CHAPTER 3

MATERIALS AND METHODS

3.1 Materials

3.1.1 Microorganism

The strain *Monascus* sp. CMU001 isolated from commercial red yeast rice and identified by conventional methods was provided by the department of Biology, Faculty of Science, Chiang Mai University, Thailand.

3.1.2 Raw material

Chinese red yeast rice

Commercial Chinese red yeast rice, available in local traditional shops (Jip Aung Tung drug store, Waroros market, Chiang Mai).

Rice varieties

Non-glutinous rice; *Oryza sativa* L. cv. Mali105

Glutinous rice; *Oryza sativa* L. cv. Kam (Kam), *Oryza sativa* L. cv. Kor Kho 6 (RD6) and *Oryza sativa* L. cv. Sanpatong1 (SPT1), which are abundantly available in the north of Thailand, were used to prepare red yeast rice. These rice samples were obtained from the same batch of processing from one rice supplier and stored in the same conditions.

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3.1.3 Nitrogen source nutrient

Sterilized soybean milk (250 g soybean in 1000 ml of water) was prepared by soaking the grains over night. After grinding the grain to a very fine paste and mix again with water, soybean milk was obtained by filtering. The soybean milk was finally sterilized by autoclaving before use.

3.2 Methods

3.2.1 Isolation of *Monascus purpureus*

Commercial Chinese red yeast rice was used for *M. purpureus* isolation. Surface sterilization (Fig 3.1) (Miche and Balandreau, 2001) is the preferred method to eliminate the contaminants. The experiment was carried out by preliminary soaking the rice in 95 % ethanol, washing by 0.1% - 0.5% sodium hypochlorite solution for 1-4 min and finally washing by 95% ethanol for 30 seconds. Twelve washed red rice grains were placed on Rose Bengal agar plates. One week culture was used for isolation of uncontaminated grains by cultivation on potato dextrose agar (PDA) plates. The pure culture were kept in mineral oil, and refrigerator at 5 ^{o}C as a stock culture.

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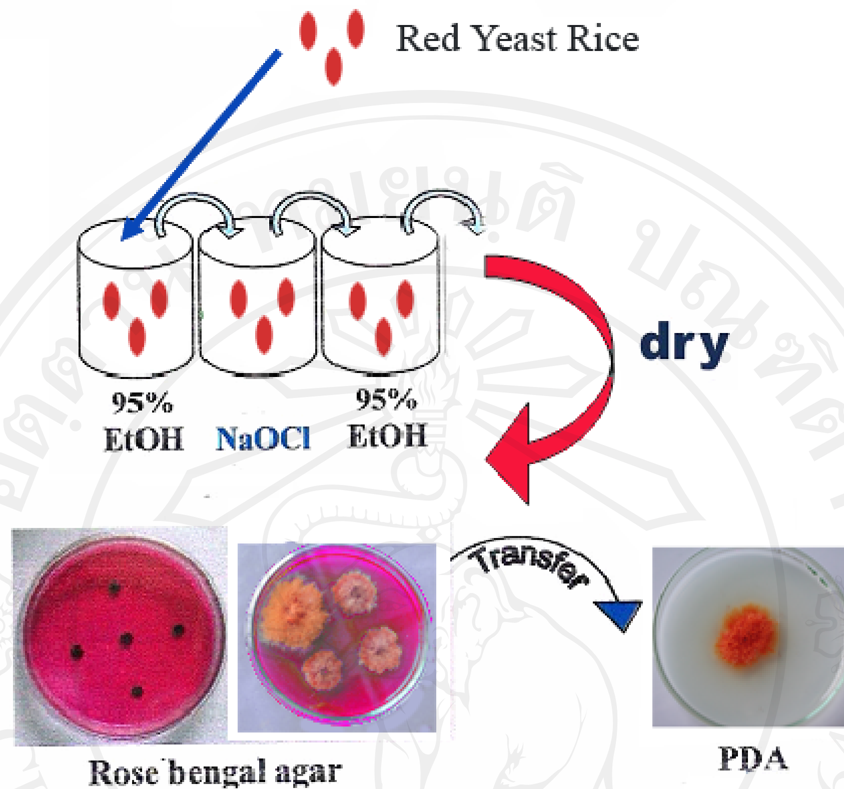

Figure 3.1 Surface sterilization

Source; Modified from Promputtha, 2006

3.2.2 Identification of *Monascus purpureus*

The strain named CMU001 isolated from commercial red yeast rice were identified by conventional methods and compared with The description in The Compendium of Soil Fungi (Domsch, 1980). The strain was inoculated on PDA plates and incubated at room temperature to observe morphological characteristics such as colonies, ascospores and conidial stage. Pigment production was also observed as well as 6% sodiumchloride and 30% ethanol tolerance. The starch hydrolyzing enzyme activity was studied by cultivation of the strain on medium following by clear zone detection using iodine solution (Yongsmith, 1997).

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3.2.3 Preparation of red yeast rice

Inoculation and cultivation of isolated *M. purpureus* CMU001 were done using different varieties of glutinous rice (*Oryza sativa* L.). The glutinous rice used were Kor kho 6 white glutinous rice (RD6), Kam purple glutinous rice (Kam) and Sanpatong 1 white glutinous rice SPT1. For comparison, non glutinous rice, Khao Hom Mali105 (Mali105) was used to make red yeast rice. The controlled condition with appropriate weight, culture age, inoculation volume, temperature, humidity and pH according to Boonsangsom *et al.* (2004) was used. Sample after 2 and 3 weeks cultivation were finally obtained and used for further study. Stepwise preparation of red yeast rice is shown in Figure 3.2 Glutinous rice grains were immersed in water for 6 hours following by steaming for 20 minutes. After cooling, 50 grams of steam rice was put in 250 ml flask and sterile at 15 psi and 121 ^{o}C for 15 minutes. One week old precultured *M. purpureus* CMU001 was used as inoculum. The inoculated rice was incubated at 30 ^{o}C for 2 or 3 weeks. Humidity and pH were measured before and after inoculation. The end-product was dried in the oven at 65 ^{o}C for 6 hours to obtain dried red yeast rice. In case of non glutinous rice (Mali105) which was used for comparison, the red yeast rice preparation was done without immersion of rice grains in water. In order to study the effect of adding nitrogen compounds, addition of 1 ml of 0.25 g/ml soybean milk solution was also performed. The yield of red yeast rice was evaluated as percentage yield. The percentage yields were obtained by using the following equation.

$$\text{Percentage yield} = \frac{\text{Weight of dried end product}}{\text{Weight of steamed rice used}} \times 100$$

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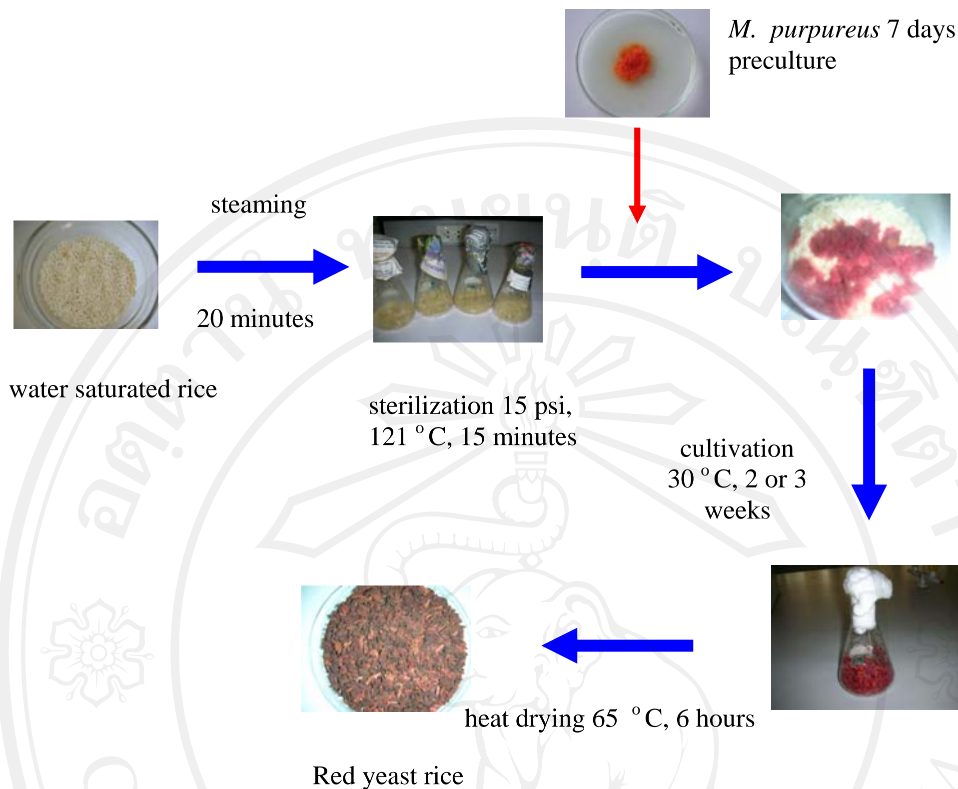

Figure 3.2 Overall used to prepare red yeast rice in this study

3.2.4 Measuring the content of red pigments

The red pigment content was measured by determination of absorbance at 500 nm (Boonsangsom *et al*., 2004; Babitha *et al*., 2007; Yongsmith *et al*., 2000; Singhapol, 2005). 0.5 g of red yeast rice powder was used for extraction by 10 ml of 75% HPLC grade ethanol (Li *et al*., 2004). The mixture was vibrated in ultrasonic bath for 60 minutes.

The supernatant liquid was obtained by centrifugation at 3000 rpm at 4 °C for 10 minutes. The extraction procedure was repeated 3 times to get 30 ml solution which was then made up to 50 ml in a volumetric flask with 75% ethanol. After

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standing for 30 min, the solution was filtered through 0.2 µm membrane and its absorbance measured over the wavelength rage of 400-700 nm.

3.2.5 Analysis of monacolins by HPLC and LC-MS

Sample extraction

An extraction of the sample was carried out using 0.5 g of ground rice. It was put into a 20 ml centrifugal tube. 10 ml of 75% HPLC grade ethanol was added and it was degassed in an ultrasonic bath for 60 min. The supernatant was collected after centrifugation using 3,000 rpm speed at 4 ^{o}C (Li *et al*., 2004). The extraction was repeated three times and all of the extracts were mixed together and made up to 50 ml using 75% ethanol. Finally, the extract was filtered through a 0.2 µm membrane and kept in a vial before being analyzed.

The extracts were analyzed for monacolins by HPLC and LC-MS. The chemical profiling procedure conducted on the HPLC (Agilent HP 1100) with a photodiode array detector was optimized by testing various system conditions. The symmetry and resolution were increased by lowering the pH value of elution. The results suggested a system composed of 0.1 % trifluoroacetic acid (TFA) and acetronitrile as an ideal system for the separation of the monacolin compounds. For the consideration of resolution, running time and solvent-saving, the column of Hypersil ODS (250 mm x 4.0 mm i.d., 5µm) was used. The chromatography was performed using a gradient of acetonitrile (eluent A) and 0.1 % TFA (eluent B). Linear gradient elution (1 ml/min) from 35 to 75 % A in 20 min and keeping at 75 % A from 20 to 28 min was applied. The total analysis time was 35 min, including column stabilization. The photo-diode array detector was set at 210-350 nm and the

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chromatogram was detected at 237 nm. The column temperature was set at 35 ^{o}C, and the injection volume was 10 µl (Li *et al.*, 2005).

For LC-MS analysis, the apparatus used were Agilent HP 1100 and MSD VL model. The eluents used and injection volume were the same as the HPLC analysis only the flow rate was set at 0.5 ml/min from 35 to 75% of solvent A in 30 min and kept at 75% of solvent A for 5 minutes. The total analysis time was 50 min, including column stabilization. The length of the column used was 125 mm instead of 250 mm.

3.2.6 Determination of antioxidant properties

The antioxidant properties in the sample were studied in two assays as described below. Each assay refers to the different characterization and antioxidant properties in the samples. The assays include;

- Antioxidant capacity

- β-carotene bleaching activity

Extracts preparation

An extraction of the sample was carried out using 0.5 g of ground rice. It was put into a 20 ml centrifugal tube. 10 ml of 75% HPLC grade ethanol was added and it was degassed in an ultrasonic bath for 60 min. The supernatant was collected after centrifugation using 3,000 rpm speed at 4 ^{o} C (Li *et al*., 2004).

The extraction was repeated three times and all of the extracts were mixed together and made up to 50 ml using 75% ethanol.

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Antioxidant capacity

The assay is based on the reduction of Mo (VI) to Mo (V) by the extracts and subsequent formation of a green phosphate/Mo (V) complex at acidic pH (Banerjee *et al.,* 2005). The extracts were combined with the reagent solution (0.6M sulphuric acid, 28mM sodium phosphate and 4mM ammonium molybdate). The tubes were incubated at 95 ^{o}C for 90 min. After cooling to room temperature, the absorbance of the solution was measured at 725 nm (Prieto *et al*., 1999). The total antioxidant capacity was expressed based on gallic acid equivalents.

Preparation of standard calibration curve for total antioxidant capacity was carried out using standard gallic acid. 0.2 mg/ml Gallic acid solution was prepared and diluted to give the different concentrations (0.01, 0.02, 0.03, 0.04, 0.05, 0.06 mg/ml). The reagent solution was as shown in Table 3.1. The tubes were incubated at 95 ^{o} C for 90 min. After cooling the absorbance was measured at 725 nm.

To determine the total antioxidant capacity of the extracts, the samples 0.1 ml were placed in test tubes and made up to 1 ml with distilled water. The tubes were incubated at 95 ^{o} C for 90 minutes and after cooling, the absorbance was measured at 725 nm.

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Table 3.1 The volume of chemical reagents for standard total antioxidant capacity graph

Solutions	Volume (ml)					
	1	2	3	4	5	6
0.2 mg/ml gallic acid	0.05	0.10	0.15	0.20	0.25	0.30
Distilled water	0.95	0.90	0.85	0.80	0.75	0.70
Reagent solution	3.0	3.0	3.0	3.0	3.0	3.0
Stirring						
Incubated at 95 ^{o}C for 90 minutes						
Measurement the absorbance at 725 nm						

β-carotene bleaching method comparing to standard BHT (Butylated hydroxytoluene)

The β-carotene method was carried out on the sample according to the rate of β-carotene bleaching (Nsimba *et al.,* 2008). β-carotene emulsion was transferred into test tubes containing the test samples in different concentrations. The mixture was shaken and placed in a water bath at 50 ^{o} C for 2 hours while the absorbance of samples were measured at 470 nm.

The antioxidant activity was calculated based on the following equation.

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$$\text{Inhibition percentage for β-carotene oxidation} = \left\{ 1 - \frac{(A_0 - A_t)}{(A^0_0 - A^t_0)} \right\} \times 100$$

Where

A_0 = absorbance values measured at initial time of the incubation for samples

A^0_0 = absorbance values measured at initial time of the incubation for control

A_t = absorbance in the samples at t min.

A^t_0 = absorbance in the control at t min.

Rate of reaction in β-carotene bleaching activity for standard (BHT)

0.1 mg/ml standard BHT was used to investigate the rate of reaction. The reagent solution was added to test tubes as shown in the following Table 3.2 The tubes were incubated at 50 ^{o} C for 2 hours while the absorbance of the tested sample was repeatedly measured every 15 minutes at 470 nm.

The results were plotted in the first graph between absorbance versus time and then, the percent inhibition was calculated for the second graph. The second graph was plotted between inhibition percentages of β-carotene oxidation versus the time of the reaction.

Table 3.2 Reagents and preparation for determining the rate of the reaction in β-carotene bleaching activity of standard (BHT).

Reagents	Volume (ml)
0.1 mg/ml standard solution (BHT)	0.20
β-carotene solution emulsion	5
Stirring and incubated at 50 ^{o}C for 2 hours	
Measurement of absorbance every 15 minutes at 470 nm	

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Preparation of standard calibration curve for β-carotene bleaching activity of standard BHT

0.05 mg/ml standard BHT solution was diluted in the different concentrations with methanol. The reagent solution was added to test tubes as shown in the following Table 3.3 The tubes were incubated at 50 ^{o} C for 2 hours. The mixtures were shaken; the absorbance of the solutions were measured at 470 nm.

β-carotene bleaching activity of the samples

The extracts of the samples were taken in test tubes and made up to 0.2 ml with methanol. The samples were combined with β-carotene solution emulsion. The absorbance at 470 nm was determined after incubating the test tubes at 50 ^{o} C for 2 hours. The percent inhibition activity was calculated.

The percent inhibition of all experiment was calculated and plotted between % inhibition of β-carotene oxidation versus concentration of standard or the samples to find IC_{50} values (concentration of sample required to scavenge 50% of free radicals).

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Table 3.3 Reagents and preparation of standard calibration curve for β-carotene bleaching activity of standard BHT.

Reagents	Volume (ml)						
	1	2	3	4	5	6	7
0.05 mg/ml standard BHT	0.01	0.02	0.03	0.04	0.05	0.06	0.07
MeOH	0.19	0.18	0.17	0.16	0.15	0.14	0.13
Stirring							
β-carotene solution emulsion	5.0	5.0	5.0	5.0	5.0	5.0	5.0
Incubated at 50 ^{o}C for 2 hours							
Measurement of absorbance at 470 nm							

3.2.7 Analysis of volatile aroma compounds by GC-MS

Solid-Phase Micro extraction

This technique is to extract organic compound in aqueous or non- aqueous sample. The compounds will be adsorbed by fuse silica fiber coated at the end of the SPME needle. When the fiber move into injection port of GC at high temperature the compounds will be desorbed and pass through the GC column.

The technique can be used for both volatile and non volatile compound, gas or liquid sample and it is a portable and low cost technique.

To take the volatile sample in the head space and to analyze the volatile aroma compounds, the steps of performing and condition are as follows;

1. Fill the sample in 15 ml vial.
2. The PDMS (100 μm) fiber is used.

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3. Extraction temperature is 70 $^\circ$ C.
4. Extraction time is 20 minutes.
5. Injection temperature for GC analysis is 240 $^\circ$ C.
6. Desorption time is 5 minutes.

The condition for GC-MS analysis are ;

Initial temperature	40 ^{o} C
Initial time	2 minutes.
Rate of temperature increasing	3 ^{o} C/ minutes.
Final temperature	70 ^{o} C
Rate of temperature increasing	7 ^{o} C/ minutes.
Final temperature	210 ^{o} C
Final time	8 minutes.
Running time	40 minutes.
Injection temperature	250 ^{o} C
Carrier gas flow rate (He)	1 ml/ minutes.
Mode	splitless

The capillary column used is Model Number: Agilent 19091S-933E, HP-1MS , Methyl Siloxane, length 30.0 m, diameter 250.0 µm, film thickness 0.25 µm and initial pressure 7.01 psi.

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3.2.8 Analysis of citrinin by HPLC

Sample extraction

An extraction of the sample was carried out using 2.5 g of ground red rice. It was put into a 250 ml Erlenmeyer flask. 15 ml of 70% HPLC grade ethanol was added and it was shaken on rotary shaker at 200 rpm for 12 h, and then allowed to stand in a water bath at 40 ^{o} C for 5 h. The supernatant was collected after centrifugation using 2,000 g speed at 25 ^{o} C for 15 min (modified from Pattanagul *et al.*, 2008), made up to 5 ml using evaporation, and pipette 1 ml of supernatant made up to 0.5 ml using the same method. Finally, the extract was filtered through a 0.2 µm membrane and kept in a vial before being analyzed.

The extracts were analyzed for citrinin by HPLC. The chemical profiling procedure conducted on the HPLC (Agilent HP 1100) with a photodiode array detector was optimized by testing various system conditions. The symmetry and resolution were increased by lowering the pH value of elution. The results suggested a system composed of acetronitrile (eluent A) and formic acid (eluent B) as an ideal system for the separation of the citrinin compounds. For the consideration of resolution, running time and solvent-saving, the column of Phenomenex (150 mm x 4.6 mm i.d., 5µm) was used. The chromatography was performed using a gradient of acetonitrile (eluent A) and formic acid (eluent B). Linear gradient elution (1 ml/min) from 60 - 25 % B in 0-20 min, 25 - 60 % B in 20 - 28 min was applied. The total analysis time was 30 min, including column stabilization. The photo-diode array detector was set at 210-350 nm and the chromatogram was detected at 345nm. The column temperature was set at 25 ^{o} C, and the injection volume was 10 µl.

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