CHAPTER 3
MATERIALS AND METHODS

Materials

1. Chemicals

Acetonitrile (Merck, Germany)

Ammonium solution (Merck, Germany)

Citrinin (Sigma, St. Louis, MO, U.S.A.)

Ethanol (Merck, Germany)

Formic acid (Merck, Germany)

Glucosamine hydrochloride (Sigma, St. Louis, MO, U.S.A.)

Glucose (Merck, Germany)

Hydrogen chloride (Merck, Germany)

Isopropanol (Merck, Germany)

Lactose (Merck, Germany)

Methanol (Merck, Germany)

Mevinolin (Sigma, St. Louis, MO, USA)

Peptone (Merck, Germany)

Phosphoric acid (Merck, Germany)

Potato Dextrose Agar (Merck, Germany)

Yeast extract (Merck, Germany)

2. Commercial angkak samples

Rice angkak from traditional drug store, Chiang Mai, Thailand

Rice angkak from Chinese grocery, Philadelphia, U.S.A.

Xuezhikang Jiaonang from pharmacency store, Beijing, China

3. Glassware

50 ml Polypropylene centrifuge tube (Corning, USA)

20 ml Polypropylene centrifuge tube (Corning, USA)

4. Instruments

Autoclave (Gallenkamp, England)

Centrifuge (Z200A, Hermle, Germany)

Colorimeter (Minolta Camera Co., Ltd., Osaka, Japan)

pH meter (Precisa 900, Switzerland)

Incubator (Gallenkamp, England)

Hot air oven (Haereous, England)

HPLC/DAD/MSD (Agilent1100, U.S.A.)

Spectrophotometer (Agilent 8453, Germany)

Vacuum dryer (N-Evap111, Organomation Association Inc., U.S.A.)

Water bath (Heto SBD50, Scientific promotion Co.Ltd., Thailand)

Methods

3.1 Validation of analysis methods

***3.1.1* Development and optimization of the HPLC/DAD/MSD**

Glucosamine standard were prepared at concentration of 0, 0.5, 1.0, 2.0, 5.0, 8.0 and 10.0 ppm. It was analyzed by HPLC/MSD using an 250X4.6 mm i.d.; C-18; 5 μm Nucleosil column. The mobile phase consisted of 55:35:10 (v/v/v) water/acetonitrile/isopropanol (added with conc. formic acid to 0.1% of mixed solution) as solution A and 100% acetonitrile as solution B (Appendix F). Both solution A and B were used to run gradiently, starting from 0 to 4 min with 100% of solution A with flow rate of 1.0 ml/min followed by decreasing the ratio of solution A to 50% from 4.1 to 12 min .The volume of 20 μl. either of samples or standards were injected into the column under room temperature (25 ^{O}C). The optimum condition of HPLC/MSD could detect glucosamine with retention time of 1.30 min.

Citrinin standard were prepared at 0, 0.2, 0.5, 0.8, 1.0, 1.5, 2.0 and 5.0 ppm. The optimum condition of HPLC was according to glucosamine standard and the retention time of citrinin was 4.45 min.

Mevinolin standard were prepared at 0, 5.0, 10.0, 20.0, 30.0, 40.0 and 50.0 ppm. It was analyzed by HPLC/DAD using an 250X4.0 mm i.d.; 5 μm Hypersil ODS column with water (pH 2.5)/acetonitrile/isopropanol (55:35:10 v:v:v) as solution A and 100% acetonitrile as solution B. Both solution A and B were run isocratically at ratio of 50:50 (v:v) for 20 min. The injection volumn was 20 μl. Diode array chromatogram of mevinolin standard at 238 nm with retention time of 11.14 min.

3.1.2 Precision and Accuracy determination

A system suitability test by validation of the chromatography system was performed before each run. The accuracy was demonstrated by determinining for %recovery. The sample blanks (dried plain unfermented adlay powder) were prepared by additionally spiked with 0.075 ppm glucosamine standard, 0.025 ppm citrinin standard respectively. The sample blanks spiked with 0.15 ppm mevinolin standard was prepared individually. The original sample blank (dried plain adlay powder) without any additional standards and with the spiked samples were prepared in triplicate, and inject three times for each sample. Each sample detection was from the average of three injection.

Relative standard deviation (%RSD) is demonstrated as the precision of analysis method for detection of the substance in samples. Tripical replications of standards of substances to be analyzed spiked in sample blanks was prepared according to the recovery examination above but did not determine for the sample blank alone.

3.1.3 Linearity

The calibration curve was constructed by plotting the peak area against the concentration using linear regression analysis. Glucosamine standards were prepared at: 0, 0.5, 1.0, 2.0, 5.0, 8.0 and 10.0 ppm and injected to HPLC/MSD with the optimum condition. Eight citrinin standards at 0, 0.2, 0.5, 0.8, 1.0, 1.5, 2.0 and 5.0 ppm were prepared for constructing the calibration curve and detected by HPLC/MSD with the same condition of glucosamine standards. Mevinolin standard at concentrations of 0, 5.0, 10.0, 20.0, 30.0, 40.0 and 50.0 ppm and detected by HPLC/DAD with the optimum condition.

3.1.4 Limit of detection and limit of quantitation determination

The LOD and LOQ were measured as the concentrations corresponding to signal-to-noise ratio of 3:1 and 10:1, respectively. Standard deviation of ten replications were used for LOD determination as 3SD and LOQ as 10SD.

3.2 Selection of *Monascus* strains for adlay angkak production

3.2.1 Microorganisms

The strains of *Monascus purpureus* (ATCC 16365, BCC 6131, DMKU, FTCMU) and *Monascus ruber* (TISTR 3006) were obtained from Thailand Institute

of Sciencific and Technological Research. All of these strains were cultivated on potato dextrose agar (PDA) (Merck, Darmstadt, Germany) for 10 day at 32-35°C before transferred to the adlay substrate.

3.2.2 Preparation of substrate and fermentation

Solid-state cultivation proceeded as follows: 25 g of polished adlay (obtained from Thai Cereals World Co. Ltd., Bangkok, Thailand) and 25 ml of water were put in 8″ X 12″ autoclavable polypropylene bag. The shape of polypropylene bag opening was manipulated similarly to Erlenmeyer flask's neck which was later plugged with cotton wool. The bags were then autoclaved at 121°C for 15 min prior to cooling down at room temperature. Each bag was inoculated with 1 cm diameter of each *Monascus* strain incubating on PDA plate. The cultures were cultivated for 28 days at room temperature (32-35°C). After incubation, the fermented adlay was transferred to a plastic bag and dried in hot air oven (Haereous, England) at 55°C for 3 days. Dried adlay angkak was ground to a fine powder (80 mesh).

3.2.3 Mevinolin analysis

3.2.3.1 Extraction of mevinolin from the samples

Sample of 1.0 g of adlay angkak was extracted with 5 ml of 68% ethanol, mixed well and placed in shaking water bath (Heto SBD50, Sciencific promotion Co.Ltd., Thailand) at 40°C for 12 h. The solution was centrifuged (Gallenkamp, England) at 1,780 g (3500 rpm), 15 min at 25°C. The filtrate was filtered through Whatman filter paper No.1 into 50 ml polypropylene centrifuge tube to be subsequently evaporated to dryness in vacuum dryer (N-Evap111, Organomation Association Inc., U.S.A.) at 50°C for 12 h. The dried extract was re-dissolved in 1 ml of mobile phase solution (solution A:solution B, 1:1 (v/v)), followed by filtering through 0.45 mm nylon membrane into 2 ml vial. Pure mevinolin was obtained from Sigma (St. Louis, MO, USA) and dissolved in methanol to prepare the standard curve.

3.2.3.2 HPLC conditions for mevinolin analysis

The concentration of mevinolin solution was analysed using Agilent 1100 high-performance liquid chromatograph (HPLC) equipped with a photodiode array detector. Analysis was perfomed using a Hypersil ODS column (Agilent technologies

250×4.0 mm i.d.; 5 μm) connected to two high precision pumps (Agilent 1100) set at a flow rate of 1.0 ml/min. The photodiode array detector set at 238 nm. The mobile phase consisted of water (pH is adjusted to 2.5 with conc. H_3PO_4)/acetonitrile/isopropanol (55:35:10 v:v:v) (solution A) and acetonitrile (solution B), running isocratically at ratio of 50:50 (v:v) for 20 min after 20 μl of extracted solution was directly injected into the HPLC system (Modified from Friedrich *et al.*, 1995; Ganrong *et al.*, 2000).

3.2.4 Citrinin and glucosamine analysis

3.2.4.1 Extraction of citrinin from the samples

Sample of 0.5 g of adlay angkak was extracted with 5 ml of 68% ethanol in 50 ml centrifuge tube by placing in shaking water bath (Heto SBD50, Scientific promotion Co.Ltd., Thailand) at 40°C for 12 h. The solution was centrifuged (Gallenkamp, England) at 1,780 g (3,500 rpm), 15 min at 25°C. The filtrate was filtered through the Whatman filter paper No.1 into 50 ml polypropylene centrifuge tube to be subsequently dried in vacuum dryer (N-Evap111, Organomation Association Inc., U.S.A.) at 50°C for 12 h. The dried extract was re-dissolved in 1 ml of mobile phase solution (solution A:solution B, 1:1 (v/v)) into 2 ml vial. Pure citrinin was purchased from Sigma (St. Louis, MO, USA) and dissolved in methanol (Modified from Sabater-Vilar *et al.*, 1999).

3.2.4.2 Extraction of glucosamine from the samples

Sample of 0.5 g adlay angkak was acid-hydrolyzed in 5 ml of 6N HCl in 50 ml polypropylene centrifuge tube to be subsequently incubated in hot air oven (Haereous, England) at 80°C for 16 h. After that the samples were neutralized by NH_3 solution (Merck, Germany) to pH 7. The filtrate was filtered through the Whatman filter paper No.1 into 50 ml polypropylene centrifuge tube to be subsequently evaporated to dryness in vacuum dryer (N-Evap111, Organomation Association Inc., U.S.A.) at 50°C for 12 h. The dried extract was re-dissolved in 1 ml of mobile phase solution (solution A:solution B, 1:1 (v/v)), followed by filtering through 0.45 mm nylon membrane into 2 ml vial. Glucosamine hydrochloride (Sigma, St. Louis, MO, U.S.A.) was acid-hydrolyzed to glucosamine and used as a standard solution (Modified from Vignon *et al.*, 1986).

3.2.4.3 HPLC condition for glucosamine and citrinin analysis

The concentration of glucosamine and citrinin solutions were analysed using Agilent 1100 high-performance liquid chromatograph (HPLC) equipped with a Mass spectrometer detector. Analysis was perfomed using a Nucleosil column (250×4.6 mm i.d.; C-18; 5 μm) connected to two high precision pumps (Agilent 1100) set at a flow rate of 1.0 ml/min. The mobile phase consisted of water/acetonitrile/isopropanol (55:35:10 v:v:v) added formic acid to 0.1% (solution A) and acetonitrile (solution B), running gradient continuously at ratio of 100% of (solution A) from 0 to 4 min and the ratio of solution A was decreased gradiently from 100% at 4.1 min to 50% at 12 min. The column was then reconditioned by using the initial solvent composition after 20 μl of extracted solution was directly injected into the HPLC system (Modified from Hajjaj *et al.*, 2000).

3.2.5 Pigment measurement

Sample of 0.5 g adlay angkak was extracted with 5 ml of 95% ethanol in 50 ml polypropylene centrifuge tube and rotated at 200 rpm for 2 h at room temperature (30°C). After that, the mixtures in 50 ml polypropylene centrifuge tube to be subsequently were centrifuged (Gallenkamp, England) at 1,780 g (3,500 rpm) for 15 min. Pigment extracts were measured by a spectrophotometer (Agilent 8453, Germany). Absorbance at 400, 470 and 500 nm represented yellow, orange and red pigments, respectively (Johns and Stuart, 1991).

3.2.6 Color measurement

The absorption spectra of adlay angkak were measured by a CR-300 colorimeter with the HunterLab color system (Minolta Camera Co., Ltd., Osaka, Japan). L value indicates lightness from 0 (dark) to 100 (light). Positives and negatives in value of a represented red and green, respectively, whereas positives and negatives in b value represent yellow and blue, respectively.

3.2.7 Moisture and pH determination

3.2.7.1 Moisture determination

Moisture content was calculated based on wet weight (Gangrong, 2000) as followed,

Moisture content of adlay angkak (%) = 100 * (wet weight of adlay angkak – dry weight of adlay angkak) / wet weight of adlay angkak

3.2.7.2 pH determination

Sample of 1.0 g adlay angkak was diluted with 20 ml of deionized water and the pH of the solution was determined by a pH meter (Precisa 900, Switzerland).

3.2.8 Statistical analysis

3.2.8.1 Statistical analysis for Monascus strain selection

This experimental design used completely randomized design (CRD). For each adlay angkak product cultivated by *Monascus* strain, three replications were used for the determination of every quality attribute. Method of analysis of variance (ANOVA) was applied to the data obtained from each treatment to detect differences at 5% level of significance ($P<0.05$). P values of 0.05 or less were considered significant and Duncan's New Multiple Comparison Method was applied for comparison of treatment means (SPSS ver.13.0, SPSS Inc., Chicago).

3.3 Methods to study kinetic behavior of *M. purpureus* DMKU cultured on adlay angkak

The solid-state fermentation of *M. purpureus* DMKU inoculated in sterilized adlay was carried out in bags for 57 days. Adlay angkak samples were taken every three days. Glucosamine, citrinin and mevinolin were analyzed according to 3.2.3-3.2.4. Pigments, color, moisture content and pH were determinated according to 3.2.5-3.2.7. Three replications were averaged as Mean±SD with trend line on graph.

3.4 Optimization of carbon and nitrogen sources on adlay angkak

3.4.1 Glucose and peptone were supplemented in adlay

3.4.1.1 Studies of glucose and peptone on adlay angkak cultivated with M. purpureus DMKU

The solid-state fermentation of *M. purpureus* DMKU inoculated in sterilized adlay for 28 days at room temperature (29-32°C). In order to identify the optimum conditions, a Central Composite Design was selected (Design-Expert ver.6.0.2, Stat-Ease, Inc., Minneopolis). The crucial factors involved were glucose (1-5%), peptone (0.1-0.5%). These factors, and the level at which the experiment was carried out, is given in Table 3.1. A total of 13 runs with 5 center points were generated. The central point of the design arrangement decided were 3% glucose, 0.3% peptone. Glucosamine, citrinin and mevinolin were analyzed according to 3.2.3-3.2.4. Pigments, color, moisture content and pH were determinated according to 3.2.5-3.2.7.

The analysis of data was carried out using Response surface methodology in statistical analysis system (Design-Expert ver.6.0.2, Stat-Ease, Inc., Minneopolis). A second order model was employed to fit the data individually for the responses Y by the general model, with two factors, each factor coded to be in the range of -1.414, -1, 0, +1, +1.414. Axial (α) of any experiment was assigned to $\pm 2^{n/4}$. n is number of factors in the experiments of these studies. Therefore, there experiments showed axial equal to $\pm 2^{2/4}$ or ±1.414.

$$\text{Axial } (\alpha) = \pm 2^{n/4} \qquad \text{Equation 3.1}$$

The model was evaluated in terms of statistically significant coefficient, R^2 and *P*-value. The quality to fit the second-order model equation was expressed by the coefficient of determination R^2, and its statistical significance was determined by an F-test. The response surface of the equation obtained $R^2>0.7$ were demonstrated with response surface plot (Statistica ver.5.0, Statsoft Inc., Tulsa).

$$Y = A_0 + A_1X_1 + A_2X_2 + A_{12}X_1X_2 + A_{11}X_1^2 + A_{22}X_2^2 \qquad \text{Equation 3.2}$$

Y is the predicted response; A_0, A_1, A_2, A_{12}, A_{11}, A_{22} are constant coefficients and X_1, X_2, X_1X_2, X_1^2, X_2^2 are the coded independent variables or factors.

Table 3.1 Levels in the glucose and peptone on response surface design of secondary-order model

Run	Nutrients	
	Glucose (%)	Peptone
1	1.59 (-1)	0.16 (-1)
2	5.00 (1.414)	0.30 (0)
3	3.00 (0)	0.30 (0)
4	3.00 (0)	0.30 (0)
5	3.00 (0)	0.30 (0)
6	1.59 (-1)	0.44 (1)
7	4.41 (1)	0.16 (-1)
8	4.41 (1)	0.44 (1)
9	3.00 (0)	0.30 (0)
10	1.00 (-1.414)	0.30 (0)
11	3.00 (0)	0.50 (1.414)
12	3.00 (0)	0.30 (0)
13	3.00 (0)	0.10 (-1.414)

3.4.1.2 Studies of glucose and peptone on adlay angkak cultivated with M. ruber TISTR3006

The solid-state fermentation of *M. ruber* TISTR3006 inoculated in sterilized adlay for 28 days at room (29-32°C). All experimental design, chemical, physical and statistical analyzes followed the same methods as 3.4.1.1.

3.4.2 Lactose and yeast extract were supplemented in adlay

3.4.2.1 Studies of lactose and yeast extract on adlay angkak cultivated with M. purpureus DMKU

The solid-state fermentation of *M. purpureus* DMKU inoculated in sterilized adlay for 28 days at room temperature (29-32°C). All experimental design, chemical, physical and statistical analyzes followed the same methods as 3.4.1.1. The crucial factors involved were lactose (1-5%), yeast extract (0.1-0.5%). These factors, and the level at which the experiment was carried out, is given in Table 3.2. A total of 13 runs

with center points were generated. The central point of the design arrangement decided on were 3%lactose, 0.3%yeast extract.

Table 3.2 Levels in the lactose and yeast extract on response surface design of secondary-order model

Run	Nutrients	
	Lactose (%)	Yeast extract (%)
1	4.41 (1)	0.44 (1)
2	3.00 (0)	0.10 (-1.414)
3	5.00 (1.414)	0.30 (0)
4	1.59 (-1)	0.16 (-1)
5	4.41 (1)	0.16 (-1)
6	3.00 (0)	0.30 (0)
7	1.59 (-1)	0.44 (1)
8	3.00 (0)	0.30 (0)
9	3.00 (0)	0.30 (0)
10	3.00 (0)	0.30 (0)
11	1.00 (-1.414)	0.30 (0)
12	3.00 (0)	0.50 (1.414)
13	3.00 (0)	0.30 (0)

3.4.2.2 Studies of lactose and yeast extract on adlay angkak cultivated with M. ruber TISTR3006

The solid-state fermentation of *M. ruber* TISTR3006 inoculated in sterilized adlay for 28 days at room temperature (29-32°C). All experimental design, chemical, physical and statistical analyzes followed the same methods as 3.4.1.1.

3.5 Studies on effect of carbon and nitrogen sources on adlay angkak

Combination of glucose-peptone or lactose-yeast extract was added in adlay as supplement for producing adlay angkak. *M. purpureus* DMKU and *M. ruber* TISTR3006 were cultivated on adlay added supplement according to Table 3.3. The solid-state fermentation of each strain was inoculated in sterilized adlay for 28 days at

room temperature (29-32°C). Glucosamine, citrinin and mevinolin, pigments, color, moisture content and pH were determinated according to 3.4.1.1. This experimental design used completely randomized design (CRD). For each adlay angkak product of *Monascus* strain, two replications were used for the determination of every quality attribute. Method of analysis of variance (ANOVA) was applied to the data obtained from each treatment to detect differences at 5% level of significance ($P<0.05$). P values of 0.05 or less were considered significant and Duncan's New Multiple Comparison Method was applied for comparison of treatment means (SPSS ver.13.0, SPSS Inc., Chicago).

Table 3.3 The formula of carbon-nitrogen source as the supplement in adlay

Nutrients
1. 1%Glucose+0.3%Peptone
2. 3%Glucose+0.1%Peptone
3. 3%Glucose+0.5%Peptone
4. 5%Glucose+0.3%Peptone
5. 1%Lactose+0.3%Yeast Extract
6. 3%Lactose+0.1%Yeast Extract
7. 3%Lactose+0.5%Yeast Extract
8. 5%Lactose+0.3%Yeast Extract

3.6 Comparison of adlay angkak with commercial red rice

Adlay angkak prepared from combinations of 1% glucose and 0.3% peptone, 3% lactose and 0.5% yeast extract obtained from experiment 3.5 were compared with adlay angkak without carbon-nitrogen source added (blank), red rice from Thailand, red rice from U.S.A. and Xuezhikang Jiaonang (drug). Glucosamine, citrinin and mevinolin, pigments, color, moisture content and pH were determinated according to 3.4.1.1. This experimental design used completely randomized design (CRD). For each adlay angkak product of *Monascus* strain, two replications were used for the determination for every quality attribute. Method of analysis of variance (ANOVA) was applied to the data obtained from each treatment to detect differences at 5% level of significance ($P<0.05$). P values of 0.05 or less were considered significant and

Duncan's New Multiple Comparison Method was applied for comparison of treatment means (SPSS ver.13.0, SPSS Inc., Chicago).