An Investigation on Mass Cultivation of *Monascus purpureus* Using the Commercial Scale Koji Making Equipment

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**ABSTRACT**

Red mold rice (also known as red fermented rice or red yeast rice) had been extensively used in Chinese processed foods for red color enhancement and nutraceutical supplements since thousands years ago. However, traditional method for producing red mold rice takes very long fermentation time and very high labor costs, in addition to a very large space need, and the quality and efficiency are not consistent and satisfactory.

In this research, a commercial white koji making equipment with a rotary perforated bed of 5m diameter was modified for the studies of red mold rice production. *Monascus purpureus* CCRC 31499 was selected for its high production capacities of monacolin K and red pigment. The selected strain was first cultivated in a 120L submerged type fermentor at 34 °C and 2vvm aeration with 60rpm agitation for 5 days using 20% liquefied rice porridge as carbon source. The high concentration red mold rice broth (> 3.5 g/mL) was harvested and well mixed with cooked rice to an initial inoculums concentration of 2% v/w. The inoculated cooked rice then was directed into the modified koji making equipment, in which the temperature and humidity were kept at 37~38 °C and 90% RH, respectively. Air was circulated to remove fermentation heat while the rotary rotated slowly. Lag phase in the large scale fermentation was determined as 16 hours, when koji temperature increased rapidly. Water was added by water curtain into koji at 36th hour for keeping moisture content of the rice koji at 50% or above. At the final stage of fermentation, temperature was adjusted to 34 °C for increasing red pigment production. After 7 days, 1200kg high quality red mold rice was harvested each batch. Labor costs, space, and fermentation time were reduced significantly compared with those made by traditional methods.

**Keywords**: Red mold rice; Nagata koji making equipment; *Monascus purpureus*; monacolin K; GABA.

**INTRODUCTION**
Red mold rice (red koji), produced from solid-state fermentation of cooked rice with *Monascus* sp. contains many high value substances, such as monacolin K, γ-aminobutyric acid (GABA), natural red pigment, and other unidentified active components [1,4,12]. These components are the secondary metabolites of fermentation, and are medicinally proved to possess anti-cholesterol and anti-carcinogenic activities [5-7]. The Chinese ancient pharmacopoeia, 本草纲目 (Ben Tsao Gum Mu), indicates the use of red mold rice to promote the health of the cardiovascular systems [11,17]. Red mold rice appears in many Chinese processed foods for red color enhancement and nutroceutical supplements at least for over thousands years. However, the formal written records were not discovered until two pharmacopoeias published in the Post-Han and Yuan Dynasties, respectively, which first described the medicinal functions of red mold rice [3,17]. In ca.1590, another pharmacopoeia was published and released the method for making red mold rice [3]. This method has ever since been adopted as the standard fermentation process until recent years, as shown in Figure 1.

For easy control of aeration and removal of fermentation heat, the inoculated cooked rice is put in a round shallow bamboo tray about 5~6cm in depth. Trays are stacked in shelves in fermentation room. Agitation with hands is needed for flipping over the bottom part of rice koji and removing fermentation heat. During fermentation, each tray has to be taken out at least three times from the fermentation room and soaked into water to maintain proper moisture content of rice koji. However, the traditional method needs large space for aerobic solid-state fermentation, high labor costs for koji agitation by hands and water soaking, and long process time. Fermentation is easily contaminated by the open environmental factors, which always results in inconsistent and unsatisfactory quality [3,17].

Microbiological studies of red molds was first conducted in 1884 by van Tieghem, a French microbiologist, and categorized as the genus *Monascaceae* [10,18]. Many species with similar red fugal filamentous appearance and physiological characteristics were isolated and named from different kinds of products since then. The most widely used red mold species in Taiwan was first named *Monascus anka* in 1931 by two Japanese, Misawa and Sato [18]. This finding led to the use of pure culture in commercial production of red mold rice. However, due to the limit of the “Monopoly Law for Production and Selling of Tobacco and Liquor”, production of red mold rice was only authorized to Taiwan Tobacco and Liquor Monopoly Corporation. Most researches for red mold rice production were conducted in its affiliated Taiwan Wine Research Institute, including species selection and process modification.

Literature survey on red mold rice production in a closed environment using koji
making equipment shows almost no successful report. Lin and his associates in Taiwan Wine Research Institute (TWRI) tried with a pilot scale (50kg in capacity) Nagata type rotary bed solid-state fermentor, and ended up unsatisfactory results [13-15]. The failure was attributed to the difficulties of water adding and fermentation heat removing in site. Since then, no further studies had been conducted. On the other hands, reports on microbial strains used in red mold rice production and functional properties of metabolites have been easily found [6,7,16]. Most of these works focused on the production capacities of monacolin, pigment, GABA, etc., and their applications as dietary supplements in healthy foods.

29 Monascus strains have been named and found possessing various production capacities of monacolin K, GABA, flavonoids, citrinin and red pigment, etc., as their primary and secondary metabolites, although citrinin is usually regarded as a hazardous factor to health. Among these strains, *M. purpureus*, *M. anka*, and *M. ruber* are most used for research and industrial production of red mold rice. However, their capacities for producing monacolin K, GABA, and red pigments are the major concerns [17]. Comparisons of the three strains are listed in Table 1.

In mid-1950s’, mechanization and automation on koji production were just initiated in Japan. A Nagata type rotary bed koji making equipment (as shown in Figure 2) was developed for making rice, soybean, and wheat koji for sake (Japanese rice wine) or soy sauce production with *Aspergillus* sp. as cultures. Major components of the rotary bed koji making equipment include a round bed with at least 30cm in depth and a perforated bottom plate for up-flow aeration, a set of adjustable speed mixer for plowing up rice koji during fermentation for assisting aeration and heat removal and preventing from rice block formation, and a set of screw for cooked rice feed-in and koji discharge. Aeration system includes an air sterilizer and humidifier before charging into bed, and a cyclone separator for the leaving air. Temperature and humidity sensors are needed for monitoring and process control.

However, this Nagata type koji making equipment is originally designed for making koji with *Aspergillus* sp., which is physiologically different from *Monascus* sp., especially on the aspects of moisture content and temperature rising speed of the rice koji during solid-state fermentation. Therefore, a modified process for mass production of red mold rice in a more hygienic and controllable condition with mechanized koji making facilities became the motivation of this research. Control of rice koji moisture content and fermentation temperature are definitely the cores of this research. The ultimate goals are to develop an optimal process for red mold rice mass production with high and consistent quality, and low cost to meet the healthy foods market demands.
MATERIALS AND METHODS

**Microbial strain**

*Monascus purpureus* CCRC 31499 was bought from Bioresources Collection and Research Center, Food Industry Research and Development Institute, Hsinchu, Taiwan.

**Media and Substrates**

*Indica* rice powder medium was a mixture of *Indica* rice powder 100g, KH$_2$PO$_4$ 0.5g, Monosodium glutamine (MSG) 1.5g, and lactic acid 2mL in 850 mL deionized water. Bread *koji* medium used steamed white bread as the raw material. *Indica* rice 15kg, MSG 110g, KH$_2$PO$_4$ 30g, and CaHPO$_4$ 15g were liquefied and dissolved in 65L water as liquid culture medium for 120L submerged fermentor. Cooked *Indica* rice was used for red mold rice mass production.

**Reagents and Materials**

All chemical reagents were of reagent grade. *Indica* rice was of local variety, 90% polished with 74% starch value. Novo Termamyl (Novo, South Carolina, USA) was used as liquefaction enzyme.

**Equipment**

Rice cooker with capacity of 1000kg was capable of cooking rice with steam in 30 min and cooling rice from 100 to 30°C in 5min (Miaoli Machine Shop, Miaoli, Taiwan). Submerged type fermentor was 120L with agitation, air aseptic and temperature control systems. Nagata type *koji* making equipment containing a rotary bed with max. capacity of 1500kg as shown in Figure 2 was applied as a solid state fermentor (Agro-Industrial Machine Co., Ltd., Chiayi, Taiwan). This fermentor was modified with different water-adding and temperature control systems for its possible application on red mold rice mass production.

**Analysis**

Red pigment, pH, activity of glucoamylase, specific viscosity, and total acidity of red mold rice were analyzed based on the standard methods of TWRI [2]. Monacolin K and citrinin were analyzed following the methods developed by Hsieh and Pan [9].

RESULTS AND DISCUSSIONS
Preparations of High Concentration Liquid Culture

Rice is the major raw material when preparing intermediate liquid culture for solid state fermentation. However, the viscosity of the porridge-like medium will be increased as increasing the concentration of rice porridge, which will in turn retard the growth of filamentous red mold culture. For reducing this effect, different liquefaction methods were compared in this research based on the final cell concentration, pH, total acidity, residual sugars, end point volume and specific viscosity. In the 120L submerged type fermentor, 15kg rice powder was added in 65kg water and heated to 121 °C for 50min. Lactic acid or Novo Termamyl was used as liquefaction agent and added into the medium according to different designs. 120g bread koji was inoculated when medium temperature was lowered to 39 °C. Agitation at 60rpm and aeration of aseptic air at 2vvm were maintained during the cultivation period.

Results were shown in Table 2, which concluded that preparation based on method F would provide the best harvest of Monascus purpureus after a 5-day cultivation period. Method F was hereafter adopted for preparing the liquid culture for mass production in this research.

Quantities of Liquid Inoculums on Lag Phase

Solid state fermentation is difficult to control, especially the aspect on prevention of microbial contamination from surroundings. The growth rate of Monascus sp. is much slower than that of other two fungi, Aspergillus sp. and Rhizopus sp., which are commonly and easily found contaminant strains in red mold rice fermentation. For providing an environment for Monascus purpureus to prevail over other competitors, the optimal quantities of liquid inoculums were tested by comparing the lag phase of the solid state fermentation. Table 3 showed that the inoculums at 2.0% v/w would provide an acceptable lag phase of 16 hours.

Water Sorption Capacity of Red Mold Rice

Moisture content of the red mold rice koji decreased significantly during solid state fermentation. This would hamper the growth of Monascus purpureus. As indicated in Figure 3, the traditional method for making red mold rice involves at least three times water soaking during fermentation to maintain the moisture content of the rice grains. Therefore, it is crucial to decide water absorption capacity of the rice grain when developing the water adding system in the solid state fermentor, which is one of the key steps in this research. Table 4 revealed that soaking time of 6 min would reach at a moisture content of around 50%. Extending soaking time longer than 6min did not increase moisture content significantly. Understanding of this characteristic would help developing a suitable water adding system for keeping the rice koji at the optimal
moisture content for fermentation.

**Water Adding Timing on Red Pigment Production**

Rice *koji* has to be taken out from the fermentation room 3 days after inoculation and soaked into water for keeping moisture content at a certain level. This is the most labor-needed step in the traditional method of red mold rice production. However, when a large scale *koji* making equipment is used, aeration as well as the fermentation heat generated from growth speed up the loss of water content. Therefore, the time for water adding to make up the moisture content for further fermentation must be earlier than that of the traditional method.

Based on the analysis of moisture contents of red mold rice *koji* during fermentation at the time of water adding, and the relationship between water adding time and final red pigment production, it was decided according to Figure 4, that the 36th hour after inoculation was chosen as the optimal water adding time when using the large scale *koji* making equipment.

**Effects of Different Ways of Water Adding**

Three different ways of prototype water adding systems were tested with the Nagata *koji* making equipment, namely atomized water spray, direct water and water curtain, respectively. Result of these tests would be used later to modify the original Nagata *koji* making equipment as an invention. Water was added at the 36th hour after fermentation began, red mold rice *koji* sampled from different spots of the rotary bed were analyzed moisture content. Data shown in Table 5 suggested that the method of water curtain was the most efficient way for water adding. However, a water collecting and recycling device was needed when using this method since water was added in great excess of the amount absorbed by the *koji*. In addition, this water recycling system also served recycling the washed off mold filaments back to the *koji*.

**Agitation for Lowering White Rice Koji Percentage**

White rice *koji* is the rice grain that is not fermented by *Monascus purpureus*, and is regarded as a major defect of red mold rice. The most possible reasons are block formation due to cooked rice agglomeration, and loss of moisture of the cooked rice. Factors resulting in white rice *koji* in the production of red mold rice could be eliminated through improved agitation timing and method. A built-in perforated paddle type blender capable of both co- and counter-current agitation relative to the direction of bed rotation was applied. Data in Table 6 showed the effects of various agitations and timings on reducing the percentage of white rice *koji*, and suggested that one round of bed rotation with counter-current agitation at the 12th hour after
fermentation began could produce red mold rice with higher red pigment content and the less percentage of white rice koji in the product as shown in Figure 4.

**Temperature Control at the Final Stage of Fermentation**

Temperature has been found the influencing factor for productions of red pigment and other secondary metabolites, such as monacolin K and citrinin. Literature shows that temperatures at the range of 25 ~ 35°C is good for productions of red pigment and monacolin K by Monascus purpureus [5]. However, in the solid state fermentation of Monascus purpureus using a large scale koji making equipment, temperature was kept at 37 ~ 38°C for facilitating reproduction and growth. For increasing the productivity of red pigment and monacolin K, temperature at the final stage of fermentation has to be kept at an optimum. Results were listed in Table 7. When temperatures at the 86th hour till the end of fermentation were maintained at 26, 30, and 34°C, respectively, there was not much significant difference in the productions of red pigment, monacolin K and citrinin. From energy point of view, 34°C was accepted as no need for lowering too much the temperature at the final stage.

**CONCLUSIONS AND SUGGESTIONS**

Red mold rice production using the modified Nagata type koji making equipment was proved feasible with appropriate hardware modifications, and optimal operating conditions concluded from this research. Further investigation was suggested to integrate with a PLC/PC based on-line control system for more automatic and accurate temperature and timing control.

**ACKNOWLEDGMENT**

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**BIBLIOGRAPHY**

Taiwan. (in Chinese)

Rice soaking 6-8 Hours, cooked and Cooled to 40°C

1st day, inoculated with *Monascus koji*, controlled at 33-35 °C

2nd day, stirring and mixing of koji, controlled at 34 °C

3rd Day, 1st time water soaking for 30 minutes, Moisture: 50%

4th day, 2nd time water soaking Moisture: 47%

5th day, Last time water soaking Moisture: 48%

6th day, Post maturing, stirring every 10 hours, Temperature: 30 °C

7th day, Drying at 45 °C for 22 hours

8th day, Harvest of dried red mold rice Moisture: 10%

Figure 1. Red mold rice production by traditional process
Figure 2. The Nagata type large scale koji making equipment (Source: Lin, T. F. 1987)
Figure 3. Effect of time to add water on production of red mold rice pigment
Figure 4. Reducing white-koji production using different ways of agitation
Table 1-1. The high value products of Monascus species

1. Extracellular hydrolases
2. Primary metabolites (unsaturated fatty acids, alcohols, and esters)
3. Secondary metabolites
   a. Pigments (red, orange, yellow)
   b. Bone enhancement (glucosamine)
   c. Inhibitors of cholesterol synthesis (monacolin)
   d. Blood pressure depressant (γ-Aminobutyric acid)
   e. Antioxidant (flavonoids)
   f. Blood sugars depressant and other un-identified physiologically active agents.

Source: Su, Y. C., 2001

Table 2. High concentration liquid koji culture for 5 days

<table>
<thead>
<tr>
<th>Methods</th>
<th>Cell Conc. % (w/v)</th>
<th>pH</th>
<th>Total acids (mL)</th>
<th>End point volume (L)</th>
<th>Residual sugars (g/100mL)</th>
<th>Spec. Visc.</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>3.59 ± 0.70b</td>
<td>4.59</td>
<td>40.5 ± 0.5d</td>
<td>3.17 ± 0.44a</td>
<td>3.90 ± 0.38a</td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>3.78 ± 0.85ba</td>
<td>5.30</td>
<td>34.4 ± 0.4b</td>
<td>2.51 ± 0.45ba</td>
<td>2.90 ± 0.12abc</td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>4.44 ± 0.43ba</td>
<td>5.37</td>
<td>70.0 ± 0.5a</td>
<td>1.50 ± 0.69bc</td>
<td>1.50 ± 0.06c</td>
<td></td>
</tr>
<tr>
<td>D</td>
<td>4.32 ± 0.20ba</td>
<td>4.51</td>
<td>43.0 ± 0.5d</td>
<td>3.00 ± 0.34a</td>
<td>3.60 ± 0.25ba</td>
<td></td>
</tr>
<tr>
<td>E</td>
<td>4.63 ± 0.12ba</td>
<td>5.29</td>
<td>34.7 ± 0.1b</td>
<td>1.72 ± 0.33ba</td>
<td>2.20 ± 0.69bc</td>
<td></td>
</tr>
<tr>
<td>F</td>
<td>4.86 ± 0.27a</td>
<td>5.31</td>
<td>33.8 ± 0.8b</td>
<td>1.11 ± 0.77b</td>
<td>1.70 ± 0.25c</td>
<td></td>
</tr>
</tbody>
</table>

Liquefaction methods: A. 2% (wt) lactic acid and rice powder koji; B. 0.2% (wt) Termamyl and rice powder koji; C. 0.1% + 0.1% (wt) Termamyl and rice powder koji; D. 2% (wt) lactic acid and bread koji; E. 0.2% (wt) Termamyl and bread koji; F. 0.1% + 0.1% (wt) Termamyl and bread koji.

Specific viscosity.

Data were all triplicates ( \( \alpha < 0.05 \).)
Table 3. Effect of inoculums amounts on lag phase during solid state fermentation of *Monascus purpureus*

<table>
<thead>
<tr>
<th>Inoculums ratio ( %V/W )</th>
<th>Lag phase (hr)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.7</td>
<td>25.70±0.26^a</td>
</tr>
<tr>
<td>1.0</td>
<td>20.80±0.53^b</td>
</tr>
<tr>
<td>2.0</td>
<td>16.50±0.38^c</td>
</tr>
<tr>
<td>4.0</td>
<td>16.00±0.30^c</td>
</tr>
</tbody>
</table>

1. Cell concentration of the liquid inoculums was 4.78% (w/v).
2. Data were triplicates (α<0.05).

Table 4. The influence of soaking time on koji moisture content

<table>
<thead>
<tr>
<th>Soaking time</th>
<th>Moisture content (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 sec</td>
<td>30.00 ±0.00^a</td>
</tr>
<tr>
<td>10 sec</td>
<td>40.30 ±0.58^b</td>
</tr>
<tr>
<td>30 sec</td>
<td>44.60 ±0.46^c</td>
</tr>
<tr>
<td>60 sec</td>
<td>47.20 ±0.40^d</td>
</tr>
<tr>
<td>2 min</td>
<td>48.70 ±0.21^e</td>
</tr>
<tr>
<td>4 min</td>
<td>49.80 ±0.35^f</td>
</tr>
<tr>
<td>6 min</td>
<td>50.30 ±0.31^gf</td>
</tr>
<tr>
<td>10 min</td>
<td>51.00 ±0.15^gh</td>
</tr>
<tr>
<td>15 min</td>
<td>51.40 ±0.30^h</td>
</tr>
<tr>
<td>20 min</td>
<td>51.60 ±0.17^h</td>
</tr>
<tr>
<td>25 min</td>
<td>51.70 ±0.12^h</td>
</tr>
</tbody>
</table>

Data were triplicate (α<0.05)
Table 5. Different ways of water addition to red mold rice *koji* during solid state fermentation

<table>
<thead>
<tr>
<th>Water adding</th>
<th>Original moisture (%)</th>
<th>Moisture content (%)</th>
<th>1st round</th>
<th>2nd round</th>
<th>3rd round</th>
</tr>
</thead>
<tbody>
<tr>
<td>Atomized</td>
<td>30.1 ± 1.2&lt;sup&gt;aA&lt;/sup&gt;</td>
<td>37.7 ± 1.9&lt;sup&gt;cB&lt;/sup&gt;</td>
<td>41.6 ± 2.6&lt;sup&gt;cC&lt;/sup&gt;</td>
<td>45.1 ± 2.3&lt;sup&gt;bD&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Direct water</td>
<td>30.0 ± 1.2&lt;sup&gt;bD&lt;/sup&gt;</td>
<td>39.8 ± 1.6&lt;sup&gt;bC&lt;/sup&gt;</td>
<td>46.5 ± 1.6&lt;sup&gt;bB&lt;/sup&gt;</td>
<td>50.0 ± 1.3&lt;sup&gt;aA&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Water falls</td>
<td>30.3 ± 1.0&lt;sup&gt;cC&lt;/sup&gt;</td>
<td>46.1 ± 1.7&lt;sup&gt;aB&lt;/sup&gt;</td>
<td>51.2 ± 2.2&lt;sup&gt;aA&lt;/sup&gt;</td>
<td>51.6 ± 2.5&lt;sup&gt;aA&lt;/sup&gt;</td>
<td></td>
</tr>
</tbody>
</table>

1. Rotating time per round is 12 minutes.
2. Data were triplicates (α < 0.05)

Table 6. Influence of agitation on white rice koji percentages

<table>
<thead>
<tr>
<th>Agitation</th>
<th>White rice koji (%)</th>
<th>Red pigment (OD&lt;sub&gt;500nm&lt;/sub&gt;)</th>
<th>Broken rice koji (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>3.70 ± 0.28&lt;sup&gt;a2&lt;/sup&gt;</td>
<td>0.85 ± 0.07&lt;sup&gt;a&lt;/sup&gt;</td>
<td>29.10 ± 0.42&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>B</td>
<td>3.10 ± 0.21&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.90 ± 0.02&lt;sup&gt;a&lt;/sup&gt;</td>
<td>30.10 ± 0.35&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>C</td>
<td>0.90 ± 0.14&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.01 ± 0.14&lt;sup&gt;a&lt;/sup&gt;</td>
<td>31.20 ± 0.77&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

1. A: Co-current agitation for three rounds right after feed in of cooked rice.
   B: Co-current agitation for three rounds 12 hours after feed in of cooked rice.
   C: Counter-current agitation for one round 12 hours after feed in of cooked rice.
2. Data were triplicate (α < 0.05).
Table 4-7. Effect of temperature control at the final stage of fermentations on metabolites productions

<table>
<thead>
<tr>
<th>Temp</th>
<th>Glucoamylase (units)</th>
<th>Pigment (OD500nm)</th>
<th>Monacolin K (ppm)</th>
<th>Citrinin (ppb)</th>
</tr>
</thead>
<tbody>
<tr>
<td>34℃</td>
<td>135.0 ± 18.2a2</td>
<td>0.91 ± 0.27a</td>
<td>46.5 ± 9.5a</td>
<td>616.5 ± 10.8a</td>
</tr>
<tr>
<td>30℃</td>
<td>78.0 ± 11.4ba</td>
<td>0.87 ± 0.18a</td>
<td>53.0 ± 20.3a</td>
<td>467.0 ± 85.1a</td>
</tr>
<tr>
<td>26℃</td>
<td>48.5 ± 7.0b</td>
<td>0.75 ± 0.50a</td>
<td>53.5 ± 5.7a</td>
<td>331.5 ± 263.6a</td>
</tr>
</tbody>
</table>

1. Temperature was lowered at 86th hour to 26, 30, 34℃, respectively, in 4 hours, and maintained until the end of fermentation.
2. Data were triplicate (α < 0.05).