การศึกษาสารสีแดงและซิตรินินในข้าวแดงที่เตรียมจากข้าวไทย

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บทคัดย่อ

งานวิจัยนี้เตรียมข้าวแดงโดยการหมักข้าวไทยชนิดต่างๆ โดยเฉพาะพันธุ์ที่รู้จักกันดี ได้แก่ ข้าวเหนียวขาว กข6 (RD6) และสันป่าตอง 1(SPT1) ข้าวเหนียวสีม่วงหรือข้าวก่ำ (Kam) รวมทั้งข้าวเจ้าหอมมะลิ (Mali 105) โดยใช้เชื้อราโมแนสคัสเพอพิวเรียส CMU 001 และศึกษาผลของสารอาหารต่างๆ เช่น น้ำนมถั่วเหลือง ที่เติมลงไปในการเพาะเลี้ยงเพื่อเป็นแหล่งของกรดอะมิโน จากนั้นจึงศึกษาข้าวแดงที่ได้ ในแง่ของความเข้มข้นของสารสีแดง ผลที่ได้แสดงให้เห็นว่าสีแดงมีความเข้มสูงสุดในกรณีการเพาะเลี้ยงบนข้าว กข6 (RD6) เป็นเวลา 3 สัปดาห์โดยไม่เติมน้ำนมถั่วเหลือง ในทางตรงกันข้ามหากเติมน้ำนมถั่วเหลืองพบว่าสีแดงจะเข้มที่สุดภายใน 2 สัปดาห์เท่านั้น ในส่วนการวิเคราะห์ปริมาณซิตรินิน พบว่าซิตรินินมีปริมาณสูงสุดเมื่อเพาะเลี้ยงบนข้าวสันป่าตอง1 (SPT1) เป็นเวลา 2 สัปดาห์ ข้าวแดงที่มีซิตรินินต่ำเป็นกลุ่มข้าวเหนียว กข6 (RD6) ที่เวลา 3 สัปดาห์และเติมน้ำนมถั่วเหลือง

คำสำคัญ : ซิตรินิน / เอชพีแอลซี / *Monascus purpureus* / สารสีแดง / ข้าวแดง

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Study on Red Pigment and Citrinin Present in Red Yeast Rice Prepared from Thai Rice

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Abstract

Red yeast rice was prepared using *Monascus purpureus* CMU001 by fermentation on various kinds of Thai rice, especially, the most well known varieties of white glutinous rice such as Kor Kho 6 (RD6) and Sanpathong 1 (SPT1), purple glutinous rice (Kam), and non-glutinous rice (Mali105). In order to study the effect of nutrient variation, soybean milk was also added during cultivation as amino acids source. The red yeast rice products obtained were studied for the intensity of red pigment. The results indicated that the most intense red pigment was present in RD6 at 3 weeks of cultivation without soybean milk. On the other hands, the highest intensity was achieved when RD6 used was only at 2 weeks of cultivation with soybean milk. For the analysis of citrinin content, the results suggested higher amount when glutinous rice was used. The highest amount was found in SPT1 red yeast rice fermented for 2 weeks. The best result with the lowest amount was found, among glutinous rice, in RD6 fermented for 3 weeks in the presence of soybean milk.

Keywords : Citrinin / HPLC / *Monascus purpureus* / Red Pigment / Red Yeast Rice

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1. Introduction

Red yeast rice is a product obtained by fermentation using *Monascus purpureus* [1]. Solid culture of the fungus on rice grain produces intense red pigment as well as characteristic odor after drying. The product is called differently according to the local language. Chinese calls “Ankak”, “Anka”, “Ang Khak” or “Hong Qu” while Japanese calls “Beni-Koji” or “Anka-Koji” [1-4].

Chinese red yeast rice is used in Thailand as food additive to make pleasant colored food such as red soybean curd, sausage, Nham, and sour fish. Sausage and Nham made from pork give stable product without dissociation of red color comparing to conventional product treated with E-249 (nitrite salt) and E-252 (potassium nitrate). The nitrogen compounds mentioned is one of the carcinogenic compounds causing cancer [5]. Nham is one of the most popular foods that contain potassium nitrate as additive to give good appearance. The use of red yeast rice in place of potassium nitrate is a safer way. However, the difficulty of red yeast rice use as colorant is the presence of a mycotoxin, citrinin, which is a compound causing renal failure [5].

Citrinin is the secondary metabolites which are formed after maximum use of nutrients in normal growth. The rice that reaches the stage faster may start to produce citrinin earlier. The polyketide pathway is the major route for the formation of secondary metabolites [6] including various mycotoxins [7] in most of the filamentous fungi. Citrinin is a typical toxin also isolated in *M. rubber* [8]. It is known that in *Aspergillus*, citrinin is formed by the condensation of one acetyl coenzyme A (acetyl-CoA) molecule with four malonyl-CoA molecules, followed by the addition of three methyl units [9].

Since citrinin is a toxic product, it is essential that the production of red pigments as food additives from *Monascus* spp. avoids the occurrence of citrinin. Regulation and control of the condition of fermentation is also studied in order to decrease citrinin. The presence of fatty acids, such as hexanoic and octanoic acid, favors the production of pigments as well as citrinin. The citrinin content becomes higher when using aeration. Malic acid addition decrease pigments concentration but has no effect to citrinin content. In case of amino acids addition, it was found that histidine increase the red pigment for six times, while citrinin was not increased. The fermentation of hydrogen peroxide caused by histidine addition may break down the citrinin structure. Apart from controlling of the cultivation condition, the amount of citrinin can be blocked by using peroxidase enzyme (Blanc *et al*.) [10]. This result agree with the work of Hajjaj *et al*. [11] who studied the effect of 6-8 carbons fatty acids to the formation of citrinin in *Monascus*. The studied was carried out by liquid fermentation with 5.20 g/l of glucose, 5 g/l glutamate adding with octanoic acid which was the precursor of red pigment synthesis via polyketide synthesis pathway. The fatty acid increased the red pigment for 30-40 % while the amount of citrinin was decreased. Chen and Hu [12] studied on red fermented rice with high concentration of monacolin K and low concentration of citrinin, found that, a mutation strain, *Monascus* spp. M12-69, was acquired by treatment with mutagenic agents from a wild strain M12 of Monascus screened from red fermented rice samples gathered around China. According to the classification guide of Hawksworth and Pitt on *Monascus* genus, they belong to *M. pilosus* Sato. The conditions of the solid state fermentation of M12-69 were optimized. At the optimum conditions, the concentrations of monacolin K and citrinin in

red fermented rice, which was dried at 50°C to a constant weight, were 2.52 mg/g and 0.13 ng/g, respectively. These results reveal that Strain M12-69 is a potential strain, which can be used to produce red fermented rice with high concentration of monacolin K and low concentration of citrinin.

According to rice used to prepare red yeast rice, there are 2 main varieties of rice grown and consumed by most people in different parts of Thailand. They are non-glutinous rice and glutinous rice. The amylopectin content in glutinous rice is higher (95%) than in non-glutinous rice (70-90%). The latter contains about 10-30% of amylose. Some varieties of glutinous rice have very small amounts of amylose or without amylose [13]. The difference in their main composition may affect the content of useful fermentation metabolites. Both kinds of rice compose of various cultivars. Many works use non-glutinous rice for making red yeast rice. Palo *et al*. [14] prepared red yeast rice from non-glutinous rice and glutinous rice. Comparing of the color between different cultivars, using the condition reported, it was found that glutinous rice and non-glutinous rice, Mali, had the same red color which was similar to Japanese rice. The color of glutinous red yeast rice was more pleasant than Mali rice. Hanpongkittikul [15] claimed that the amylose content affected the production of red color. He reported that rice cultivars with more than 24% amylose such as Luang 148, Kor Kho 23 and Kor Kho 25 were better than Kor Kho 7 and Mali 105 for red color. In the year 2002, red yeast rice used as red colorant of sausage was studies (Pattanagul) [16]. Red yeast rice using Chai Nat rice was the most suitable for the sausage. Recently, Boonsangsom *et al*. [17] reported that Mali rice fermented by *M. purpureus* ATCC 16365 could reach 623 unit/g of red color value. Therefore, the glutinous rice abundantly grown in the northern part of Thailand is interesting in our work to be used as potential raw materials for making red yeast rice.

Considering the effect of other alternative nutrient sources to the color production by *Monascus*, Lin and Iizuka [18] found that bread had better red color than potato. Other nutrient sources such as maize grain, green bean and cassava gave unsatisfied product. According to the condition of culture, Palo *et al*.[14] found that the optimum pH was 3-0-7.5. Concurrently, the optimum temperature at 27-30 °C was proposed by Yongsmith [19].

Moisture is also one of the important factors. Palo *et al*. [14] also mentioned the moisture of below 50 % was the good condition. Han [20] found that the higher initial moisture (50-56%) gave the intense color within 8 days. Nitrogen source may affect the production of the pigments. Dussa et al. [21] found that the increases of monascorubamin and rubropunctatin correlated to the decrease of valine, methionine, isoleucine, glycine, glutamic acid and alanine.

This research work aims to study the amount of red pigment and citrinin found in red yeast rice made from Thai rice. The results obtained will be used to improve red yeast rice products and scaling up for industrial application.

2. Materials and methods

2.1 Materials

Monascus purpureus CMU001 strain [22], non-glutinous rice, *Oryza sativa* L. cv. Mali 105 and glutinous rice; *Oryza sativa* L. cv. Kam (Kam), *Oryza sativa* L. cv. Kor Kho 6 (RD6) and *Oryza sativa* L. cv. Sanpathong1 (SPT1) abundantly available in the north of Thailand were used to prepare red yeast rice. These rice samples were purchased from the same rice supplier and the same batch of

processing and kept in the same condition.

2.2 Preparation of red yeast rice

Stepwise preparation of red yeast rice is carried out by firstly immersion of each rice cultivar in water for 6 h followed by steaming for 20 min. After cooling, 50 g of steam rice was put in 250 mL flask and sterilized at 15 psi and 121°C for 15 min. One week old pre-cultured *M. purpureus* CMU001 was used as inoculums. The inoculated rice was incubated at 30 °C for 2 and 3 weeks. The end-product was dried in the oven at 65 °C for 6 h 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 before streaming. In order to study the effect of adding nitrogen compounds, addition of 1 ml of 0.25 g/ml soybean milk solution was done.

2.3 Measuring the content of red pigments

The red pigment content was measured by determination of absorbance at 500 nm [23-25]. 0.5 g of red yeast rice powder was used for extraction by 10 mL of 75% HPLC grade ethanol [1]. The mixture was sonicated in ultrasonic bath (Branson 2510E, 42 kHz.) for 60 minutes.

The supernatant liquid was obtained by centrifugation at 3,000 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 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.

2.4 Analysis of citrinin by HPLC

2.4.1 Sample extraction

An extraction of the sample was carried out using 2.5 g of ground red yeast 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°C for 5 h. The supernatant was collected after centrifugation using 2,000 g speed at 25°C for 15 min (modified from Pattanagul *et al*., 2008) [26], 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 C18 (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 345 nm. The column temperature was set at 25°C, and the injection volume was 10 µl.

2.4.2 Sample quantification

The standard calibration curve of standard citrinin was prepared using 150, 313, 625, 1250, 2500, 5000, 10000, 50000, 100,000 and 150,000 ppb. The solutions were filtered with 0.20 μm nylon membrane before use. All of the standard citrinin was injected in triplicates time in the volume of 10 μl.

To determine the citrinin content in the samples, the content of citrinin in all of the samples were quantified using HPLC technique. The amount of citrinin content was determined from the peak area and calculated from the standard calibration curve.

3. Results and disscusion

3.1 Measuring the content of red pigment

The content of red pigment in the 75% ethanol extract is expressed as absorbance unit per gram (AU/g) of red yeast rice powder. The absorbance was measured at 500 nm which is the maximum wave length for red color (Table 1).

Table 1 Red pigment content from fermented 1 cultivar of non-glutinous rice and 3 cultivars of glutinous rice with *M. purpureus*.

Time	AU(AU/g)							
	Mali105		Kam		RD6		SPT 1	
	a	b	a	b	a	b	a	b
2 weeks	4.51 ±0.00	3.60 ±0.01	3.63 ±0.01	6.99 ±0.01	30.11 ±0.00	45.04 ±0.01	29.63 ±0.02	36.09 ±0.01
3 weeks	1.42 ±0.00	0.91 ±0.00	6.06 ±0.02	5.06 ±0.01	39.90 ±0.02	42.16 ±0.00	34.13 ±0.00	42.70 ±0.01

a = Red yeast rice fermented without soybean milk
b = Red yeast rice fermented with soybean milk
The result were reported with ± SD.

After preparation of red yeast rice, the difference varieties gave red yeast rice products varying from pale red to darker red depending on the kind of rice used.

Many reports indicated the absorbance of red pigment from *M. purpureus* at 400-500 nm [26-29]. The results show maximum absorbance around 500 nm that was used for measuring of red pigment. The red color is due to the presence of rubropunctamine and monascorubramine which are derived from orange colored compounds rubropunctatin and monascorubrin, respectively [19]. Amine compounds seem to play an important role for derivatization of orange colored compounds by ring opening and shift rearrangement reaction. Consequently, from Table 1, most of the rice variety expressed more intense red color when adding soybean milk solution during red yeast rice preparation.

The red pigment produced from glutinous rice seems to be higher than non-glutinous rice, Mali105. The result is controversial to the one reported earlier by Pinthong *et al*. [4]. This is, possibly, due to the higher moisture content of rice in the present study, leading to higher level of starch gelatinization.

In this report, it was found that, under control condition, glutinous rice gave more intense

red color products. The result that shows the most intense red color was observed from using RD6 with addition of soybean milk harvested within 2 weeks. The most intense red color obtained in RD6 may be due to the content of carbon and nitrogen source as well as the growth rate of fermentation. This may be supported by the state of secondary metabolites production was obtained faster.

3.2 Mycotoxin (citrinin) in red yeast rice

The results obtained by detecting at 345 nm shows. The chromatograms of 3 weeks period of fermentation without and with the addition of soybean milk using non-glutinous rice, *Oryza sativa* L. cv. Mali105, glutinous rice; *Oryza sativa* L. cv. Kam, *Oryza sativa* L. cv. RD6 and *Oryza sativa* L. cv. SPT1 are shown in Fig. 1 and Table 2.

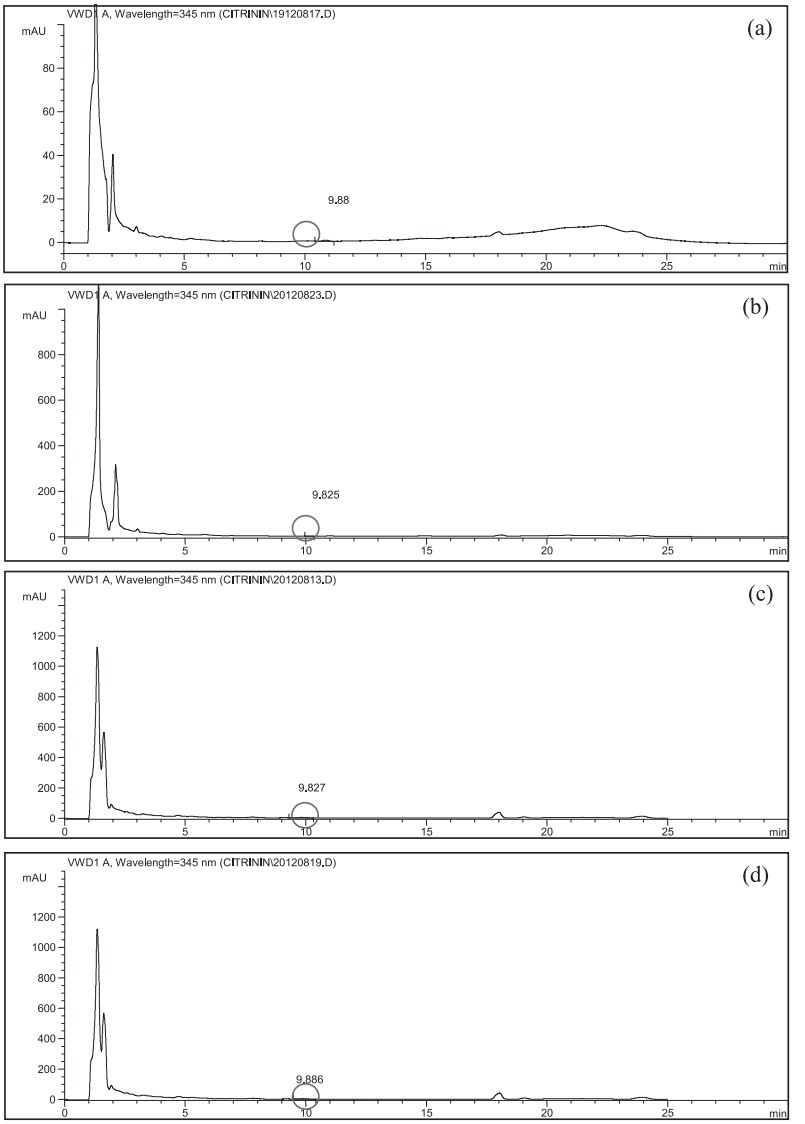

Fig. 1 Chromatographic chemical profiling of citrinin in fermented red yeast rice from non-glutinous rice and glutinous rice, (a) and (b) *Oryza sativa* L. cv. Mali105 without and with soybean milk, (c) and (d) *Oryza sativa* L. cv. Kam without and with soybean milk, (e) and (f) *Oryza sativa* L. cv. RD6 without and with soybean milk, (g) and (h) *Oryza sativa* L. cv. SPT1 without and with soybean milk.

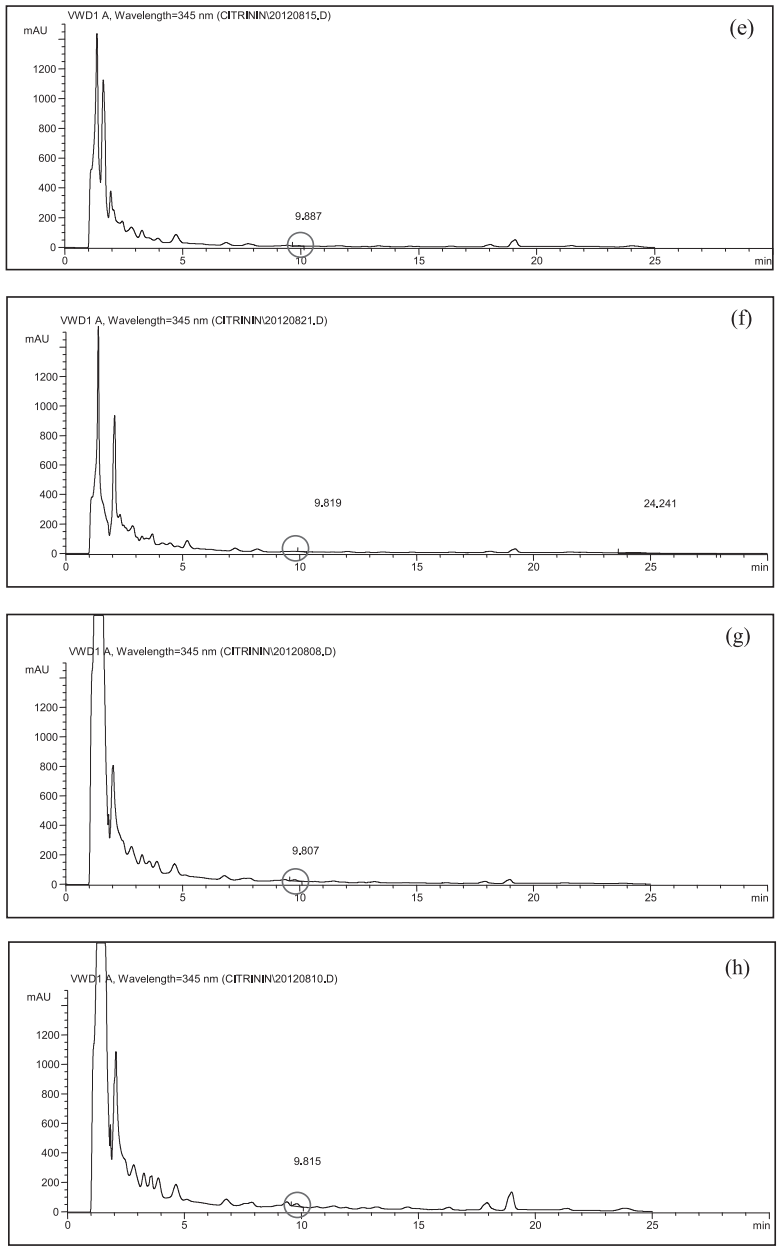

Fig. 1 (cont.) Chromatographic chemical profiling of citrinin in fermented red yeast rice from non-glutinous rice and glutinous rice, (a) and (b) *Oryza sativa* L. cv. Mali105 without and with soybean milk, (c) and (d) *Oryza sativa* L. cv. Kam without and with soybean milk, (e) and (f) *Oryza sativa* L. cv. RD6 without and with soybean milk, (g) and (h) *Oryza sativa* L. cv. SPT1 without and with soybean milk.

Table 2 Citrinin contents in red yeast rice.

Time	Citrinin (ng/g)							
	Mali105		Kam		RD6		SPT 1	
	a	b	a	b	a	b	a	b
2 weeks	608.14 ±0.02	697.67 ±0.01	9084.88 ±0.01	6430.23 ±0.01	13415.12 ±0.01	10282.91 ±0.01	39312.20 ±0.02	49851.16 ±0.01
3 weeks	1136.25 ±0.01	405.81 ±0.03	9620.93 ±0.02	5681.39 ±0.01	7862.62 ±0.02	4372.09 ±0.01	14221.71 ±0.03	29220.81 ±0.01

a = Red yeast rice fermented without soybean milk
b = Red yeast rice fermented with soybean milk
The result were reported with ± SD.

The results in Table 2 shows that, among the glutinous rice the citrinin content of RD6 at 3 weeks with an addition of soybean milk was the lowest (4,372.09 ng/g). The addition of soybean milk during cultivation in most of the rice shows decreasing of citrinin content for glutinous rice except in the case of SPT1. The non-glutinous rice, Mali 105, with an addition of soybean milk at 3 weeks shows lowest citrinin concentration of 405.81 ng/g. Anyway, the citrinin content is still higher than acceptable amount. In Japan, authorized amount of citrinin is under 200 ng/g, while more than 1,000 ng/g of citrinin was found in Chinese products [23].

The possibility to decrease the amount of citrinin is to use peroxidase enzyme in the presence of lipids, especially, fatty acids. The amount of citrinin can be blocked by using peroxidase enzyme [24]. This result agree with the work of Hajjaj *et al*. [25] who studied the effect of 6-8 carbons fatty acids to the formation of citrinin in *Monascus*. The studied was carried out by liquid fermentation with 5.20 g/l of glucose, 5 g/l glutamate adding with octanoic acid which was the precursor of red pigment synthesis via polyketide synthesis pathway. The fatty acid increased the red pigment for 30-40 % while the amount of citrinin was decreased. The degradation of the newly synthesized citrinin (or an intermediate in the citrinin pathway) is done by hydrogen peroxide resulting from peroxisome proliferation induced by medium-chain fatty acids or methylketones. The results in this work showed the tentative decrease of citrinin when adding soybean milk. This may be due to the presence of lipids in soybean milk as well as the peroxidase activity.

4. Conclusions

The results show the most intense red pigment using RD6 fermented for 3 weeks without soybean milk. On the other hands, the highest intensity was achieved when RD6 used was only at 2 weeks of cultivation with addition of soybean milk. For the analysis of citrinin content, the results suggested higher amount when glutinous rice was used. The highest amount was found in SPT1 red yeast rice fermented for 2 weeks. The best result with the lowest amount was found, among glutinous rice, in RD6 fermented for 3 weeks in the presence of soybean milk.

The optimum condition for the preparation of red yeast rice from glutinous rice should be studied to decrease citrinin. Anyway, the citrinin content is still higher than acceptable amount.

The improvement of the production process should be studied to decrease citrinin concentration. A possible way is to find a mutant strains. The variation of some nutrients such as histidine is another method to be done as well as the control of aeration.

Further experiment is suggested to be done to know how the contents relate to the intense color. Among the glutinous rice used, the lowest quality product was obtained with Kam rice (purple rice). Many further interesting works should be studied concerning red yeast rice production. The optimization for the manufacturing condition using glutinous rice should be carried out. The variable parameters such as oxygen supply and the content of amino acid such as histidine, glycine and luecine are also important.

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