CHAPTER 3

Optimization of Monacolin K Production of *Monascus purpureus* mutant under Solid State Fermentation by Response Surface Methodology

3.1 Introduction

Solid state fermentation (SSF) of *Monascus* on rice has a long tradition in East Asian countries (Hesseltine, 1965). In China, red mold rice (RMR), red yeast rice (RYR) or "ankak" has been used as a food preservative and natural food coloring of fish, Chinese cheese, red wine and sausage (Lin, 1973). During the Ming Dynasty (1368-1644), RMR was used as a medicinal food to promote blood circulation (Li *et al.,* 1998). It is used as a dietary supplement in USA, Indonesia, Japan, Taiwan and Philippines. RMR contains various biologically active components, such as red pigment, γ-aminobutyric acid (GABA), monacolin, glucosamine, lecithin, flavonoid and sterols (Lin *et al.,* 2008). The most useful compound is monacolin K, a secondary metabolite that an inhibitor of the enzyme hydroxymethylglutaryl coenzyme A reductase (HMG-COA) which can limit the rate of cholesterol biosynthesis (Endo, 1979).

Monascus species can produce citrinin, which can be a contaminant of *Monascus* products. Citrinin is a secondary metabolite which was first found in 1931 from *Penicillium citrinum* and is also produced by other species of *Penicillium*, *Aspergillus* and *Monascus*. On account of its antibacterial effects, citrinin was investigated as an antibiotic (Wong and Koehler, 1981). It is a mycotoxin, reported to be hepatotoxic and nephrotoxic in mammals. It has a negative impact on the acceptable of *Monascus* fermented product that has limited its use as food additive, colorant and health food. Therefore, reduction of citrinin contents is the most important issue for consumer safety.

Monacolin K and citrinin production by *Monascus* species would be directly affected by type of substrates (Lee *et al.,* 2007a). Several substrates have been used in *Monascus* SSF such as coconut oil, grape waste, jackfruit seed powder, palm kernel cake, sesame oil cake and wheat bran (Babitha *et al.,* 2006; Silverira *et al.,* 2008). Agricultural waste such as coconut residue, corn meal, peanut meal and soybean meal were also reported as substrates (Nimnoi and Lumyong, 2009). Moreover, previously study indicated that type of substrate and *Monascus* strain affected on the level of monacolin K and citrinin production by *Monascus* species. This aimed to investigate the effect of various substrates and fungal strains of *M. purpureus* both wild type and mutant strains on the red pigment, monacolin K and citrinin production in SSF with the final goal for the best substrate and strain which gives the highest content of monacolin K and the lowest content of citrinin.

3.2 Materials and methods

3.2.1 Isolation and Identification *of M. purpureus*

Pure cultures of *M. purpureus* were isolated from red mold rice (RMR) purchased from local markets in Thailand and Hong Kong by the surface sterile technique. RMR grains were washed in 95% ethanol for 30 s, then soaked in 0.1% sodium hypochlorite solution for 1 min and finally soaked in 95% ethanol for 30 s (Figure 3.1). After that, the rice grains were placed on potato dextrose agar (PDA, Labscan®) plus 0.05% of rose bengal and incubated at 30°C for 7 days. The mycelia emerging from samples were aseptically transferred to fresh PDA. Pure cultures were maintained in PDA slant at 4°C and kept at the Sustainable Development of Biological Resources Laboratory, Faculty of Science, Chiang Mai University.

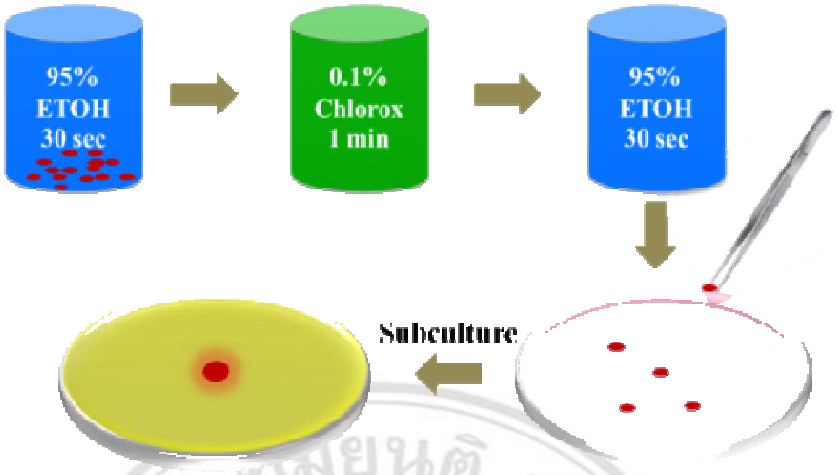

Figure 3.1 The process of surface sterile technique for isolation of *M. purpureus* from RMR

The strains isolated from commercial RMR were identified. The strain was inoculated on PDA and incubated at 30°C to observe morphological such as mycelium, conidiospore and ascospore. Pigment production was also observed as well as 6% sodium chloride and 30% ethanol tolerance. A molecular technique was use to identification. About 1 ml of culture medium including mycelium and spores was used for DNA extraction by Wizard® Genomic DNA Purification Kit (Promega, Madison, WI, USA) and stored at -20°C until use. The 18S rRNA gene was amplified by polymerase chain reaction (PCR) from these isolates as the templates using GoTaq®Flexi DNA Polymerase (Promega, Madison, WI, USA), with primers ; ITS4 and ITS5 in 25 µl reactions. The PCR products of 18S rRNA gene were purified using a Wizard® SV Gel and PCR Clean-Up System (Promega, Madison, WI, USA). 18S rRNA gene was sequenced using an ABI Prism 310 DNA sequencer (Perkin-Elmer Applied Biosystems, Foster City, Calif.), using the ABI Prism dye terminator cycle-sequencing ready-reaction kit (Perkin-Elmer Applied Biosystems). All sequence chromatograms were edited using Sequence Scanner Software version 2 (Applied Biosystems, Foster city, Calif.) and were compared with 18S rRNA gene sequences available in the DNA Data Bank of Japan.

3.2.2 Mutagenesis

3.2.2.1 Preparation of spore suspension

The spores of three wild type *M. purpureus* strains were collected from pure cultures grown on PDA at 30°C. After 7 days, added 10 mL distilled water (with 0.05 % Tween 80) in the plate and swirl handily. After that, the aliquot were filtered through wool glass and spore suspension was transferred to a sterile tube (Figure 3.2).

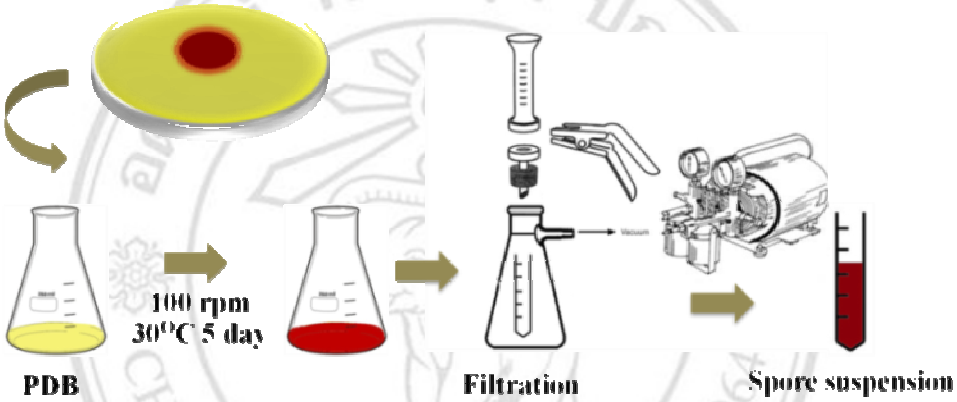

Figure 3.2 The preparation of spore suspension for mutagenesis

3.2.2.2 Induction of mutation

Ultraviolet rays (UV) and N-methyl-N'-nitro-N-nitrosoguanidine (NTG) were used in this study. Four milliliters of spore suspension (10^6 spore/ml) was spread on a Petri dish and placed under an ultraviolet lamp irradiated with different time from 0 to 30 min. In addition, treatment with the chemical mutagen NTG was conducted. One milliliter of NTG was added to 1 ml of spore suspension (Figure 3.3).

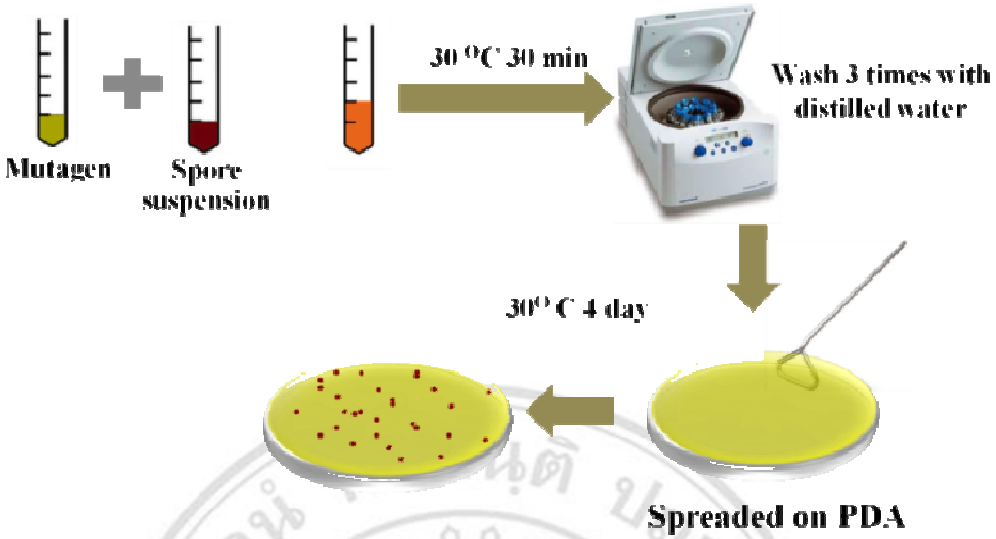

Figure 3.3 Induction of mutation by NTG treatment

The mixtures were incubated at 30°C for different concentration intervals ranging from 0.5-8%. The viable spores from both induced mutagenesis were harvested by centrifugation, washed three times with sterile distilled water, and spread on PDA and incubated at 30°C for 72 h. The colonies were counted and calculated for the survival rate after treatment. The optimal condition resulted at 0.1-1% survival was selected. The single colonies of survivors were screened by bioassay to select the target mutants.

3.2.3 Screening of lower citrinin producing strains

The bioassay method follows the reported (Wang *et al.,* 2004) using *Bacillus subtilis* as tested bacteria. Potato dextrose broth (PDB) was inoculated with one loop of *B. subtilis* and grown overnight at 37°C. The top of the agarose were melted before use by heating in a microwave. Aliquots of the melted agar were stored in a water bath at 47°C to keep the solution molten. Aliquot of molten agar were added to the first tube. The contents of the tube were mixed. The entire content of the tube was poured onto the center agar plate and swirled gently to ensure an even distribution of bacteria and top agarose. After that, hyphae of mutant strains were placed on PDA for 24–48 hour to observe whether an

inhibition zone formed. The inhibition ratio was determined using the following Equation 3.1,

$$\text{Inhibition ratio (\%)} = \left(\frac{P-M}{P}\right) \text{x } 100 \qquad (3.1)$$

where P is the average diameter of the inhibition zone of the parental strain and M is the average diameter of the inhibition zone of the mutant strain.

3.2.4 Solid state fermentation (SSF)

3.2.4.1 Preparation of Inoculum

Inoculumn was prepared from 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 (Figure 3.4).

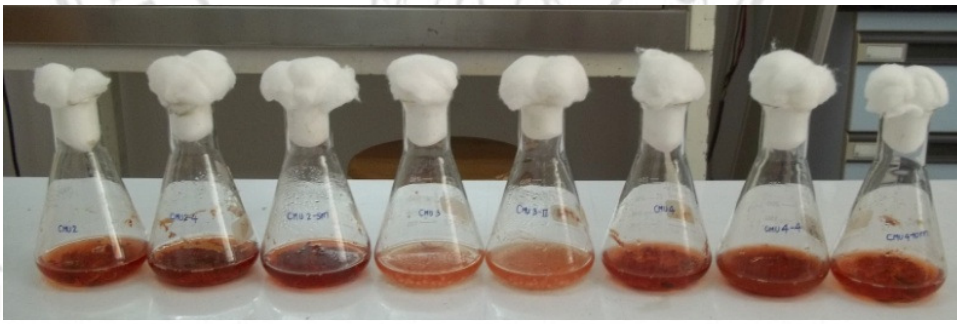

Figure 3.4 Inoculum of *M. purpureus* both wild type and mutant strains

3.2.4.2 Selection of substrate and *M. purpureus* strain

Sanpatong glutinous rice, RD 6 glutinous rice, dioscorea, sweet potato and taro were purchased from a local market in Chiang Mai, Thailand. Twenty gram of rice that was pre-soaked for 3 h and 20 g of non-soaked rice were autoclaved at 121°C for 15 min. After cooling, The moisture content of rice and other substrates were adjusted to 60% (w/w) on a wet basis. The inoculumn size was measured at

5% (10^6 spore/ml) and the specimens were incubated at 30°C for 14 days. The end products were dried in an oven at 60°C overnight to obtain dried RMR.

3.2.5 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 Chairote *et al.* (2007).

3.2.5.1 Monacolin K detection

Half gram of RMR were extracted with 10 ml of 75% ethanol. The supernatants were collected after centrifugation using 3,000 rpm speed at 4°C. The extract volume was increased to 50 ml using 75 % ethanol and filtered through a 0.2 µm nylon membrane and kept in a vial before being analyzed by HPLC. The samples were analyzed using Shimadzu Prominence UFLC system equipped with LC-20AD pump, SIL-20ACHT autosampler, CTO-20AC column oven, CBM-20A system controller and SPD-20A UV/VIS detector (Shimadzu, Japan) as well as a RP-C18 column (Mightysil : 150 × 2.0 mm, 5 µm) was used at 30°C. Acetonitrile–0.5% phosphoric acid (65:35 v/v) was used as mobile phase. The eluent was pumped at a flow rate of 0.7 ml/min. UV detection was set at 238 nm.

3.2.5.2 Citrinin detection

One gram of RMR was extracted with 10 ml of acetone: ethyl acetate (50:50, V/V) at 65°C for 90 min. The upper phase was evaporated to dryness and was then dissolved in acetronitrile, followed by filtering with a 0.2 µm pore size nylon membrane filter and analyzed by HPLC. Citrinin was determined by HPLC on a RP-C18 column (Mightysil: 150 × 2.0 mm, 5 µm) using the mobile phase with the composition of water: acetonitrile: triflouacetate (450:550:0.5). The flow rate was set at 0.6 ml/min, and the detector used was a fluorescence detector. The excitation and emission wavelength was 330 and 500 nm, respectively.

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 sample extract and calibration curve.

3.2.6 Optimization of monacolin K production

3.2.6.1 Plackett-Burman experiment design

To determine the variables significantly on monacolin K production, a Plackett-Burman design (PBD) was formulated. An experimental design of 15 runs containing 3 central points and 5 independent variables were tested at 3 levels, high (+1), medium (0), low (-1) and range of different level for the variables were listed in Table 3.1. The other factors including initial moisture (50%), amount of rice grain (20 g) and temperature (30°C) were set at constant. Response value was measured in term of monacolin K yield.

Table 3.1 Range of different factors studied in PBD

Factors		Level		
Name	Code	-1	0	+1
Soak (hr)	A	3	4.5	6
Fermentation time (day)	B	15	16.5	18
Yeast extract (g)	C	0.15	0.225	0.3
Peptone (g)	D	0.15	0.225	0.3
Inoculumn size (%)	E	5	7.5	10

3.2.6.2 Central composite design

Preliminary study of Plackett-Burman experiment design indicated that monacolin K production was influenced by soak time and fermentation time. These factors were selected for further optimization by response surface methodology using 5 levels of Central Composite design (CCD).

Table 3.2 Range of the independent variable used in CCD

Factor		Level				
Name	Code	-α	-1	0	+1	+α
Fermentation time (day)	X_1	10.9	15	25	35	39.1
Soak (hr)	X_2	0.2	1.2	3.6	6	7

A total number of 13 runs were carried out with three replicates at the center point and axial point located at a specified distance α from the design center in each direction each axis. The actual value of the factors at various levels was showed in Table 3.2. The model was represented by the following quadratic Equation 3.2,

$$Y = \beta_0 + \sum \beta_i x_i + \sum \beta_{ij} x_i x_j + \sum \beta_{ii} x_i^2 \quad (3.2)$$

where, Y is the predicted response, β_0 is the offset term, β_i is the linear offset, β_{ii} is the squared offset, β_{ij} is the interaction effect and x_i is the dimensionless coded value of x_j. The relative effect of two factors on monacolin K production was identified from the contour and response surface plot. All experimental designs and statistical data were analyzed by using software Design-Expert® 7.0.0

3.2.6.3 Validation of the model

Validation experiment was conducted to determine the maximum monacolin K production when the variables were set at the optimum levels established above, through Plackett-Burman experiment design and CCD. Percentage of validation was calculated according to Equation 3.3,

$$\% \text{ Validation} = \frac{\text{Actual value}}{\text{Predicted value}} \times 100 \quad (3.3)$$

3.3 Results and discussion

3.3.1 Isolation and identification

Three wild type strains, CMU002 and CMU003 were isolated from Thailand commercial RMR whereas wild type strain CMU004 was isolated from Hong Kong commercial RMR. The fungal were identified based on morphology (macroscopic and microscopic characteristics) and molecular technique to confirm the classification (Iizuka and Lin, 1981). The colour and characteristic of colonies were observed initially white colonies, became orange on PDA after 7 days at 30°C (Figure 3.5) and changed to red colour after 2 weeks of incubation. The results according to Nimnoi and Lumyong (2009) who studied the growth of *M. purpureus* on PDA, the colony were compacted and secreted the red pigment into medium. The colour of mycelium was depended on media type. The conidiospore, ascospore and cliestothecia were observed under a light compound microscope and the morphology characteristics of all strains were shown in Table 3.3. Analysis of ITS gene sequence confirmed that the strain CMU002, CMU003 and CMU004 were *M. purpureus*.

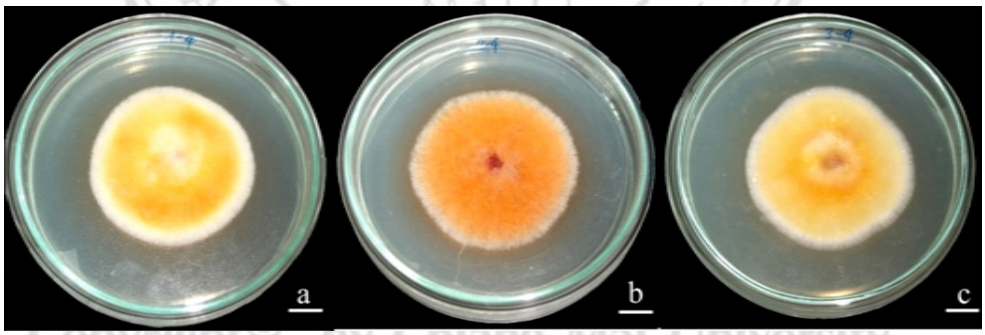

Figure 3.5 Isolation of *M. purpureus* from commercial RMR; 7 days old colonies on PDA; (a) CMU002, (b) CMU003, (c) CMU004 (bars = 1 cm)

Table 3.3 Morphological, biochemistry test and molecular confirmation of *M. purpureus* CMU002, CMU003 and CMU004

Identification	7 days of cultivation on PDA		
	CMU002	CMU003	CMU004
Morphological			
Colony			
Diameter	48 mm	47 mm	46.5 mm
Colour	Yellow-orange	Orange	Yellow-orange
Shape	Regular raised	Flat	Regular raised
Aerial mycelium	Short, abundant, white	Short, rare, white	Short, abundant, white
Conidia			
Shape	Subglobose	Subglobose	Subglobose
Colour	No	No	No
Ascospore			
Shape	Oval	Oval	Oval
Colour	No	No	No
Biochemistry test	-*	-	-
30 % Ethanol	-	-	-
6% NaCl			
Accession number	LC057318	LC057319	LC057320

* No growth

3.3.2 Mutagenesis

Three wild types of *M. purpureus* were induced by UV and NTG. The spore suspension was spreaded on PDA plates for observed the viable spores. The survival rate was selected according to previous study. Zambare (2010) suggested that 0.1-1% was appropriated for primary selected the mutant strain. The results

showed that 0.1-1% of survival rate were found to be at 20 to 30 min for UV rays and 4 to 8% (w/v) concentration of NTG**.** The single colonies of survivors were selected for primary screening (Figure 3.6).

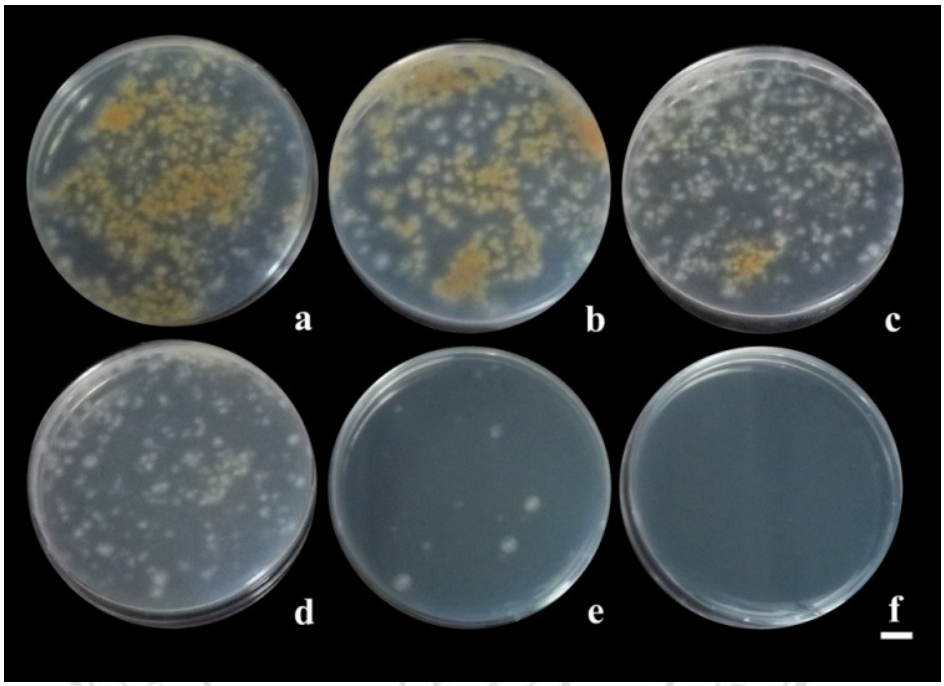

Figure 3.6 Survival of *M. purpureus* CMU002 at different exposure times of UV rays (a) control, (b) 5 min, (c) 10 min, (d) 15 min, (e) 20 min and (f) 30 min (bar = 1 cm)

3.3.3 Screening of lower citrinin producing strains

The purpose of mutation was to improve the monacolin K yield and obtain a better strain than wild type. Screening on PDA with *B. subtilis*, demonstrated the citrinin could inhibit the bacterial growth (Wang *et al.,* 2004). The inhibition zone formed around the fungal colony due to the antibacterial activity of citrinin. Large clear zone related to high citrinin production and small clear zone associated to lower citrinin production. The *Monascus* strain with no or small inhibition zone were selected. Five of the fungal colonies from a total of 225 showed the inhibition zones smaller than the wild type strains. *M. purpureus* strain CMU002U, CMU003U and CMU004U were obtained from UV rays and CMU002N and CMU004N were obtained from NTG treatment. All mutant strains

showed the significant difference inhibition zone from wild type strain (Table 3.4). The smallest inhibition zone was found in *M. purpureus* strain CMU003U, and the other colonies shown in Figure 3.7.

Table 3.4 Average diameter of inhibition zone

Strains	Average diameter (mm)
CMU002	9.00 ± 1.73*[b]
CMU002U	4.00 ± 2.18[a]
CMU002N	3.67 ± 0.58[a]
CMU003	16.17 ± 2.75[c]
CMU003U	3.50 ± 0.87[a]
CMU004	10.00 ± 0.50[b]
CMU004U	2.67 ± 0.58[a]
CMU004N	3.50 ± 1.50[a]

*Average ± standard error from triplicate samples. Means within column followed by the same letter(s) are not significantly different at ($P \leq 0.05$) by ANOVA analysis using Turkey test.

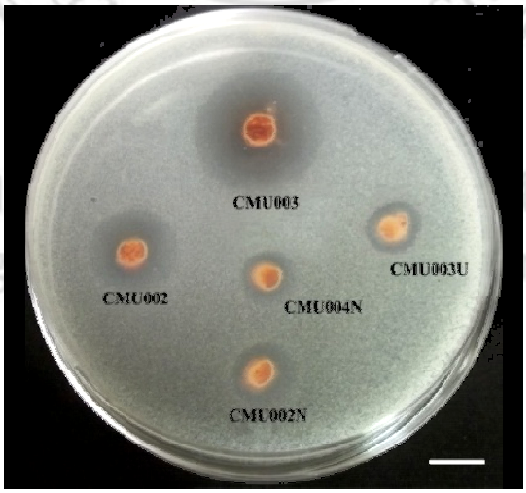

Figure 3.7 Wild type and mutant strains of *M. purpureus* showed the antibacterial activity to against *B. subtilis* when culture on PDB medium incubated at 30°C in the dark for 24 hour (bar = 1 cm)

3.3.4 Solid state fermentations

Five substrates including Sanpatong glutinous rice, RD 6 glutinous rice, dioscorea, sweet potato and taro were used in this study. The eight strains of *M. purpureus* were selected from previous parts of this experiment. Three wild types and 5 mutant strains were employed on these substrates under solid state fermentations at 30°C for 14 days. SSF was incubated at a similar temperature used in many previous reports which stated that the highest yield of monacolin K was obtain at 30°C (Su *et al.,* 2003). After fermentation, RMR from Sanpatong and RD 6 glutinous rice revealed a soft texture, orange-red colour and a pleasant odor. Products of RMR had a similar texture and color to the products described by Chairote *et al.* (2008). *Monascus* fermented products from dioscorea sweet potato and taro had a crumbly texture and dark red colour (Figure 3.8).

Figure 3.8 The *Monascus* fermented products from various substrates by *Monascus purpureus* CMU002; (a) Sanpatong glutinous rice (b) RD6 glutinous rice (c) dioscorea (d) sweet potato and (e) taro (bar = 1 cm)

The suitable substrate and strain were chosen from the high yield of monacolin K (Table 3.5). *M. purpureus* CMU002U gave the highest monacolin K

value on Sanpatong glutinous rice at 12,273 ppm and 439 ppb of citrinin which was a 17.7% monacolin K increase and a 17.2% citrinin decrease when compared with the *M. purpureus* CMU002 wild type strain. The contamination of citrinin is a major problem that influences consumer acceptance. This toxin damages the liver and kidneys of mammals. The present study agrees with previously published data (Wang *et al.,* 2004) that reported that UV rays and NTG could induce mutations causing decreased citrinin levels. The Food and Drug Administration (FDA) has recommended that citrinin in agricultural products for sale do not exceed 20 ppb. Japan stipulates that the citrinin concentration limit is 200 ppb. In Taiwan and the European Union (EU), the standard specification on the citrinin concentration level for functional food products should be less than 2000 ppb (Xu *et al.,* 2006; Le Bloc'h *et al.,* 2015). However, it was reported that the toxicity test of 200 ppm citrinin on Wistar rats for 90 day did not affect the function of the kidneys and liver or cause nephrotoxic and hepatotoxic reactions (Lee *et al.,* 2010). According to safety considerations, the concentration of citrinin in our RMR products may be considered a safe concentration.

Table 3.5 Effect of substrates and *M. purpureus* strains on monacolin K and citrinin production

Monacolin K (ppm)	Sanpatong glutinous rice	RD6 glutinous rice	Dioscorea	Sweet potato	Taro
CMU002	10431±289*	8024±313	2422±110	2135±215	0
CMU002U	12273±365	8152±232	2178±137	2130±156	0
CMU002N	8142±344	10124±36	2175±232	2292±132	0
CMU003	5078±160	7343±174	2941±171	3125±175	0
CMU003U	4692±238	4377±262	2911±245	5411±236	0
CMU004	5937±113	5269±167	2242±136	2263±119	0
CMU004U	11062±332	6089±234	2173±218	2167±152	1424±135
CMU004N	7933±275	6632±133	2226±187	2439±162	0
Citrinin (ppb)					
CMU002	530±15	605±31	16±1	26±6	79±15
CMU002U	439±12	572±12	14±0	0	195±12
CMU002N	339±15	536±50	6±0	0	151±15
CMU003	728±26	784±15	339±3	31±2	276±26
CMU003U	264±1	387±35	7±0	0	448±57
CMU004	691±1	620±21	222±11	0	205±10
CMU004U	435±38	312±10	0	0	143±37
CMU004N	330±15	443±46	0	0	234±15

In other studies, mutant strains were obtained by gamma irradiation to increase monacolin K yield (Suh *et al.,* 2007). Different factors on SSF including raw materials, the strain of fungus and the cultivation time affected the secondary metabolite production (Carvalho *et al.,* 2007). Using a co-culture of *M. purpureus* and *M. ruber* on long grain rice (non-glutinous) as the substrate under optimum conditions, monacolin K increased to 2,830 ppm (Panda *et al.,* 2010). Moreover, monacolin K could be produced by other strains of fungi or other substrates. SSF under suitable conditions on wheat bran by *Aspergillus flavipes* increased the monacolin K value to 16,780 ppm (Valera *et al.,* 2005).

Therefore, Sanpatong glutinous rice and the mutant strain *M. purpureus* CMU002U were selected for the optimization of monacolin K production. This determination was made according to the results of Chairote *et al.* (2008) who used the Sanpatong glutinous rice as a substrate fermented with *M. purpureus* CMU001 that was able to obtain a monacolin K yield at about 33,540 ppm.

3.3.5 Optimization of monacolin K production

From preliminary study, Sanpatong glutinous rice fermented *M. purpureus* CMU002U was a good condition for monacolin K production. Sanpatong glutinous rice was available and cheap substrate in our country. For screening the significant factors to monacolin K yield. A total of 5 factors were screened through 15 experimental runs by the Plackett Burman design. The predicted and experimental monacolin K yield was shown in Table 3.7.

Table 3.7 Experimental design and response of Plackett-Burman study

Run	A (hr)	B (day)	C (g)	D (g)	E (%)	Monacolin K (ppm)	
						Actual	Predicted
1	6	15	0.3	0.3	10	6734.8	5037.8
2	3	15	0.15	0.15	5	11639.5	10805.8
3	6	18	0.3	0.15	5	12059.3	12479.3
4	3	18	0.15	0.3	10	16020.4	15065.9
5	6	18	0.15	0.3	10	10372.9	11798.4
6	4.5	16.5	0.225	0.225	7.5	9672.1	9796.2
7	3	15	0.15	0.3	5	9182.1	10566.7
8	6	15	0.3	0.3	5	5516.1	6389.4
9	3	18	0.3	0.3	5	16539.4	15507.7
10	3	15	0.3	0.15	10	8061.9	8544.4
11	4.5	16.5	0.225	0.225	7.5	8841.4	9796.2
12	3	18	0.3	0.15	10	13442.2	14395.2
13	6	18	0.15	0.15	5	14201.3	13389.1
14	4.5	16.5	0.225	0.225	7.5	10875.1	9796.2
15	6	15	0.15	0.15	10	6396.2	6186.7

The *P*-value less than 0.05 were considered to be significant. Among these variables, soak time and fermentation time had *P*-value below the significance level (0.05). Therefore, these variables were estimated to affect the monacolin K production. This model was significant and statistical analysis of the model was shown in Table 3.8.

Both lack of fit and curvature of model were not significant, indicating that the models fit the data appropriately. Fermentation time had a positive significance, so increased this factor would be increased monacolin K yield. From the model, inoculumn size, peptone and yeast extract were not significant factors and showed a negative effect.

Table 3.8 Analysis of significant independent variables on monacolin K production

Source	Sum of squares	df	*F* value	*P* > *F*	
Model	142859858	5	15.90	0.0006	Sig
A-Soak	32029508	1	17.83	0.0029	
B-Time	102695403	1	57.15	< 0.0001	
C-Yeast extract	2483115	1	1.38	0.2736	
D-Peptone	171532	1	0.10	0.7652	
E-Inoculumn size	5480300	1	3.05	0.1189	
Curvature	2650973	1	1.48	0.2591	
Residual	14374443	8			
Lack of Fit	12283268	6	1.96	0.3760	Non sig.
Pure Error	2091174	2			
Cor Total	159885274	14			
Statistical analysis					
Std. Dev.	1340.45		R-Squared		0.909
Mean	10636.98		Adj R-Squared		0.851

Inoculumn size at 5% (v/w) was used for SSF in further experimental. Low inoculumn size led to insufficient biomass and high inoculumn related to over production of biomass leading to quick depletion of nutrients thus less monacolin K production, (Latha *et al.,* 2012). The supplement of additional nitrogen sources including peptone and yeast extract had shown a negative impact on the monacolin K production. Our results were contrary to some report showed that nitrogen source was significant limiting factor influencing the regulation of monacolin K production (Miyake *et al.,* 2006). In 2010, the experimental of Seraman *et al.* showed that peptone had a positive effect on monacolin K production. However, the difference may be caused from the different growth conditions, strain and fermentation patterns.. Therefore, nitrogen sources both peptone and yeast extract were eliminated from next experiment. Furthermore, the model could be checked by the determination of multiple correlation coefficient (R^2). R^2 of the first order model was found to be 0.909 which described that

90.9% of the variability in the response could be explained the model. However, the difference between the adjusted R^2 (0.851) and predicted R^2 (0.663) explained to a first order model was not an adequate mathematical equation for demonstration the significant independent variable and monacolin K yield. The response surface methodology was performed to evaluate the optimum level of soak time and fermentation time for monacolin K production. The level of the variables for this experiment was selected from the result of Plackett-Burman design. A total of 13 experiment runs with different level of variables, the predicted and experimental monacolin K yield were shown in Table 3.9.

Table 3.9 CCD of factors in coded levels with monacolin K as response

Run	X_1 (day)	X_2 (hr)	Monacolin K (ppm)	
			Actual	Predicted
1	15.0	1.2	2742.0	597.2
2	25.0	3.6	35394.8	35758.4
3	25.0	3.6	38886.9	35758.4
4	15.0	6.0	11466.5	12736.4
5	25.0	0.2	9531.2	12091.4
6	25.0	3.6	33632.8	35758.4
7	25.0	3.6	37218.9	35758.4
8	25.0	7.0	21238.3	18969.5
9	35.0	1.2	28601.9	27040.5
10	10.9	3.6	2858.9	3417.2
11	35.0	6.0	22775.2	24628.5
12	39.1	3.6	30791.3	30524.5
13	25.0	3.6	33658.7	35758.4

The second order polynomial Equation (3.5) obtained for monacolin K production was as follow,

$$\text{Monacolin K yield} = 35758.42 + 9583.87\,(X_1) + 2431.77\,(X_2) - 3637.79\,(X_1)(X_2) - 9393.79\,(X_1^2) - 10113.97\,(X_2^2) \quad (3.5)$$

where X_1 and X_2 were the fermentation time and soak time, respectively. The fitting of the model was calculated to be 0.978 by the coefficient of determination (R^2), which indicated that 97.8% of the variability in the response could be explained the model (Table 3.10). The three-dimensional graphs for the response surface model were shown in Figure 3.5.

Table 3.10 Analysis of variance (ANOVA) for response surface quadratic model obtained from experimental design

Source	Sum of squares	df	*F* value	*P* > *F*	
Model	2008051686	5	62.27	< 0.0001	Sig.
A-Time	734803888	1	113.92	< 0.0001	
B-Soak	47308114	1	7.33	0.0303	
AB	52934099	1	8.21	0.0242	
A^2	613866275	1	95.17	< 0.0001	
B^2	711598831	1	110.33	< 0.0001	
Residual	45149778	7			
Lack of Fit	24170071	3	1.54	0.3353	Non sig.
Pure Error	20979707	4			
Cor Total	2053201464	12			
Statistical analysis					
Std. Dev.	2539.68		R-Squared	0.978	
Mean	23753.64		Adj R-Squared	0.962	

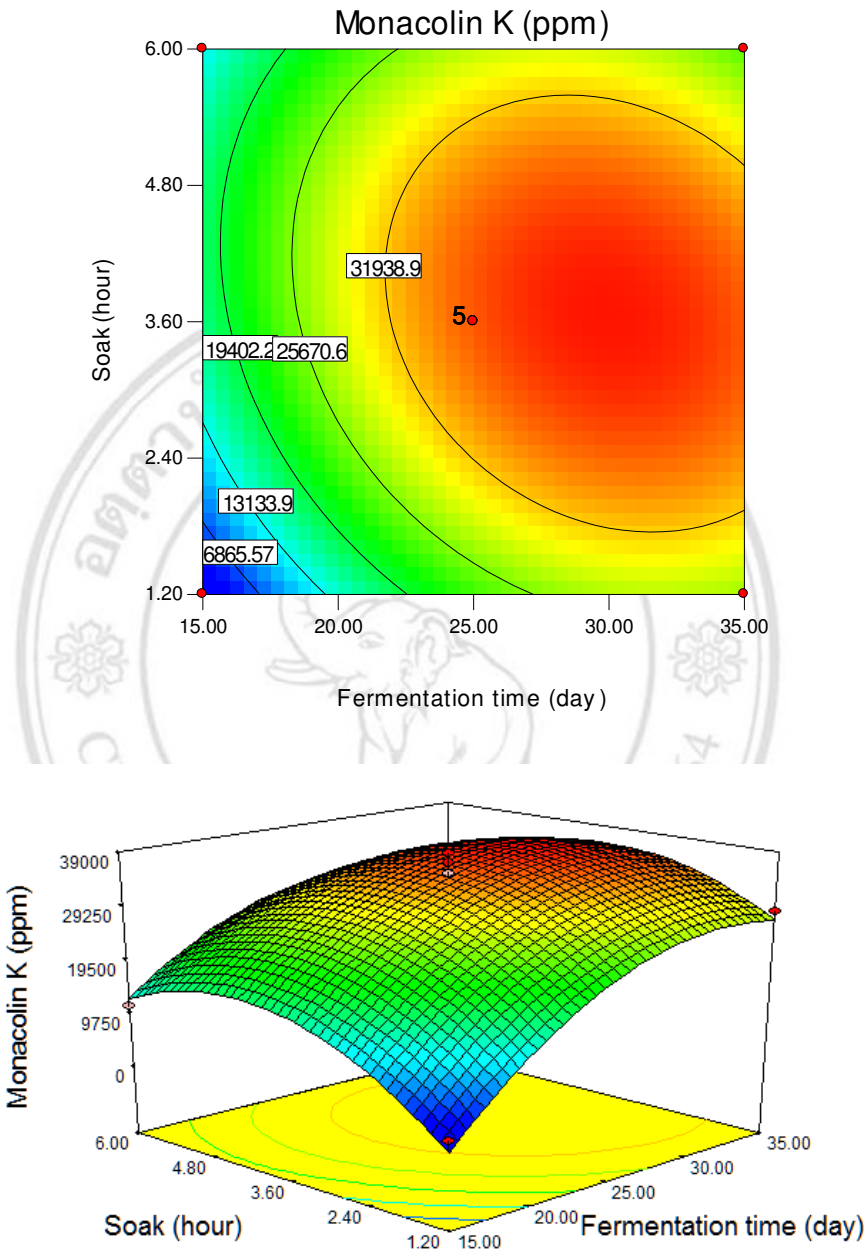

Figure 3.9 Contour and response surface plot indicating the effects of interaction between soak and fermentation time on monacolin K production from Sanpatong glutinous rice by *M. purpureus* CMU002U

To confirm the correctness of the model, 3 experiments were conducted using the optimum conditions from statistical analysis. The maximum monacolin K yield was 34,758 ppm, calculated to 96.6% validation and enhanced to 2.83 folds after optimization (Table 3.11).

Table 3.11 Validation of Quadratic model

Factors		monacolin K (ppm)		% Validation	Fold after optimization
Fermentation time (day)	Soak (hr)	Predicted	Actual		
35	3.46	35984.5	34758.6	96.59	2.83
35	3.42	35982.4	34211.7	95.08	2.79
35	3.78	35806.5	34156.2	95.39	2.78