High \(\gamma\) Amino Butyric Acid and Low Citrinin Produced by Monascus purpureus Serasi Strain

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Abstract. The analysis bioactive production by Monascus purpureus SERASI strain was carried to know its potency for industrial application as herbal medicine. Previously, the fungus was cultured on malt extract agar for 12 days and harvested for inoculum purpose. For Monascus fermented rice (MFR) production, previously sterilized rice strain 24R after soaking treatment of plain water for a night was inoculated by the inoculum. Afterwards, the rice was incubated at 30°C for 6, 8, 10, and 12 days of incubation time period. The MFR was analysed for its GABA, lovastatin and citrinin content by using High Performance Liquid Chromatography. The result showed that the highest amino butyric acid (GABA) content of MFR was at 452 \(\mu\)g/g after 10 days of incubation time period. But it was decreased to 129 \(\mu\)g/g and 316 \(\mu\)g/g after 12 days and 14 days, respectively. While, lovastatin produce was at 315 \(\mu\)g/g after 10 days but decrease dramatically to 16 \(\mu\)g/g and 26 \(\mu\)g/g after 12 days and 14 days. In regard to citrinin, the acceptable content was reached at 0.12 \(\mu\)g/g after 12 days. At day 10 or 14 days, the citrinin content was higher at 13.39 \(\mu\)g/g and 14.18 respectively. Based on high GABA and low citrinin produces, this result showed that M. purpureus Serasi strain might be more potency for industrial purpose as an herbal medicine for anti hypertensive agent. Therefore, further study is needed to confirm MFR with high content of GABA as anti hypertensive agent.

1. Introduction
Monascus purpureus, a fungus belonged to Monascaceae [1, 2], is mostly used for making Monascus red rice (MFR) which is also familiar as Red yeast rice, Red Koji, Hong Qu, Ang-kak, red mold rice, and Beni-Koji. This is a traditional Chinese food product commonly used as a flavoring, colorant, and preservative in cooking and medication for its blood circulation and food digestion promoting properties. It is believed that the development of red yeast rice already ages more than a thousand years and its use has been documented at the Chinese Tang dynasty (around 800 AD) and up today numerous Asian countries continues using as a dietary staple [3, 4, 5, 6, 7]. Red yeast rice is made traditionally which this fermentation processes let take place naturally on cooked-non glutinous rice. This traditional solid-state fermentation under controlled conditions produces monacolins, a family of polyketides, that it can inhibit cholesterol production [3, 8]. MFR consists of several monacolins, all of which have known for their ability to inhibit the enzyme 3-hydroxy-3-methylglutaryl co-enzyme A (CoA) reductase, which is a critical step in cholesterol biosynthesis. Specifically, monacolin K, also
known as mevicolin or lovastatin, is the monacolin in red yeast rice that has the same chemical structure as the purified, Aspergillus-derived monacolin K known as the pharmaceutical drug Mevacor (Merck & Co, Inc)[3, 5, 6]. Closely related to monacolin K is the hydroxy acid form called monacolin KA, which is the activated form of lovastatin after passes through the liver. Monacolin K (lovastatin) and monacolin KA (hydroxyl acid form of lovastatin) are typically the predominant active ingredients in most commercially available red yeast rice preparations [3]. Other active ingredients in red yeast rice include plant sterols (b-sitosterol, campesterol, stigmasterol, and sapogenin), Isoflavones and isoflavone glycosides, zinc, selenium and mono unsaturated fatty acids [3, 6].

2. Materials and Methods

2.1. Preparation of Monascus purpureus culture
This study used M. purpureus Serasi, a selected strain which was maintained on a freezer at -80°C. The fungus was then revived by cultivation on MEA 2% (Difco) medium at room temperature 25°C.

2.2. Preparation of Monascus Inoculum
Monascus Inoculum was prepared by cultivating on MEA 2% plate for 12 days at 30°C.

2.3. Making Monascus Red Rice (MFR)
An amount of 25 g of rice IR42 variety soaked in water for overnight before sterilized by using autoclave for 15 minutes, at 121°C and at 1 atm. An amount of 5 ml of Monascus inoculum (10% of rice medium) was inoculated onto sterile rice which was earlier placed on Petri dish and subsequently mixed well. This culture was incubated at 30°C for five, seven, nine, 12, and 14 days of the period. After harvest, the rice was oven dried at 50°C for 24 hours before keeping at 4°C.

2.4. Analysis of Pigment
A procedural analysis of pigment was used [9]. Pigment extract was obtained by extraction of 0.05 g of powdered MFR by 10 ml of methanol for 24 hours and then homogenized by using an electric shaker. The extract was filtered subsequently by using nylon filter paper and was then measured spectrophotometrically for yellow pigment at $\lambda=390$ nm and red pigments at $\lambda=500$ nm.

2.5. Analysis of Citrinin
Analysis of citrinin content was performed by using HPLC according to Sakai et al. method [10]. Standard citrinin from Sigma was purchased. Citrinin was extracted by dilution of 1.25 g of powdered angkak with 50 ml of 70% ethanol (pH 8.0). Subsequently, the extract was homogenized by using the magnetic stirrer for 3 hours, at 15-25 °C, and filtered through nylon filter of 0.45µm in diameter. The filtrate (20µl) was injected into HPLC by using Column C18 and detector UV-Vis and the mobile phase which was the mixture of acetonitrile/water/ trifluoroacetic acid (55 : 45 : 0.1) was used at a flow rate of 1.0ml/min.

2.6. Analysis of Lovastatin
To do analysis of lovastatin, following procedures were conducted [11]. Lovastatin extract was obtained by mixing a one gram of powdered MFR with solution of 2 ml of acetonitrile and 0,1 ml 0,1% phosphoric acid and incubated for 30 minutes and later centrifuged at 10.000 rpm for 10 minutes at 4°C. The supernatant was concentrated by freeze drying and diluted subsequently with a mobile phase solution (acetonitrile +0.1% phosphoric acid (65:35). A 20µl of lovastatin extract were injected to HPLC by using C18 column and a $\lambda=235$nm UV detector with elusion rate at 1 ml/minute at 45°C.

2.7. Analysis of GABA
Analysis of GABA was performed based on the form of phenylthiocarbamyl-GABA (PTC-GABA) according to a method of Rossetti & Lombard [12]. MFR, which has been crushed with a mortar, was weighed to 1 g, then extracted in 5mL of water at 70°C for 2 hours. A 200µL of extract was to freeze
dried. To the residue, a 40 µL of ethanol-water-thriethylamine solution (2:2:1) was added. The residue was homogenized and dried again with a freeze dryer. Subsequently, 60 µL of ethanol-water-triethylamine-p-phenylothiocyanate solution (7 : 1 : 1 : 1) were added into the residue and letting for 20 minutes at room temperature to become PTC-GABA. The excess reagent is removed using a freeze dryer. For a standard curve GABA is treated the same as the above procedure to become a standard GABA solution. The dry residue containing PTC-GABA was dissolved by 80% of the mobile phase solution containing 8,205g sodium acetate, 0.5mL triethylamine, 0.7mL acetic acid, 5mL acetonitrile with adjustment to 1000mL by adding sterile water) and 200µL of B 20% solution (acetonitrile-water, 60:40). Hence, the pH mixture of solutions A or B was 5.8. The samples were analyzed using PTC-GABA using HPLC, Discovery Supelco C18 column, 0.6mL/minute flow rate, with UV detector at $\lambda$ 254nm.

3. Result and Discussion

3.1. Pigment content of MFR

Table 1. Pigment content of Monascus Red Rice fermented by *Monascus purpureus* Serasi strain.

| Day of harvest | Red Pigment Content | Yellow Pigment Content |
|----------------|---------------------|------------------------|
|                | Absorant Unit (A $\lambda$500nm) | Absorant Unit (A $\lambda$390nm) |
| Sample A       | Sample B            | Mean                   | Sample A       | Sample B            | Mean                   |
| 5              | 1.99                | 1.78                   | 1.89           | 2.32                | 2.12                   | 2.22                   |
| 7              | 2.34                | 2.2                    | 2.27           | 2.15                | 2.02                   | 2.09                   |
| 10             | 5.42                | 5.7                    | 5.56           | 5.37                | 5.73                   | 5.55                   |
| 12             | 2.12                | 2.62                   | 2.37           | 1.72                | 2.13                   | 1.93                   |
| 14             | 1.41                | 1.64                   | 1.525          | 0.97                | 1.15                   | 1.06                   |

The high Monascus pigment content was at day 10 of the day incubation both red and yellow pigments and then decreased.

*Monascus* species, mostly *M. pilosus*, *M. purpureus*, and *M. ruber* [13] has been applied in East Asian countries for many years as coloring agents in the manufacture of traditional foods (yoghurt, sausages, red wines, tofu, hams, meats, and other products) [13, 14, 15]. Chinese traditional medicine practitioners utilize MFR to treat abdominal pain due to stagnant blood and dysentery, as well as external and internal trauma [13, 16].

Pigments produced by Monascus are secondary metabolites called azaphilones, which its molecular structures are similar and its chemical properties as well. These pigments are formed essentially in the cell-bound state [12, 17, 18, 19]. There are three main pigments such as yellow, orange, and red pigments [13,19, 20, 21] and six main compounds characterized in Monascus pigments are rubropunctamine and monascorubramine (red), rubropunctatin and monascorubrin (orange), and monascin and ankaflavin (yellow). Currently, more than fifty further compounds in Monascus pigments were reported. These include xanthomonasin A, yellow II pigment, monascopyridines A and B, monascusones A and B, xanthomonasin B, compounds R3 and Y3, monasfluor A and B, monafilones A, B, C, monarubrin and rubropunctin, purpureosone, monasnicotinates A, B, C, D, a new red pigment and monapurpyridine A. Some of these minor pigments have been found in pairs, having side chains containing six or eight carbons, the same as the main pigments. The most frequently occurring minor pigments are xanthomonasins A and B (furanoisophtalides). Some of these minor pigments might be intermediates or degradation products of the main pigments, and a possible relationship between monascusones A and B and the major yellow pigment, monascin, has been proposed. Published results have showed that conditions of fermentation could affect the composition of Monascus pigments such as pH, carbon source, nitrogen source and temperature [18, 19, 20].

Because of the affinity by amine groups, it is frequently that the Monascus pigments are linked to proteins or to the cell wall, establishing complex pigments, causing difficulty of extraction. Many
factors can effect on Monascus pigment production such as strain selection, substrate, pH, nitrogen source, light intensity, temperature, broth rheology and oxygen [14, 18].

Monascus pigments have been known for its high economic value mainly as a coloring agent. The pigments have many benefits such as easy production by using non-expensive substrates, good solubility in water and ethanol, many bioactive metabolites and safe when produced under definite conditions. Today, many researchers investigate to replace synthetic food colorant by Monascus natural pigments. Besides the application are being to use as a natural coloring agent in foodstuffs and texture industries, the pigments also produce aroma and flavor savoury and used as condiment. In pharmacology and medicine Monascus pigments are widely used in prevention and treatment of various human diseases and also in cosmetics [21].

3.2. Citrinin content of MFR

Citrinin standard was calculated and resulted a regression equation: $y = 62531x + 39123$ ($R^2 = 0.9988$) for calculation HPLC analysis result. The result showed that the minimum citrinin content of MFR was 0.12 µg/g at day 12 of harvest.

**Table 2.** Citrinin content of Monascus Red Rice fermented by *Monascus purpureus* Serasi strain.

| Day of harvest | Citrinin Content (µg/g) | Mean of Citrinin Content (µg/g) |
|---------------|-------------------------|---------------------------------|
|               | 1                       | 2                               |
| 5             | 2.73                    | 2.73                            |
| 7             | 7.23                    | 7.37                            |
| 10            | 13.54                   | 13.25                           |
| 12            | 0.09                    | 0.14                            |
| 14            | 14.07                   | 14.3                            | 14.18                      |

This result showed that citrinin production tended increasingly along day of incubation. But at day 12 of the incubation period, the citrinin was drastically decrease to 0.12 µg/g and again increased drastically produced to 14.18 µg/g.

Citrinin, a mycotoxin, was first found from culture of *P. citrinum* [22] in 1931. Citrinin is nephrotoxic and potential genotoxic, carcinogenic, embryotoxic, and teratogenic toxin[23]. Later, an active compound termed monascidin A was isolated from the cultures of *M. purpureus* strains in 1977 during a research on its antibacterial activities [24]. Monascidin A was found effectively against bacterial genera such as Bacillus, Pseudomonas, and Streptococcus [25]. Monascidin A was later identified as the same compound with citrinin [26]. Comprehensive studies of various Monascus species based on the use of different culture media and conditions revealed that both culture conditions and genetic attributes could influence citrinin production [27, 28].

The both pigments and citrinin is synthesised by the polyketide pathway which uses a tetraketide as its precursor. This precursor was formed by condensation of one acetyl-CoA molecule and three malonyl-CoA molecules. Citrinin is formed by addition one acetyl-CoA molecule to tetraketide, followed by a sequences of reactions involving methylation, condensation, reduction, O-alkylation, cleavage between C-1 and C-2 bonding, oxidation, and dehydration. Pigments were formed by addition two malonyl-CoA molecules to tetraketide then followed by esterification. Therefore, the pigment production and the production of citrinin were synthesized alongside by the polyketide pathway [29, 30, 31].Citrinin is nephrotoxic and hepatotoxic properties [31, 32]. Accordingly, critical attention to Citrinin has addressed to the safety of Monascus products in all countries. The limitation of citrinin in Monascus pigment firstly. The limit index of citrinin in Monascus pigment was determined to be 0.2 ng/kg (color value standard was 500 U/g), which was the lowest dose of citrinin that was detected at that time. The U.S. Food and drug administration has made it clear that Monascus products are recognized as food additives and must be evaluated for citrinin. In
China's exports of Monascus products, Europe and the United States require the production of Monascus strains have been identified, and the citrinin content was limited strictly. Monascus imported from China must be certified for safe production, strains, and citrinin free in Germany. In recent years, standards have been formulated for citrinin Monascus products. “Determination of citrinin in Monascus products (GB/T 5009.222-2008)” was established in which the method for determination of citrinin in Monascus products was laid down, the limit of citrinin in liquid sample was 50g/L and the limit of citrinin in solid sample was 1 mg/kg [33].

3.3. Lovastatin content of MFR

Lovastatin standard was calculated and resulted a formula: \( y = 18778x - 689.6; \) (R\(^2\) = 0.9945) for calculation HPLC result. The result showed that the high lovastatin content of MFR was 0.032% at day 10 of harvest.

### Table 3. Lovastatin content of Monascus Red Rice fermented by Monascus purpureus Serasi strain.

| Day of harvest | (ppm) Sample 1 | (ppm) Sample 2 | Mean (ppm) |
|----------------|----------------|----------------|------------|
| 5              | 65.663         | 65.673         | 65.668     |
| 7              | 128.953        | 132.699        | 130.826    |
| 10             | 303.248        | 327.604        | 315.426    |
| 12             | 12.309         | 19.854         | 16.082     |
| 14             | 18.141         | 34.225         | 26.183     |

This result showed much higher (327.604 ppm at day 10 of the incubation period) production of lovastatin if compared to other fungi such as Aspergillus flavus (48.4 ppm), A. niger (29.9 ppm), A. oryzae (37.6), A. terreus (59.2 ppm), Cylindrocarpon radicicola (7.1 ppm), Penicillium spinulosum (15.8 ppm), Trichoderma viridae (36 ppm), Mycellia sterilia (15.3 ppm) [34, 35], A. terreus (116.8 ppm), Monascus sp. (21.5 ppm), A. niger (4.3), A. flavus (5.9 ppm), Penicillium purpureogenum (16.9 ppm), Pleurotus sp. (18.6 ppm), Trichoderma viridae (8.6 ppm) [35, 36]; A. terreus (55.0 ppm) [35, 37], A. parasiticus (4.5 ppm), A. fischeri (2.0 ppm), A. flavus (9.0 ppm), A. lumbrosus (14 ppm), Penicillium funiculosum (79.3 ppm), Trichoderma viridae (9.0 ppm), Trichoderma longibrachiatum (1.0 ppm), Acremonium chrysogenum (2.5 ppm) [35,37].

Lovastatin is a typical secondary metabolite that is produced in the stationary growth phase, and its production is subject to glucose repression. Induction treatments have been made to get its high production. \( M.\ pilosus \) was induced to produce lovastatin (725 mg/l) in liquid medium using a mixed substrate of maltose: glycerol (1:7) and peptone as the nitrogen source [18, 38]. With solid substrate cultivation (SSC), an increase in lovastatin formation was induced by adding glycerol, soya meal, acetic acid or NaNO\(_3\) to the main substrate, rice [18, 39].

In 1987, lovastatin was the first drug isolated from a group of statins with anti-cholesterolemic effects that was approved by the U.S. Food and Drug Administration (FDA). In general, statins are competitive inhibitors of hydroxymethylglutaryl-coenzyme A (HMG-CoA) reductase, a key enzyme in cholesterol biosynthesis, due to a structural analogy between the b-hydroxy acids of statins and an HMG-CoA intermediate. The affinity of a statin towards HMG-CoA reductase is several folds higher than that of the HMG-CoA intermediate. The hypocholesterolemic effect of statins, i.e. a decrease in total blood cholesterol concentration, is significant several days after the beginning of treatment [18, 40].

In addition to their confirmed anticholesterolemic effect, statins have other positive influences on human health, such as anti-inflammatory activity, improvement in the state of the blood vessels,
decreased risk of thrombosis and accelerated healing of fractures [18]. Recent research has also shown a decreased risk of Alzheimer’s disease and cancerostatic effects [41]. Negative effects on human health (especially muscle myopathy and kidney disease) are not frequent and are usually reversible [18, 40].

3.4. GABA content of MFR

As the GABA content was based on the formation of PTC-GABA [12], the calibration curve for PTC-GABA was then calculated based on linear regression. From the calibration assay, the regression equation was y=160590x - 218381 and r=0.9919.

The result showed that the rice had already contained GABA (380.8µg/g) (Table 4). But after soaking for overnight, the GABA content of the rice was decreased (98.9µg/g). However after fermentation by M. purpureus Serasi strain, the GABA content was increase intensely. The GABA content of MFR tended to increase along with day period incubation. However, the optimum of GABA production was reached at the day 10 of the incubation period (1,744.6µg/g) (Table 4).

Table 4. Gamma amino butyric acid content based on HPLC Analysis.

| Sources                      | Sample 1 µg/g | Sample 2 µg/g | Mean µg/g |
|------------------------------|---------------|---------------|-----------|
| Fresh Rice                   | 394.0         | 367.5         | 380.8     |
| Soaked rice                  | 85.2          | 112.6         | 98.9      |
| MFR after Day of Incubation  |               |               |           |
| 5                            | 743           | 900           | 821.5     |
| 7                            | 1,118.5       | 1,519.3       | 1,318.9   |
| 10                           | 2,260.12      | 1,229         | 1,744.6   |
| 12                           | -             | 645.8         | 645.8     |
| 14                           | -             | 1,580         | 1,580     |

This result showed a good potency of M. purpureus Serasi strain as a good producer for GABA rather than pigments or lovastatin.

Gamma-amino butyric acid (GABA) is a non-protein amino acid which has a main function as an inhibitory neurotransmitter in mammalian nervous systems. Several physiological functions such as anti-epilepsy, hypotensive effects, tranquilizing excitation, enhancing memory and regulating hormone secretion are possessed by GABA activity [42]. Those properties that are beneficial to health make current research focussed on enhancement of GABA production. As demand for GABA-enriched foods has increased, many efforts have been made to develop functional foods with a high GABA content, including producing fermented food products by using lactic acid bacteria, cheese [43], tempeh-type fermented soya beans, Korean kimchi and yogurt [44]. GABA is also widely distributed in nature, including in bean sprouts, soya bean seedlings, tea leaves, grain germs and microorganisms [45]. In this study, the rice already contained GABA before fermented by M. purpureus. Besides Monascus, other microorganisms have been screened for GABA production, such as Rhizopus, Lactobacillus paracasei, Lactobacillus brevis, Lactococcus lactis and Streptococcus thermophilus [46]. Monascus strains are known to produce secondary metabolites that are beneficial to health, along with a red pigment. Traditionally, three strains of Monascus such as Monascus purpureus, Monascus ruber and M. pilosus have been used for centuries in fermentation and the food industry in East Asia [47]. Biosynthesis methods for GABA production can be more encouraging than chemical synthesis methods since they have a simple reaction procedure, high catalytic efficiency, mild reaction conditions and good environmental compatibility [48]. They involve the bioconversion of decarboxylating glutamate to GABA, catalyzed by the enzyme glutamate decarboxylase (GAD).
Various techniques such as immobilized cell technology, sourdough fermentation and batch fermentation methods have been used for GABA production from microbial sources, and fermentation factors such as pH, temperature, cultivation time and media additives in the culture are considered to be of key importance for optimizing the GABA yield [49,50].

4. Conclusion

*M. purpureus* Serasi strain might be seen more potency for industrial purpose as an herbal medicine for antihypertensive agent. However, further study is needed to confirm MFR as antihypertensive agent related to its GABA production. Optimizing for the GABA production is also still needed such as addition Monosodium glutamate, temperature, pH regulation or other condition.

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6. References

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