CHAPTER 4

Antioxidant Properties and Formation of Monacolin K, Citrinin and Red Pigments during Solid State Fermentation of Purple Rice Varieties (*Oryzae sativa*) by *Monascus purpureus*

4.1 Introduction

Rice is a staple food for over half of the world ′s population especially in Asia. The nutrient content of rice is based on the rice cultivar and nutrient quality of the soil. Brown rice is unpolished whole grain rice that is produced by removing only the outermost layer, the hull of the rice kernel. It may be distinctly brown, reddish or purplish and has a mild nutty flavor (Babu *et al.,* 2009). In the past, it was rarely eaten because it is difficult to store and becomes rancid more quickly than polished rice. However, brown rice has high dietary fiber, and is rich in B vitamins and minerals as well as protein (Oh and Oh, 2004). Also it has been reported that brown rice contains high phytic acid (antioxidant, anti-cancer) and polyphenols. It decreases serum cholesterol to prevent cardio-vascular disease and it is considered a low glycemic index food (low starch, high completely carbohydrates which decreases the risk to type 2 diabetes). Any rice, including sticky rice, long-grain rice, or short-grain rice can be eaten as brown rice. The change of staple food from polished rice to unpolished rice can maintain and promote health and improve the quality of rice (Patil and Khan, 2011).

In Thailand, purple rice is glutinous rice the genotypes of which are local races; glutinous rice is widely grown in different geographical areas. Purple rice varieties have been found to be a good source of nutrients, minerals, and phytochemicals and antioxidant compounds. The bran hull of purple rice has the unique characteristics of containing high levels of gamma oryzanol and anthocyanin antioxidants. These

substances enable the use of purple rice as in traditional herbal medicine in reducing plasma cholesterol, cholesterol absorption and decreasing early atherosclerosis and inhibition of the growth of Lewis lung carcinoma cells in vivo (Chen *et al.,* 2005).

Functional foods are defined as foods similar to conventional foods, which are consumed as part of a usual diet, and are demonstrated to have physiological benefits, and reduce the risk of chronic disease in one of the earliest regulatory guidelines suggested by Health Canada Organization. Red mold rice (RMR) is rice fermented by *M. purpureus* and has been used as a dietary supplement and a folk remedy. The main components of RMR are carbohydrates (25–73%), proteins (14–31%), water (2–7%) and fatty acids (1–5%). During fermentation, many products of the secondary metabolism are formed including pigments, γ-aminobutyric acid (GABA), monacolin, glucosamine, lecithin, flavonoids, sterols and a mycotoxin (Lin *et al.,* 2008).

Monacolin K (lovastatin, mevinolin and mevacor) is a secondary metabolite of *Monascus* strains that is an inhibitor of the enzyme hydroxymethylglutaryl coenzyme A reductase (HMG-COA) which regulates and inhibits enzymes involved in cholesterol biosynthesis. Many studies have shown that some secondary metabolites of *Monascus* species improved hyperlidemia, hypertension, hyperglycemia, Alzheimer′s disease development, oxidative stress and fatigue during exercise (Shi and Pan, 2011). However, RMR may be contaminated with a mycotoxin known as citrinin. This mycotoxin was investigated as an antibiotic and reported to be hepatotoxic and nephrotoxic in mammals, leading to a negative impact on the acceptability of RMR products (Betina, 1989).

In this experiment, we focused on the effect of various purple rice varieties on the production of monacolin K, citrinin, red pigment and antioxidant properties. This is the first report on using local pigment glutinous rice as substrate for monacolin K production.

4.2 Materials and methods

4.2.1 Microorganism and Inoculumn

Monascus purpureus CMU002U is a UV treated mutant strain that was obtained from the Sustainable Development of Biological Resources Laboratory, Faculty of Science, Chiang Mai University. This strain was carried out using the spore suspension treated with UV rays (18 W) for 20 to 30 min. The viable cells were harvested by centrifugation, washed with distilled water and suspended in potato dextrose broth (PDB) at 30°C for 6 h and subcultured to potato dextrose agar (PDA) and then incubated for 3 days. Each single colony from 0.1−1% of the survival rate was selected for the primary screening of citrinin production following the method described by Wang *et al.* (2004). The strain was cultivated on PDA at 30°C for 7 days.

Five mycelia plugs (5 mm in diameter) from the periphery of the growing colony on PDA at 30°C for 7 days were transferred to 50 ml PDB in a 250-Erlenmeyer flask. Cultivation was performed in the dark at 30°C with shaking at 150 rpm on a reciprocal shaker. After 5 days of incubation, the cultures were used as the inoculum.

4.2.2 Solid state fermentation for RMR Production from purple rice

Six purple Thai glutinous rice varieties, Doi Muser, Doi Saked, Na, Nan Phayao and Hom CMU, were used in this study. The solid state fermentation was conducted following the method described by Chairote *et al*. (2007) with some modifications. Each rice variety was soaked in water overnight, then 20 g of rice were placed in a 250 ml Erlenmeyer flask and autoclaved at 121°C for 15 min. The moisture content of each rice variety was adjusted to 60% (w/w) on a wet basis. After cooling, 1 ml of liquid fungal inoculum (10^6 spore/ml) was added and the inoculated flasks were incubated at 30°C in the darkness. After 14 days, the fermented rice was collected and dried at 60°C overnight. Three replications were made for each rice variety.

4.2.3 Detection and Quantification of Monacolin K and Citrinin

High performance liquid chromatography (HPLC) analysis was used to determine the level of monacolin K and citrinin production with a modified method based on the method propose by Wang *et al.* (2004) and Chairote *et al.* (2007). The presence of monacolin K and citrinin were confirmed by retention time and co-injection with standards (Sigma®). A standard curve was constructed with different levels of both authentic standards. Monacolin K and citrinin in fermented substrates were quantified by correlating peak area of the extracted sample and calibration curve. Three replications were made in each sample.

4.2.4 Determination of Red Pigment

The method for RMR extraction was followed by Nimnoi and Lumyong (2011). The extracts were measured at 500 nm and pigment yield was expressed as OD per gram dried RMR. Each sample was carried out in triplicate.

4.2.5 DPPH Radical Scavenging Method

The evaluation of RMR extracts was carried out using dried products diluted with methanol at different concentrations (5.0, 2.5, 1.25, 0.63 and 0.32 mg/ml). Antioxidant activity was measured using the modified method described by Brand-Williams *et al.* (1995). One milliliter of RMR extracts was added to 2 ml of 0.004% DPPH (2,2–diphenyl–1–picrylhydrazyl) in methanol. The mixture was shaken and left to stand at room temperature (25°C) for 30 mins in the dark. As DPPH radicals are scavenged, the solution turns from purple to yellow (Figure 4.1). The solution was measured at 517 nm and calculated as percentage of antioxidant activity using the following Equation 4.1,

$$\% \text{ Antioxidant activity} = 100 - \left[\frac{\text{sample absorbance} - \text{blank absorbance}}{\text{control absorbance}}\right] \text{X } 100 \quad (4.1)$$

(Blank = Methanol + RMR extracts, Control = DPPH + Methanol)

In this experiment, butylated hydroxytoluene (BHT) was used as the standard. Antioxidant activity was expressed in terms of the amount of antioxidants necessary to decrease the initial DPPH and IC_{50} value was determined by calculating the concentration of sample that is required for a 50% reduction of antioxidant activity. All extracts were carried out in triplicate.

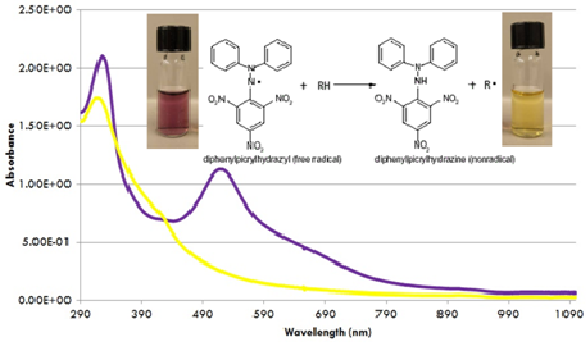

Figure 4.1 Absorption spectrum of DPPH and oxidation of DPPH radicals

4.2.6 Production of Monacolin K by Temperature Shift

Suitable purple rice from the above experiment was selected and used in this experiment based on the highest ratio of monacolin K to citrinin production following the method of Kalaivani and Rajasekaran (2014). The SSF was carried out by a temperature shift from 30°C to 25°C. Different incubation times at 30°C followed by 25°C were evaluated and compared with un-shifted temperature incubation experiments. All treatments were carried out in triplicate. (Table 4.1)

Table 4.1 The conditions of monacolin K production by temperature shifting

Temperature	Incubation duration (day)								
	A	B	C	D	E	F	G	H	I
30°C	2.5	5	7.5	10	15	20	25	30	0
25°C	27.5	25	22.5	20	15	10	5	0	30

4.2.7 Statistical Analyses

All the experimental data were analyzed with SPSS program version 11.5 for Windows. All data were subjected to analysis of variance (ANOVA) by the Tukey's test ($P \leq 0.05$).

4.3 Results and discussion

4.3.1 SSF for RMR Production from purple rice

Red mold rice (RMR) were prepared by solid state fermentation on various purple rice (*Oryzae sativa* L. Kam) with mutant strain; *M. purpureus* CMU002U. Generally, polished non-glutinous rice is used as substrate for RMR production. In addition, brown rice, black rice both glutinous rice and non-glutinous rice, cereal and agricultural waste can be used as raw material for RMR production (Velmurugan *et al.,* 2011). Several purple rice including Doi Muser, Doi Saked, Na, Nan, Phayao and Hom CMU were used as substrates. The grain characteristics were a hard texture and dark purple colour that affected the texture and colour of the products (Figure 4.2).

Figure 4.2 Purple rice varieties for red mold rice production; (a) Doi muser (b) Doi saked (c) Na (d) Nan (e) Phayao and (f) Hom CMU (bars = 1 cm)

The results showed that after being fermented, the grains had a soft texture from amylase production and a pleasant odor from esters and alcohols

(Chen *et al.,* 2013). The colour of products is represented in Figure 4.3. The products had a dark red colour from the *Monascus* pigment and the grains retained their original shape.

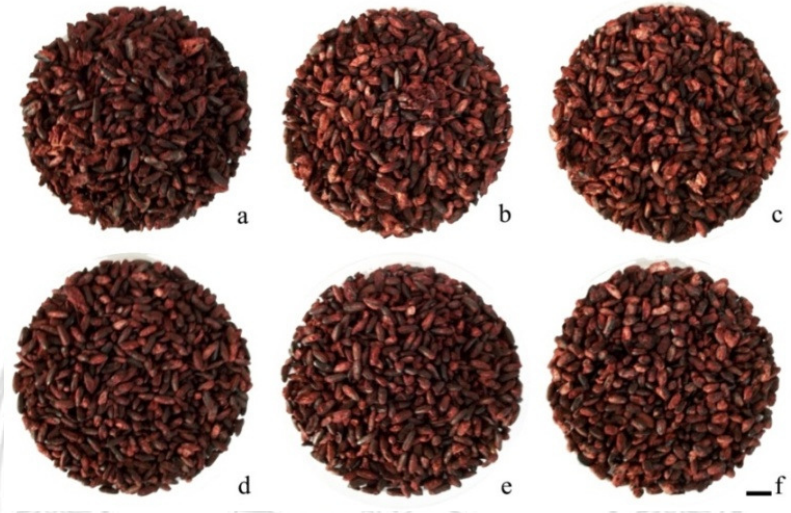

Figure 4.3 Red mold rice from purple rice (a) Doi muser (b) Doi saked (c) Na (d) Nan (e) Phayao and (f) Hom CMU (bars = 1 cm)

The yield percentage of RMR from purple rice after 2 week of fermentation was similar to the work of Chairote *et al.* (2007). RMR from Nan and Hom CMU rice gave the highest and lowest percentage of yield at 33.28 and 27.47, respectively ($P \leq 0.05$) (Figure 4.4).

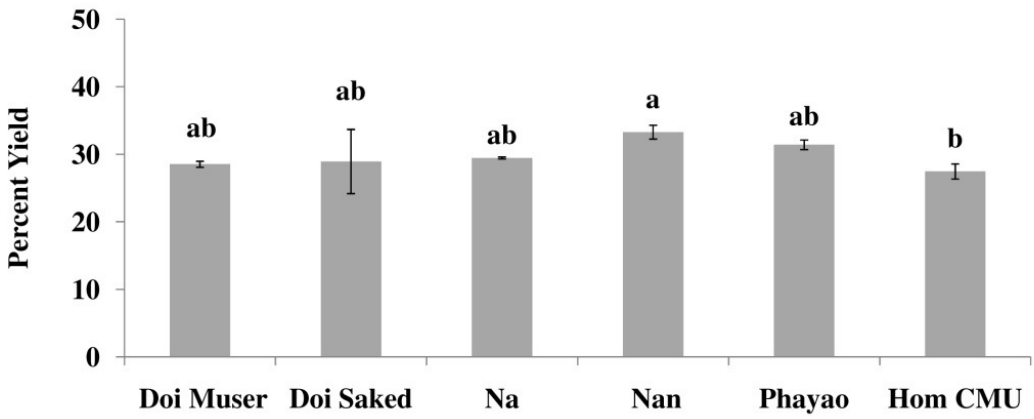

Figure 4.4 Percentage of yield of RMR from purple rice

4.3.2 Red Pigment Production

Pigments are secondary metabolites synthesized by *M. purpureus* and can be divided into three groups: orange (rubropunctain and monascoubrin), yellow (monascin and ankaflavin) and red (rubropunctaminea and monascorubramine) (Campoy *et al.,* 2006). These pigments have a high stability to pH and temperature, which are interesting properties for substituted synthetic dyes. For a long time in eastern Asia, the red pigment has been used as food additive after fermentation (Fabre, 1993). Doi Muser RMR and Na RMR have the highest and lowest red pigment yield, respectively, and are three-fold different. The red pigment yield from other purple rice substrates is shown in Figure 4.5.

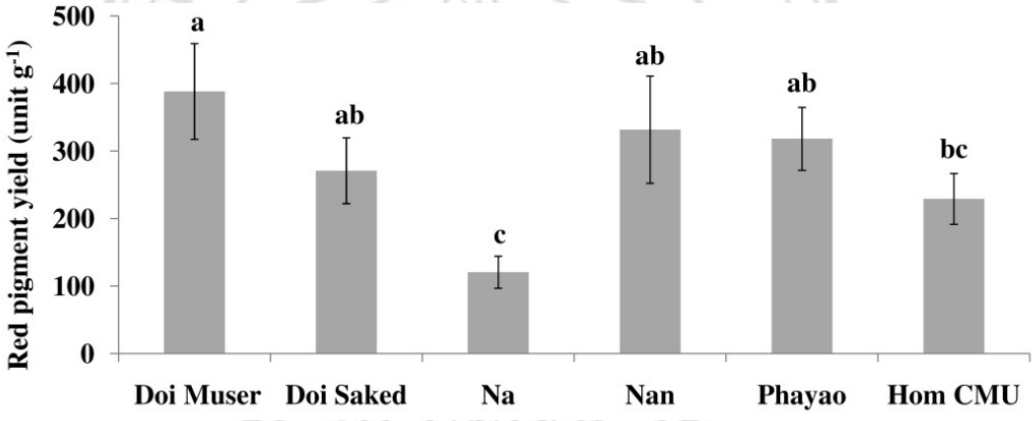

Figure 4.5 Red pigment yield of RMR from purple rice

The red pigment yield from RMR obtained from Doi saked, Nan and Phayao were not significantly different. The advantage of using purple rice varieties as substrates is higher protein, iron and zinc when compared to brown rice and white rice (Fernando, 2013). Therefore, the process does not need to be supplemented with sugar or nitrogen sources when compared with that of Chairote *et al.,* (2007) and Nimnoi and Lumyong (2011). The content of pigments in RMR varies depending on the cultural conditions such as humidity, pH as well as other nutrients and oxygen supply (Vidyalakshmi, 2009). The red pigment yield can be obtained from liquid, submerged and solid state fermentation. Solid state fermentation is the cultivation of microorganism under

controlled conditions in the absence of free water, and is similar to natural habitats of fungi (Mienda *et al.,* 2011).

4.3.3 Monacolin k and Citrinin Production

Purple rice can be used as materials for synthesis of monacolin K or other secondary metabolites through the RMR process. HPLC was used to analyze the amount of monacolin K and citrinin in RMR products. The effects of purple rice cv. on monacolin K productivity are shown in Figure 4.6.

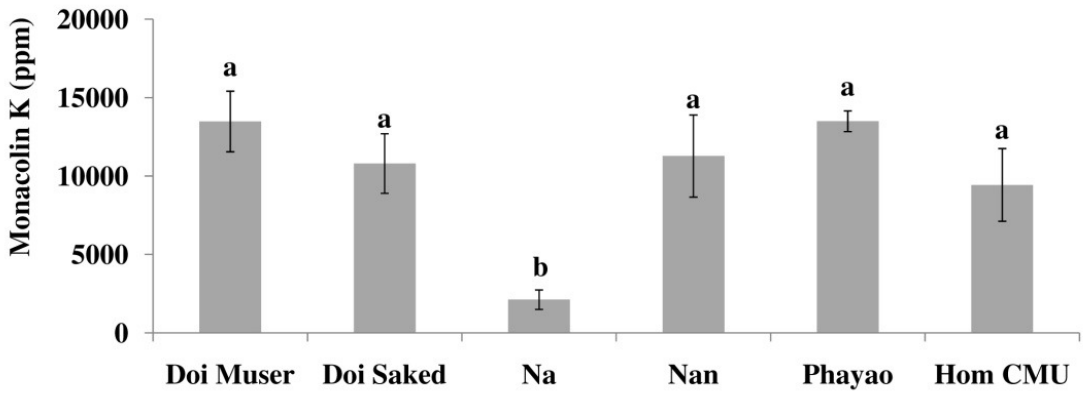

Figure 4.6 The effect of purple rice on monacolin K production

SSF on Phayao and Doi Muser purple rice achieved high monacolin K levels, 13,496 and 13,482 ppm, respectively. The lowest yield of monacolin K was found in SSF of Na purple rice (2,118 ± 619 ppm) which was 6.37−fold less than Phayao purple rice, and this result was related to the low level of red pigment yield detected in the previous part of this study. The research of Chiu *et al.,* (2006), who studied the liquid state fermentation (LSF) for monacolin K production on rice material, showed that monacolin K was produced at a lower level (46.5−53.5 ppm). Different factors on SSF including raw materials, temperature, initial moisture, strain of fungus and cultivation time affected secondary metabolite production (Carvalho *et al.,* 2007). SSF is the one type of fermentation that could improve yield of products, and has a low energy requirement which reduce the production costs. SSF was incubated at a similar temperature to Su *et al.* (2003) who stated that the highest yield of monacolin K

was obtained at 30°C. However, productions of RMR are frequently contaminated with citrinin. The contamination of citrinin is a main problem that influenced acceptability because it's a mycotoxin that damages the liver and kidneys of mammals. *Monascus purpureus* used in this study has been improved through mutation by UV radiation. This strain produced less toxin than the parental strain. The highest citrinin level was found in RMR from Phayao purple rice (1,125 ppb) and the lowest citrinin level was found in Na rice (132 ppb) (Figure 4.7.).

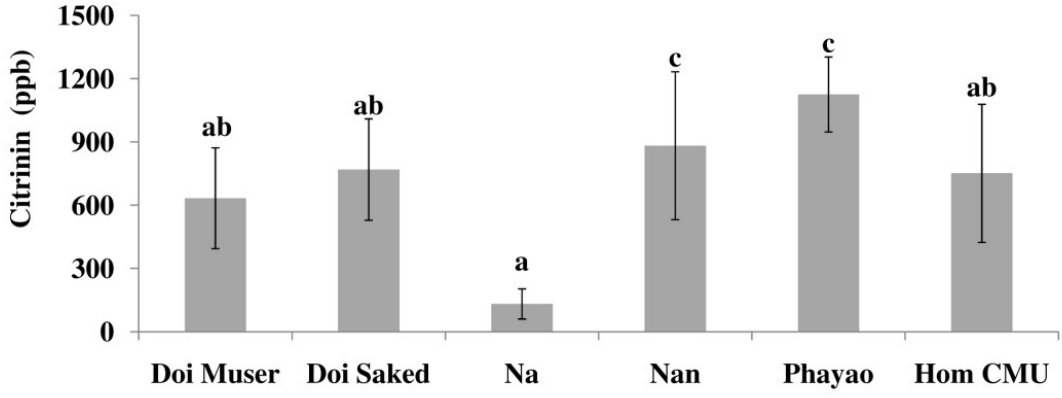

Figure 4.7 The effect of purple rice on citrinin production

The monacolin K produced by our study was higher than that obtained in other RMR research. SSF on Indian rice by *M. purpureus* MTCC1090 under optimal conditions that was supplemented with fructose, sodium nitrate and acetic acid produced monacolin K only 37 ppm (Rajasekaran and Kalaivani 2012). In addition, monacolin K can be produced by other strain of *Monascus*. Xu *et al.* (2005) studied the optimal conditions for monacolin K production using *M. ruber* on rice. Their result showed that the addition of soybean powder can be increased monacolin k to 4,020 ppm. In the research work of Park *et al.* (2014) used Korean rice varieties as raw materials for RMR production. Their results showed that the highest monacolin K content only 117 ppm from *M. ruber* KCCM60141 cultures occurred in the Sangjuchalbyeo rice variety.

The results of a comparative study of the ratio of monacolin K to citrinin production are shown Figure 4.8. RMR from Doi Muser rice varieties had the

highest ratio and monacolin K production was 21,299-fold higher than in citrinin production (13,482 ppm of monacolin K and 633 ppb of citrinin). The levels of citrinin detected in RMR were in accordance with the standards of Japan and Taiwan which have imposed citrinin concentration limits to 200 and 2,000 ppb, respectively (Chung *et al.* 2009). In 2014, the Commission Regulation under the EU No. 212/2014 accepted the limitation of citrinin concentration in rice fermented with *M. purpureus* at 2,000 ppb in the European Union (EU) (The European Commission 2014). Our fermented products from the Na rice variety have passed the standards of Japan, Taiwan and the EU but could not pass those standards of Korea and the US FDA since the acceptable levels must be less than 50 and 20 ppb, respectively (Chung *et al.* 2009; Le Bloc′h *et al.* 2015).In Thailand, there have been many reports about mycotoxin contamination in raw agricultural commodities and processed food, which can be identified as aflatoxin. However, there is no set regulation limit of citrinin contamination (Songsermsakul, 2015).

RMR is commonly used as a food supplement in many countries including the USA, China and Taiwan for the treatment of high cholesterol. Journoud and Jones (2004) demonstrated that RMR seems to be safe when compared with other available statins (cholesterol-lowering drugs), as the incidence of adverse side effects is fairly low. However, the US FDA issued a consumer warning stating that consumers should avoid RMR products because some products contained the unauthorized drugs (monacolin K) (Childress *et al.* 2013). The RMR has no clear position on the use of this substance in food supplements due to the medical regulation (EC No. 2001/83) which states that the limitation of lovastatin in products can range from 10–20 mg. Therefore, its regulatory status in the EU depends mainly on the dosage of monacolin K in consumer products (Le Bloc′h *et al.* 2015).

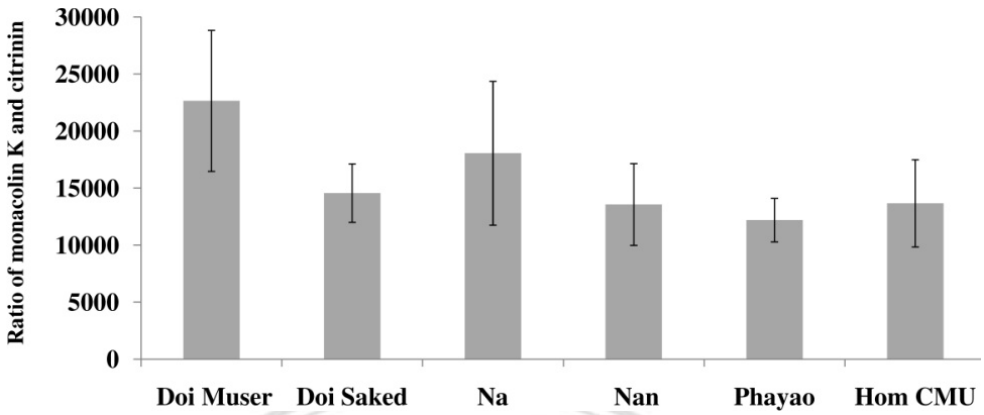

Figure 4.8 The ratio of monacolin K to citrinin production on SSF of RMR

4.3.4 DPPH Radical Scavenging

Antioxidant activity can be evaluated by measuring the DPPH scavenging activity. This method is based on the reduction of DPPH. Figure 4.9 showed the DPPH solution mixed with a substance that can donate a hydrogen atom. The solution will change from purple to yellow colour and the resulting of decolourization of DPPH is related to the percent reduction (Molyneux, 2009).

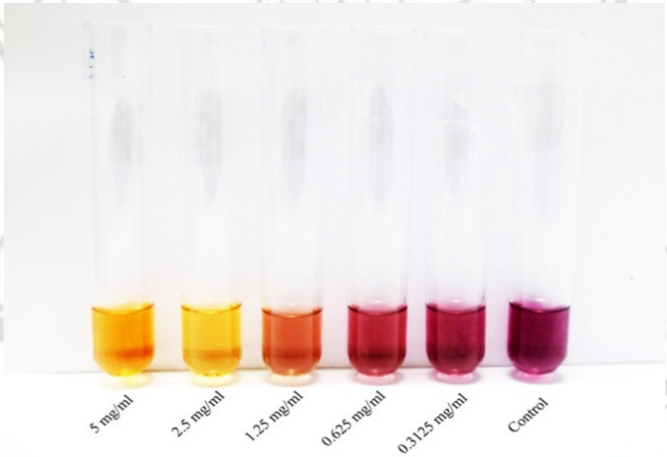

Figure 4.9 Decolourization of DPPH at various concentration of RMR

The antioxidant activity of BHT was used as the comparative standard. The initial concentration of all RMR products at 5 mg/ml, as well as 1 mg/ml of BHT could scavenge more than 90% of DPPH radicals. The antioxidant activity of the RMR extracts and BHT standard were expressed as IC_{50} values and are

presented in Figure 4.10. Doi Saked RMR had the lowest IC_{50} value, which means that the concentration of RMR at 0.727 mg/ml reduced free radical to 50% which represented a 0.776 equivalency to BHT. The comparison of DPPH radical scavenging activity of RMR products to BHT standard was showed in Table 4.2. Antioxidant activity from our RMR products had more efficiency compared to Chairote work (2009). IC_{50} value from fermented purple glutinous rice was calculated to 0.315 equivalency to BHT. Other substrates including Mali 106 (non glutinous rice), RD6 and Sanpatong glutinous rice showed IC_{50} value was 0.426, 0.333 and 0.222 equivalency to BHT, respectively. RMR is one of the dietary foods that has antioxidant properties. Al-Shahrani *et al.* (2013) studied the amount of antioxidants in nutraceuticals food products and also demonstrated that RMR has antioxidant properties. They reported that their RMR had 1.067 mesfer units (1 mesfer unit = antioxidant activity of 500 mg of ascorbic acid), which was more than contained in concentrated green tea and a curcuminoid material which have only 0.426 and 0.372, respectively.

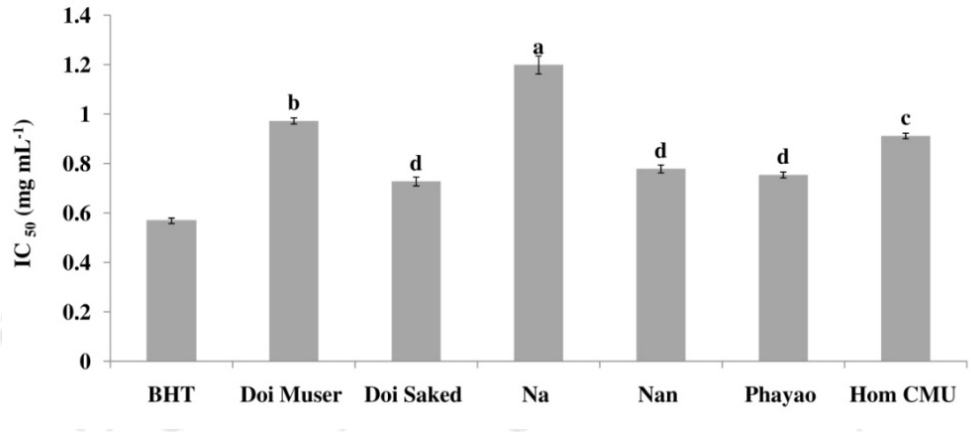

Figure 4.10 IC_{50} value of BHT and RMR extracts

Table 4.2 DPPH radical scavenging performance of the RMR extracts compared to BHT

RMR from	Efficiency of RMR extracts compared to BHT
Doi Muser	0.581
Doi Saked	0.776
Na	0.471
Nan	0.725
Phayao	0.749
Hom CMU	0.619

4.3.5 Production of monacolin K by temperature shifting

The highest ratio of monacolin K and citrinin was found in the SSF of Doi Muser purple rice. Therefore, this rice variety was used in the experiments. The optimal temperature for the growth of *M. purpureus* is 30°C (Subsaendee, 2014); mycelium covered all of the rice grains and changed the substrate colour to dark red more rapidly than when incubated at 25°C for 30 days. However, higher values of monacolin K were found when process the SSF at 25°C. The fermentation of temperature shifting has been a simple technique that has been used for the improvement of product yields (Ansorge and Kula 2000; Tsukahara *et al.* 2009). In this study, the temperature shifting was modified for SSF. The highest ratio of monacolin K and citrinin was found in the SSF of Doi Muser purple rice. Therefore, this rice variety was used in this experiment. The monacolin K contents in SSF of Doi Muser purple rice at 25 and 30°C were found to be 23,832 and 12,344 ppm, respectively. The monacolin K content at 25°C increased to 93.07%, compared to 30°C incubation. The initial temperature of fermentation was carried out at 30°C and then shifted after various periods to 25°C and the specimens were incubated until 30 days. In this part of the experiment, it was necessary to increase the fermentation time from 14 to 30 day in order to allow the fungal growth to cover all grains. The results are shown in Figure 4.11.

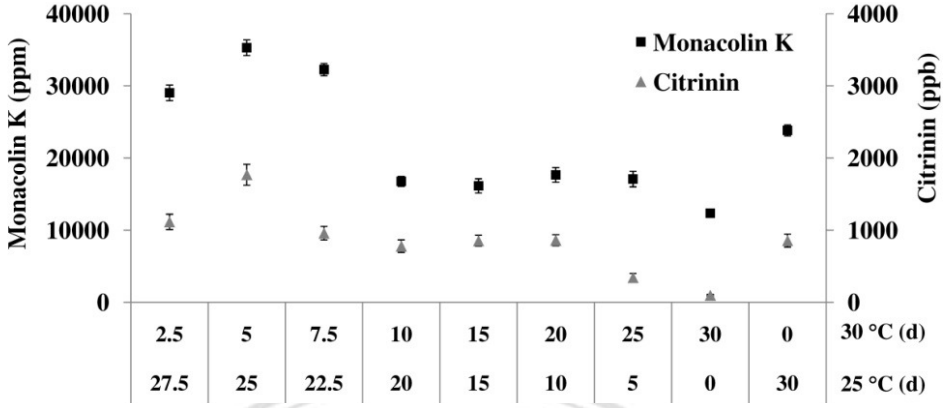

Figure 4.11 Monacolin K and citrinin production by temperature shifting (30 to 25°C)

In treatment B, fermentation for 5 days at 30°C followed by 25 days at 25°C revealed the highest monacolin K (35,292 ppm) and citrinin (1,769 ppb) (19,950:1) values. The monacolin K and citrinin values were 186% and 1695% higher, respectively when compared with treatment H (constant incubation at 30°C). Treatment G (fermentation for 25 days at 30°C followed by 5 days at 25°C) was found to be optimal for the highest ratio of the values of monacolin K and citrinin (49,677:1). The levels of monacolin K and citrinin production were 17,089 ppm and 344 ppb, respectively. This treatment produced 163% and 37% more monacolin K and citrinin, respectively, than treatment H. Moreover, treatment G was applied to the SSF of white Sanpatong glutinous rice. The results showed that the ratio of monacolin K and citrinin was 29,780:1 (monacolin K 22,097 ppm and citrinin 742 ppb). The findings of our research work agree with those of Tsukahara *et al.* (2009) who studied the effects of temperature shifting. Temperature shifting from 30 to 23°C increased monacolin K production when compared to cultivation at a constant temperature and our results agreed with Feng *et al.* (2014) who studied monacolin K production by temperature shift (30°C 2.5 days and 24°C 14 days) on non glutinous rice and soybean flour that could result in higher levels of monacolin K at 18,733 ppm.