bioactive components, e.g. monacolins, phenolic compounds, γ-aminobutyric acid and dimerumic acid. This review mainly focuses on the biosynthesis pathway of pigments and monacolin K, and the factors affecting *Monascus* fermentation on *Monascus* pigments and monacolin K. The improvement of solid-state fermentation (SSF) and submerged fermentation (SF) of *Monascus* sp. using two-step fermentation for pigments and monacolin K production and antioxidant activities of *Monascus*-pigments is also discussed.

Introduction

Monascus pigment from monascal rice has been used as a colouring agent in foodstuffs, textile industries, pharmacy, medicine and cosmetics as well as used as a folk medicine to improve food digestion, blood circulation and lowering blood cholesterol levels. Monascal rice is also known as red koji, Hung-Chu, Hong Qu, Ang-kak, Ankak rice, red mold rice and Beni-Koji. *Monascus* pigment not only has natural food colourant but also different antioxidant potentials, i.e., abilities to donate a hydrogen atom and/or an electron, to chelate redoxactive metals and to inhibit lipoxygenases (Ramarathnam *et al*., 1995; Hadjipavlou-Litina *et al*., 2010). Furthermore, Yang *et al*. (2006) and Kraboun *et al*. (2013) reported that the *Monascus* pigment extract had great antioxidant activities such as inhibition of peroxidation, reducing power, scavenging ability on DPPH radicals and chelating ability on Fe^{2+}. Moreover, there were some studies concerning monascal products, i.e. monascal adlay, having high antioxidant activities (Tseng *et al*., 2006; Yang *et al*., 2006). The substances having antioxidant capabilities obtained from the *Monascus* fermentation are monacolin K, γ-aminobutyric acid (GABA), dimerumic acid and flavonoids (Kongbangkerd *et al*., 2014). Whereas, citrinin, a mycotoxin, is also formed during *Monascus* fermentation, and is harmful to humans and animals (Dufosse *et al*., 2005). Monacolin K is commercially called Lovastatin, Mevinolin or Mevacor. It has been demonstrated as a specific inhibitor on 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase in cholesterol biosynthesis through which it helps to decrease blood pressure. The reductase enzyme is an important key in cholesterol synthesis, which produces mevalonyl-CoA (Hajjaj *et al*., 2001). Lovastatin, Mevinolin and Mevacor, as statin drugs, used in the United States have the same properties of cholesterol synthesis inhibition and the structures as monacolin K (Wang *et al*., 1997).

This review thus summarises all the available and recent publications of the pigments and monacolin K synthesis pathway. The factors affecting *Monascus* pigments and monacolin K. and the improvement of conventional fermentation (batch fermentation) of *Monascus* fermentation using two-step fermentation (modified batch fermentation or novel batch fermentation) are also discussed.

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Biosynthetic pathways of pigments and monacolin K from Monascus sp.

Monascus sp. can produce various enzymes which hydrolyse the starchy substrates during fermentation, for example, amylase, protease, glucoamylase, maltase, pectinase, α-galactosidase and ribonuclease (Lin and Demain, 1991). During *Monascus* fermentation, these enzymes lead to secondary metabolites' biosynthesis. Each production step of *Monascus* secondary metabolites is described as the following: (1) the primary metabolic products, e.g. reducing sugars, succinic acid, citric acid, gluconic acid, oxalic acid, ethanol and ester compounds are produced during the lag and the log phases of *Monascus* growth (Kao, 2004); (2) the secondary metabolic products, the complexes formed between significant antioxidants and *Monascus* pigments are produced after the primary metabolites. Pigments, citrinin and monacolin K are produced in the initial period of the stationary phase of the fermentation (Yongsmith, 1999; Kao, 2004).

Monascus pigment synthesis

The six main pigments produced by *Monascus* sp., especially *M. purpureus*, *M. ruber* and *M. pilosus*, are monascin and ankaflavin (yellow), rubropunctatin and monascorubrin (orange) and rubropunctamine and monascorubramine (red). The structures of these compounds (Figure 1), together with a probable biosynthesis of orange and red pigments by a reaction with amino group-containing compounds, are well documented (Lin and Demain, 1993; Jůzlová *et al*., 1996). The pigments are both polyketides and azaphilones, i.e., compounds with an oxygenated bicyclic nucleus and a quaternary centre (Patakova, 2013).

The formation mechanism of yellow pigments (Figure 1) such as monascin, ankaflavin, monascin C, monascidin A (citrinin), yellow II and xanthomonascin A, adjusted the structure with the chemical oxidation of monascorubrin and rubropunctatin (as orange pigments) (Yongsmith, 1999). In fact, the structures of yellow pigments (Figure 1) are obtained from not only the reductive derivatives of the orange pigments but also the biosynthetic pathways of the orange pigments (Yongsmith *et al*., 1994). Previous study showed the formation of mutant yellow pigments. Cheng *et al*. (2010) stated that the extracts of angkak fermented by *M. pilosus* contained the yellow mutant pigments, e.g., monascuspyrone, 6-(2-hydroxydodecan-2-yl)-3-(hydroxyl methyl)-4-methoxy-2H-pyran-2-one, monascin, ankaflavin, monasfluore B, 3-epi-betulinic acid, 3-epibetulinic acid acetate, α-tocospiro B, methyl isovanillate, p-dihydro coumaric acid and methylparaben as well as novel pyran-2-one derivative.

The orange pigments, monascorubrin and rubropunctatin (Figure 1), are obtained from a combination of polyketide and fatty acids which were confirmed by the experiments with an addition of radioactively labelled octanoic acid to the culture medium (Hajjaj *et al*., 2000). The pigments are synthesised in the cytosol (cytoplasmic matrix) and obtained from acetyl coenzyme A by the multienzyme complexes of polyketide synthase I (Hopwood and Sherman, 1990). They are sensitive to heat, unstable at pH <2 or >10 and fade with exposure to light. The other four compounds of orange azaphylones such as monaphilol A-D (Figure 2) could inhibit human laryngeal carcinoma and human colon adenocarcinoma, and have antioxidant activities (Ramarathnam *et al*., 1995).

Red pigments (monascorubramine and rubropunctamine) (Figure 1) are produced both extra- and intracellularly. The orange pigments react with amino acids, amino polysaccharides and amino alcohols, and are converted to a deep red color. The intensity of red pigments depends on factors such as the contents of carbon and nitrogen sources, pH, strains, and moisture contents. Red pigments are highly stable against pH, light and high temperature over 70°C. However, red pigments are converted to a blackish color at 100°C for 15 min. Nevertheless, they have high alcohol solubility and good stability in Ca, Mg, Fe, Cu and other metal ions. These *Monascus* pigments are approved as safe (non-toxic to humans and other mammals). Based on the previous literatures reviewed herein, a commercial dietary product (monacolin by Maruzen Pharmaceuticals) containing *Monascus* extract has been introduced in Japan. The Chinese Food and Drug Administration also confirmed that applications of *Monascus* metabolites was not toxic and safe for consumption (Shi and Pan, 2011; Mostafa and Abbady, 2014).

The pigments are well known to have low water solubility. To enhance water solubility of *Monascus* pigments, nitrogen sources are used. The oxygen atom in monascorubrin or rubropunctatin will be replaced by nitrogen atom of amino group (amino acid, protein and peptide), indicated by the changes from orange to purple-red. These pigments react with the reducing and oxidising agents as well as other substances, especially amino acid. The complexes formed from the reaction between the pigments and amino acids are glutamyl-monascorubine and glutamyl-rubropunctatine (Figure 3). The complex forms could be further isolated from the submerged fermentation (Dufosse *et al*., 2005).

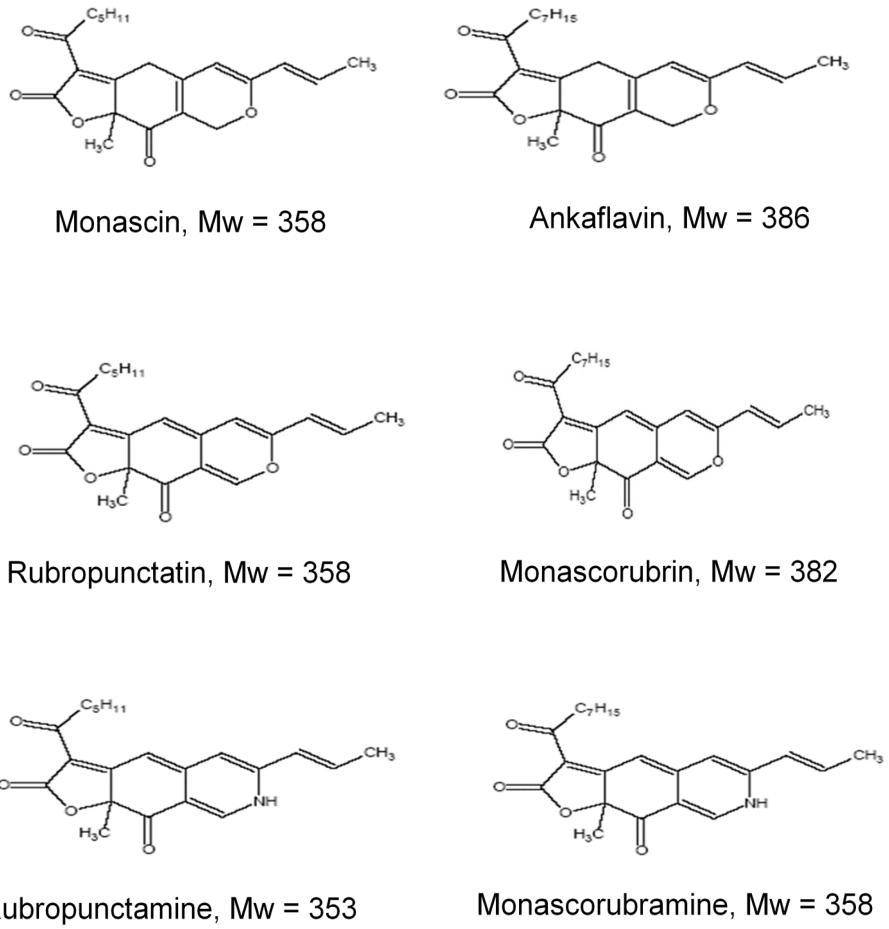

Figure 1. The structures of Monascus pigments.

Figure 2. The structures of monaphilol B-D

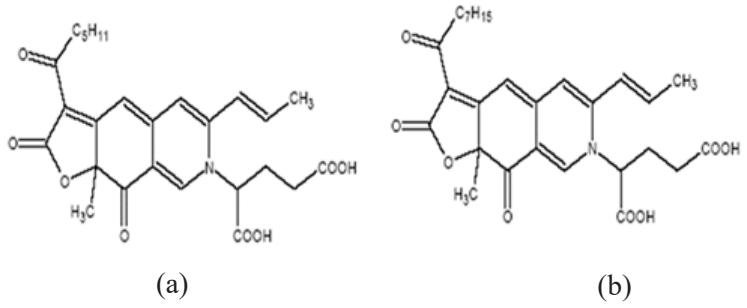

Figure 3. The structures of (a) glutamyl-rubropunctatine (mw = 483) and (b) glutamyl-monascorubine (mw = 511).

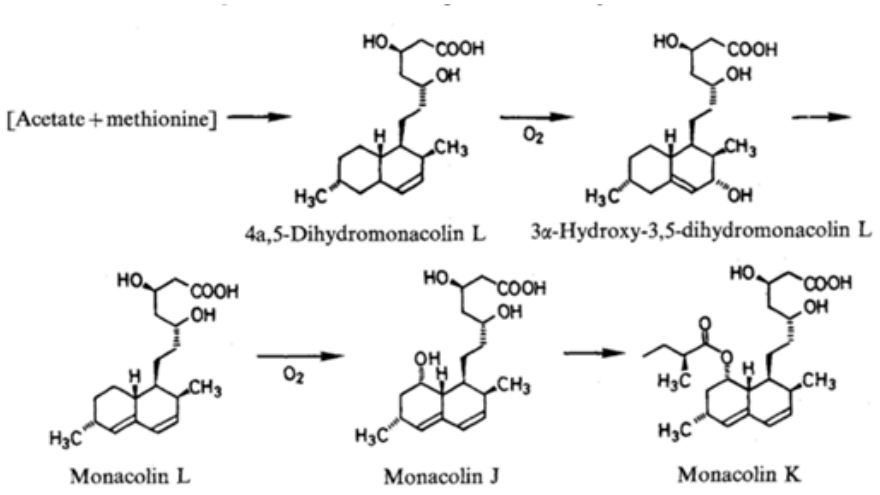

Figure 4. The biosynthesis pathway of monacolin K

Monacolin K synthesis: its antioxidant activities and health effect

The polyketide monacolin K is not only produced by the members of the genus *Monascus* but also by other filamentous fungi including *Aspergillus terreus*, some species of *Penicillium*, *Hypomyces, Doratomyces, Phoma, Eupenicillium, Gymnoascus, Trichoderma* and *Pleurotus ostreatus*. Monacolin K is [(1S,3R,7R,8aS)-8-[2-[(2R,4R)-4-hydroxy-6-oxooxan-2-yl]ethyl]-3,7-dimethyl-1,2,3,7,8,8a-hexahydronaphthalen-1-yl](2S)-2methylbutanoate (IUPAC name)]. The empirical formula and molecular weight of monacolin K is C24H36O5 and 404.55 $g.mole^{-1}$, respectively. A 2-D structure of monacolin K is shown in Figure 4. The biosynthesis begins with the conversion of 4a,5-dihydromonacolin L into 3a-hydroxy-3,5- dihydromonacolin L and monacolin L, and hydroxylation of monacolin L to monacolin J which ultimately transformed to monacolin K. Monacolin K is a hypocholesteremic agent (Chen and Johns, 1994).

Monacolin K is a mixture of a lactone and a free hydroxy acid. The lactone form of monacolin K is more lipophilic than the hydroxy acid form. However, the lactone form could be converted in vivo into the hydroxy acid form (Chen and Johns, 1994). The ratio of acid form to lactone form depends on the *Monascus* strains, pH, culture media, temperatures and initial moisture contents. Monacolin K is an effective inhibitor for an HMG-CoA (3-hydroxy-3-methylglutaryl-coenzyme A) reductase. Monacolin K significantly helps lowering the total cholesterol (TC) levels and triglyceride (TG) levels in liver and serum since high cholesterol directly affects high blood pressure (hypertension). When the arteries become hardened and narrowed with cholesterol plaque, the heart must strain much harder to pump blood through them. As a result, blood pressure becomes abnormally high. The treatment with monacolin K can reduce low-density lipoprotein cholesterol (LDL-C) levels, but high-density lipoprotein cholesterol (HDL-C) levels are also increased (Lee *et al*., 2010). Moreover, the mechanism of action of monacolin K reducing blood pressure is against the pathway belonging to synthesising cholesterol of the liver (Figure 5) so that blood pressure levels will subsequently decrease. Saito *et al*. (1991) further reported that monacolin K had hypocholesterolaemic, liver-protective and antitumor effects.

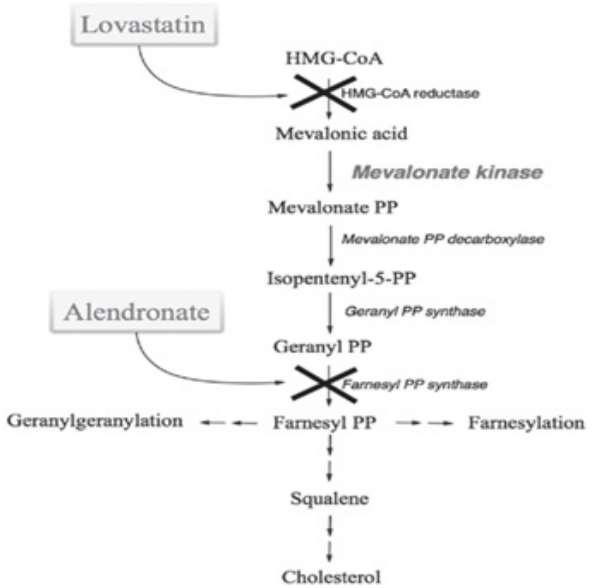

Figure 5. The mechanism of action of monacolin K to block cholesterol production pathway.

Presently, monascal rice is developed as a kind of hypolipidemic functional food based on in vivo experimental data. Monascal rice lowered the blood cholesterol and triglyceride in animal tests and clinical studies (Wang *et al*., 2000; Wei

et al., 2003; Cicero *et al*., 2005; Lin *et al*., 2005; Setnikar *et al*., 2005; Lee *et al*., 2006). In addition, monacolin K effectively exhibits antioxidative ability and against hyperlipidemia-induced oxidative stress (Lee and Pan, 2012). Monacolin K exhibited antioxidant activities through several assays such as the inhibiting ability on the peroxidation of linoleic acid, the reducing power, the scavenging ability on 1,1-diphenyl-2-picrylhydrazyl radicals (DPPH), the scavenging ability on OH- and the chelating ability on Fe^{2+} (Lee *et al*., 2008). Monacolin K abundantly occurs in *Monascus* pigment extract, and the phenolic compound, low-molecular-weight viscous substances (mw <100,000) also occurs (Lee *et al*., 2008). It is possible that phenolic tetrahydro-β-carboline alkaloids from the complexation between monacolin K and phenolic aldehydes might cause anti-radical activities (Lee *et al*., 2008). Some of the substances such as cysteine, histidine, mannitol and adenosine monophosphate (AMP) occurred in the substrates, such as soybean used in *Monascus* fermentation, helped to promote better efficiency of monacolin K for scavenging abilities on OH^- (Halliwell *et al*., 1987). With regard to antioxidant activity by DPPH assay, the scavenging ability on DPPH radical of the *Monascus* extracts at 0.1 $mg.mL^{-1}$ was in the range of 76 – 94% which was almost equivalent to the efficiency of butylated hydroxytoluene (BHA) and α-tocopherol (approximately 93% at 0.1 $mg.mL^{-1}$) (Lee *et al*., 2008).

The factors affecting the monacolin K destruction include heating, light and O_2 (Kraboun *et al*., 2018). Kraboun *et al*. (2018) further reported that pancreatic enzymes digestion was a significant cause to reduce the effectiveness of antioxidant activities of monacolin K. In *in vitro* digestion process of duodenum, a proportion of the compounds might be transformed into different structural forms and chemical properties. Moreover, in this digestive simulation, light and O_2 are two important factors that can alter the changes in structures and properties of monacolin K resulting in its denaturation with oxidative degradation and polymerisation reactions (Talcott and Howard, 1999; Rufián-Henares and Delgado-Andrade, 2009; Kraboun *et al*., 2018).

Factors affecting solid state fermentation for monacolin K and pigments

Strain

Monascus purpureus is one of the popular strains, which is used in many experiments because of a high production of the extracellular pigments and the antioxidants (Wang *et al*., 1997; Yang *et al*., 2004; Babitha *et al*., 2006; Yang *et al*., 2006; Babitha *et al*., 2007; Kraboun *et al*., 2013). The intensity of *Monascus* pigments is generally detected by a spectrophotometer at two absorption bands; 420 and 500 nm. In fact, the pigments released from different strains of *Monascus* lead to different maximum absorption of wavelengths (λ_{max}). For example, the maximal wavelengths of pigments produced by *M. purpureus* or *M. anka* were at 500 and 420 nm, while *M. barkeri* or *M. kaoliang* could produce the pigments absorbed at the maximal wavelengths of 500 and 370 nm. This might be caused by the number conjugated bonds (molecular structures) affecting the absorption band and the colour of the pigments (Yongsmith, 1999).

Different strains also influence the monacolin K synthesis. For example, *M. purpureus* produced higher monacolin K contents (25.03 $mg.kg^{-1}$ dry weight) than *M. ruber* (15.33 $mg.kg^{-1}$ dry weight) (Pattanagul *et al*., 2008), while the monacolin K content obtained from *M. kaoliang* KB9 (a wild type strain) was 13,536.61 $mg.kg^{-1}$ dry weight (Subsaendee *et al*., 2014).

Substrate

Solid-state fermentation is usually used as the culture mode for *Monascus* production. However, the selection of solid substrate is important because its nutrients, moisture and physical character would influence the growth and secondary metabolites production of *Monascus*. Several raw materials have been used as substrates for *Monascus* fermentation. It is believed that white rice as a nutrient-rich substrate (such as carbohydrate, protein and minerals) has been the most popular for monascal rice production (Yang *et al*., 2006; Subsaendee *et al*., 2014). Previous researches indicated that *Monascus* preferred glutinous rice to non-glutinous rice since the pigment intensity of monascal glutinous rice (45.04 $AU.g^{-1}$ substrate of pigment intensities at OD500) was ten times higher than monascal non-glutinous rice (4.51 $AU.g^{-1}$ substrate) (Chairote *et al*., 2008). The difference of amylose or amylopectin content affected monacolin K and derived compounds such as compactin (Chairote *et al*., 2008; Kongbangkerd *et al*., 2014). However, other raw materials have also been used as the substrates for *Monascus* pigments and antioxidants fermentation such as waxy corn, corn, wheat, soy and cassava, containing similar contents of protein, lipid, ash and phosphorus (Kongbangkerd *et al*., 2014). Kraboun *et al*. (2013) reported that the extract of monascal waxy rice contained 16.83 $mg.kg^{-1}$ dry weight of monacolin K and 0.02 mmol equivalent $trolox.mL^{-1}$ of IC_{50} (DPPH

assay). Pyo and Seong (2009) described that the pigment intensity at 500 nm of monascal corn extract was 107 unit.g^{-1} dry weight, which was less than that of monascal waxy corn (450 unit.g^{-1} dry weight) (Kraboun *et al*., 2013). Soybean was utilised as a substrate for *Monascus* fermentation in the previous study. Monascal soybean extracts consisted of high contents of isoflavone aglycones (1,515.1 ± 59.2 μg.g^{-1} sample) and monacolin K (3,635.6 ± 68.4 μg.g^{-1} sample) when compared with unfermented soybean extracts (391.6 ± 18.2 μg.g^{-1} sample of isoflavone aglycones and monacolin K not detected). This product was evaluated on the hypolipidemic effect through oral administration. It was found that doses between 200 and 400 mg.kg^{-1} body weight could significantly reduce the TC, TG, and LDL-C levels as well as increase the HDL-C levels in hyperlipidemic rats. It was further observed that monascal soybean extracts group indicated significantly lower HMG-CoA reductase activity than the unfermented soybean extracts group (Pyo and Seong, 2009).

Nitrogen source

Nitrogen source is an important supplement for *Monascus* fermentation, which consists of both inorganic and organic substances.

Inorganic nitrogen

Sodium nitrate ($NaNO_3$) can stimulate sporulation and high pigment yield of *Monascus*, but it constrains the growth. Ammonium chloride (NH_4Cl) suppresses the conidial germination and the sexual cycle of *Monascus*, but it results in high *Monascus* pigment yield during stationary phase (Chen and Johns, 1994). However, organic nitrogen sources are increasingly being used instead since they help to promote higher pigmentation and yield of *Monascus* than the inorganic ones. Chen and Johns (1994) reported that organic nitrogen peptone yielded superior *Monascus* growth and pigment when compared with sodium nitrate ($NaNO_3$).

Organic nitrogen

Organic nitrogen have also been used as supplements, i.e., monosodium glutamate (MSG), peptone and yeast extract, in monascal rice production. In a previous study, yeast extract increased the yield of pigments of monascal broken rice fermentation when compared with MSG and inorganic sources (Subsaendee *et al*., 2014). However, Dufosse *et al*. (2005) reported that MSG promoted the pigment production of *Monascus* in submerged culture. Lin and Demain (1991) confirmed that MSG was the most favourable nitrogen source for pigment production of *Monascus* when compared with other organic and inorganic sources. Vidyalakshmi *et al*. (2009) further reported that both red (rubropunctamine and monascorubramine) and yellow (monascin and ankaflavin) pigments from *Monascus* fermentation supplemented with 0.5% of MSG increased to 0.464 and 1.314 U.g^{-1}, respectively, when compared with those supplemented with peptone or yeast extract or without supplemented with nitrogen source (control) showing 0.202 U.g^{-1} of red pigment and 0.330 U.g^{-1} of yellow pigment. However, the pigmentation of *M. purpureus* medium fortified with 20 – 22.5 g.L^{-1} of peptone with independent MSG levels was in the optimal region for pigment production (Silveira *et al*., 2008). Monacolin K contents during *Monascus* fermentation are also achieved when using a lot of amino acid occurring in the nitrogen sources used. For example, soybean milk (as a source of high amino acid contents) increased the productivity of monacolin K from monascal glutinous rice (Chairote *et al*., 2008).

Carbon source

Previous studies demonstrated that various carbons affected the production of secondary metabolites. Sucrose led to sporulation and cell mass inhibition. *Monascus* sporulation was not induced by glucose alone. Using a combination of glucose and sucrose significantly enhanced sporulation and cell mass production of *Monascus* (Ajdari *et al*., 2011). In the production of monacolin K, Miyake *et al*. (2006) noted that *M. pilosus* required a proper ratio of peptone to maltose for high monacolin K production (Miyake *et al*., 2006).

pH

pH in the range of 2.5 – 10.0 is appropriate for *Monascus* pigment production (Wang *et al*., 2004). Nevertheless, various structures of *Monascus* pigments would be formed in different pH's of culture (Wang *et al*., 2004). Chen and Johns (1994) noted that the pH 2.5 of *Monascus* substrate would increase the formation of yellow and orange pigments by *Monascus* (Aniya *et al*., 1999; Babitha *et al*., 2007). However, the appropriate condition to produce monacolin K and GABA is the initial pH of 6-7 (Su *et al*., 2003). While, the low pH values of *Monascus* substrate result in not only high monacolin K level but also citrinin level (Lee *et al*., 2007). This is in agreement with Lee *et al*. (2007) who confirmed that *M. purpureus* stopped producing at 6.8 mg.g^{-1} of monacolin K contents when pH >5.0. Therefore, acidic pH of *Monascus* culture affects the production of hypolipidemic ingredients due to the alteration of *Monascus* metabolite formation.

Temperature

Temperature is an important factor that directly affects the acceleration for the activities of glucoamylase and α-amylase involved in the hydrolysis of *Monascus* substrate (Babitha *et al*., 2007). The optimal temperatures for *Monascus*-pigment production are between 27 and 37°C. To obtain high growth and high glucoamylase production, the temperature should be in the range of 35-37°C (Yongsmith, 1999). However, *Monascus* sp. prefers the temperature between 30 and 40°C for the pigmentation. Beyond 40°C, the pigmentation decreases (Babitha *et al*., 2007). Whereas, the optimal temperature for *Monascus* cultivation to produce monacolin K is between 23 and 30°C due to increasing about 20 times of monacolin K production with low citrinin production (Tsukahara *et al*., 2009).

Moisture content

Moisture content is a factor significantly influencing on *Monascus* substrate. The unsuitable moisture content of the substrates (>50%) impacts on lower pigment intensity and monacolin K production due to a reduction in oxygen transfer, heat exchange and low ventilation which leads to CO_2 accumulation, which is an index implying an improper condition for the formation of secondary metabolites (Kongbangkerd *et al*., 2014). Commonly, the optimal initial moisture of *Monascus* substrate to obtain the highest pigment intensity is in the range of 30-50% (Kongbangkerd *et al*., 2014). Kraboun *et al*. (2008) reported that the moisture content of broken rice substrate should not exceed over 30% for high pigment intensity. In monacolin K production, the initial water contents of *Monascus* substrate should be between 60 and 80 mL.100 g^{-1} while the citrinin formation also increased (Lee *et al*., 2007).

Solid state fermentation (SSF) and the correlation among secondary metabolites from Monascus sp.

Solid substrate fermentation (SSF) or solid substrate cultivation is a when microorganisms are cultured on a concentrated water-soluble substrate (usually containing polysaccharides as a carbon and energy source) with low-level moisture (Inui *et al*., 2010). For example, in China, SSF has been extensively used to produce fermented foods such as Chinese wine, soy sauce and vinegar since ancient time (Chen and Johns, 1994). In Japan, SSF has been commercially used to produce the industrial enzymes (Suryanarayan, 2003). The advantages of SSF are the following: (1) simple technique; (2) the use of a concentrated medium resulting in a small reactor volume and low capital investment costs; (3) high productivity because of the low risk of contamination with yeasts and bacteria due to low moisture levels; (4) easier product recovery; (5) good oxygen circulation and low energy consumption (Babitha *et al*., 2007). Therefore, SSF is a popular fermentation method for *Monascus* fermentation to produce food colorant, food flavor and monacolin K for a very long time. SSF has been used to study the optimisation of pigmentation and/or the antioxidant activities of monascal rice (Yang *et al*., 2004; 2006; Kongbangkerd *et al*., 2014). However, the limitation of SSF in *Monascus* fermentation is the residual reducing sugars about 2,000 $mg.kg^{-1}$ substrates in monascal products, especially glucose, at the end of the fermentation (Babitha *et al*., 2007). Kongbangkerd *et al*. (2014) stated that the reducing sugar contents remained at 8.00 $mg.g^{-1}$ dry weight after fermentation. Accumulation of residual reducing sugars such as glucose is a cause of glucoamylase repression during *Monascus* fermentation. A number of glucose contents would inhibit the transformation of acetyl CoA, acetyl-coenzyme A (CoA) and malonyl-CoA units, catalysed by polyketide synthase type I (Schümann and Herweck, 2006), in glycolysis pathways for the production of pigments and monacolin K, which these substances could not enter to the TCA cycle as well as polyketide pathway (Yu, 2006; Subsaendee *et al*., 2014). To understand the correlation of each secondary metabolite production, Pearson product-moment correlation coefficient has been used in many researches of *Monascus* fermentation to assess monacolin K production, pigment intensities and antioxidant activities (DPPH and chelating ability on Fe^{2+}). There are many researches of *Monascus* fermentation indicating that the pigmentation and antioxidants are simultaneously formed during fermentation. Kraboun *et al*. (2013), Kongbangkerd *et al*. (2014) and Pengnoi (2017) confirmed that monacolin K, phenolic compounds, vitamin E and pigments produced from *M. purpureus* were simultaneously produced during the stationary phase of *Monascus* growth. Kraboun *et al*. (2013) indicated that the Pearson's correlation coefficients among the secondary metabolites from monascal waxy corn showed good negative correlations appearing not only between pigment intensity and IC_{50} DPPH value but also pigment intensity and IC_{50} chelating ability on Fe^{2+}. A good positive correlation was further found between pigment intensity and monacolin K content. Hence, pigment intensity can be a good index for antioxidant activities of monascal products.

Submerged fermentation (SF) and its application with Monascus sp.

Submerged fermentation (SF) or submerged cultivation is a system where microbial cells are cultivated in bioreactors with controlled environment in order to establish efficient production with high-quality metabolites and to finish optimum productivity and yield. In food industries and food biotechnology industries, the bioreactors operating in batch, fed batch, or continuous mode are utilised to cultivate different types of microorganisms producing a wide range of products. Nevertheless, this review focuses on the batch mode of fermentation as it is easy to apply and popular in *Monascus* fermentation (Inui *et al*., 2010). In SF, *Monascus* produces low monacolin K contents and pigment intensities. However, the purified monacolin K and pigments obtained from SF are higher than those from SSF (Hajjaj *et al*., 1999; Dufosse *et al*., 2005). The high pigment production is successful using glucose and maltose. The nitrogen sources seem to have more significant effect than the carbon sources. Peptone as a nitrogen source gives high growth and pigment when compared with other nitrogen sources. The advantages of SF are summarised that the secondary metabolites extraction is not necessary as they can be utilised immediately. However, SF is difficult for application in the edible pigments industries due to easy cross contamination with other microorganisms (Hamdi *et al*., 1997).

Improvement of Monascus fermentation using two-step fermentation

Two-step fermentation has been designed to enhance the effectiveness of the conventional fermentation of SSF and SF, and used in few researches (Jin *et al*., 2008; Hongzhang *et al*., 2012). Two-step fermentation has several different meanings: (1) different kinds of microorganisms are used both in step 1 and 2; (2) both steps of the fermentation are adjusted differently (Jin *et al*., 2008; Hongzhang *et al*., 2012). The design of two-step fermentation depends on the kind of substrates or the appropriate conditions. Therefore, two-step fermentation is defined as the designed experiment and the appropriate condition to obtain high secondary metabolite content (Kongbangkerd *et al*., 2014). Kongbangkerd *et al*. (2014) described that monascal waxy corn from a two-step fermentation enhanced antioxidant activities. In two-step fermentation, monascal waxy corn obtained from the conventional method was reinoculated again with the same volume and spore suspension contents (10^6 spores.mL^{-1} spore suspension of *M. purpureus*) and continuously fermented with the same condition as the conventional method. At the end of the fermentation, 62.89 mg kg^{-1} dry weight of monacolin K content and 3,072.70 unit g^{-1} dry weight of pigment were extracted from monascal waxy corn and were higher than those of the conventional method (16.83 mg kg^{-1} dry weight of monacolin K and 400 unit g^{-1} dry weight of pigment) but the residual reducing sugars were exhausted. Moreover, the IC_{50} values of DPPH, FRAP and chelating ability on Fe^{2+} assays from pigment extract of monascal waxy corn from the two-step fermentation were about 50% less than those of the conventional method. Therefore, the two-step fermentation influenced the increase of the antioxidant activities through many factors: (1) molecular weight, numbers of aromatic ring and nature of hydroxyl group substitutions of the antioxidants are changed; (2) the presence of the glycone part, which masks hydrogen donation property indicating an important feature for free radical scavenging greater electron donors and can reduce more oxidised intermediates so that they can act as better primary and secondary antioxidants (Hagerman *et al*., 1998); (3) the enzymatic hydrolysis of conjugated phenolics can lead to an increase in free phenolic contents with enhanced antioxidant potentials, improve the nutraceutical value of the products and can be exploited as value-added food products (Shetty and McCue, 2003).

Conclusion

Angkak or monascal rice has been utilised as a food colorant and a folk medicine to improve digestion, blood circulation and lowering blood cholesterol levels. Commonly, during *Monascus* fermentation, monacolin K is produced higher than other antioxidants such as γ-aminobutyric acid (GABA), dimerumic acid and flavonoids. The factors affecting SSF for monacolin K and pigments are strains, substrates, moisture contents, pH, temperature, and carbon and nitrogen sources, which are considered as the important tools for the fermentation. To understand more about the improvement of *Monascus* SSF, two-step fermentation is described. For *Monascus* fermentation of step 2, *Monascus* mycelium forming from the addition of spore suspension after step 1 could exhaust primary metabolic products, e.g., reducing sugars, succinic acid, citric acid, gluconic acid, oxalic acid, ethanol alcohol, acid and ester compounds to be the substrate to increase the pigment intensities and monacolin K content.

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