Influence of Light and Temperature on Secondary Metabolites Production by Monascus Ruber in Rice Solid Cultures

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Abstract. Monascus species have been used in Chinese fermented foods such as Anka pork, and rice wine because of its bioactive substances including pigment, and monacolin K. In this study, the effect of light and temperature on red pigment, total polyphenols, DPPH radical scavenging, reducing ability and monacolin K of Monascus ruber BCRC31535 in rice solid culture was conducted. No obvious difference was observed by the DPPH radical scavenging assay whatever the cultural condition of light and temperature was performed. However, the results revealed that the red pigment, total polyphenols, reducing ability and monacolin K were the highest in the darkness at 30°C. Blue light and red light remarkably declined these secondary metabolites and antioxidant capacity, probably resulting from the induction of oxidative stress. By contrast, blue light can stimulate the production of red pigment and monacolin K at 20°C while red light can improve the reducing ability. Nevertheless, total polyphenols were not affected by light at the low temperature. Taking together, the temperature was also the interference factor in the solid-state culture of M. ruber BCRC31535, which influenced the light on the yield of secondary metabolites.

1. Introduction

Monascus species are a filamentous fungus that belongs to the class Ascomycetes and the family Monascaceae. It has been applied in the food industry for thousands of years in Asia because of the production of various secondary metabolites including monacolin K, and pigments [1]. In addition to the useful metabolites, a mycotoxin, namely citrinin, can be found in the Monascus [2]. Monacolin K, pigments and citrinin were belonging to polyketide metabolites [2, 3]. The biosynthesis process of
polyketide from the condensation of one acetyl-CoA molecule in Monascus species is similar to bacterial and mammalian fatty acid synthesis. Monacolin K is the cholesterol synthesis inhibitor and nine genes were involved in its regulation and biosynthesis in Monascus species [3]. Moreover, it has been verified with other bioactivities such as the prevention of acute myeloid leukemia, colon cancer, and neurological disorders [1]. Monascus can produce yellow, orange, and red pigments while the red pigment is regarded as the most important because it can be used as substitutes for nitrites in meat products [4]. Also, Monascus pigment, rubropunctatin, revealed the cytotoxic activity against human cervical carcinoma HeLa cells, which could be a promising natural anticancer agent [5]. By contrast, citrinin is identified as a contaminant in the foods and caused the accumulation in the mitochondria, inducing apoptosis at the cellular level [6].

The light in the environmental factor is a crucial role in the physical profile of Monascus species, such as spore germination, the yield of pigment, monacolin K, and citrinin [7]. Several kinds of research revealed that blue light inhibited pigment production due to the induction of oxidative stress by short-wavelength light [8]. On the contrary, Miyake et al. indicated that red light can improve pigment production, whereas Babitha et al. showed that pigment production was slightly affected by red light [7, 9]. Thus, the light was involved in the metabolism of Monascus with a complex regulatory relationship. In this study, the rice solid culture was used to cultivate M. ruber BCRC31535 under darkness, blue light, and red light at 20°C and 30°C, respectively. The secondary metabolites including red pigment, total polyphenols, 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging, reducing ability and monacolin K were determined. Our results demonstrated that the production of secondary metabolites was affected not only by light but also by temperature.

2. Materials and methods

2.1. M. ruber BCRC31535 cultivation
M. ruber BCRC31535 was incubated in potato dextrose broth (PDB) for 14 days at 25°C. Mycelia were harvested, ground and inoculated into the rice solid media consisting of 20-gram rice (Oryza sativa L.) and 20 mL distilled H₂O which were prepared by autoclave for sterilization. The rice solid media with M. ruber BCRC31535 were allowed to grow under red light, blue light and darkness at 20°C and 30°C, respectively, for 21 days in a constant-temperature light growth incubator with 65% relative humidity. The incubator was set with 3 W red and blue LED monochromatic lamps, while the other one was cultured in darkness.

2.2. Red pigment analysis of M. ruber BCRC31535 in rice solid cultures
To detect the red pigment, M. ruber BCRC31535 in rice solid cultures was harvested, lyophilized and ground. Five-gram powder of M. ruber BCRC31535 was extracted by 95% ethanol of 40mL. The filtrate was concentrated by rotary vacuum evaporator, and dissolved in 8mL methanol, which was further used to detect red pigment, total polyphenols, DPPH radical scavenging, reducing ability, and monacolin K were determined. Our results demonstrated that the production of secondary metabolites was affected not only by light but also by temperature.

2.3. Total polyphenolic analysis of M. ruber BCRC31535 in rice solid cultures
The content of total polyphenols was measured using the Folin–Ciocalteu method. M. ruber BCRC31535 extract (0.05mL) was mixed with 0.5mL of Folin–Ciocalteu reagent for 5min. The 20% sodium carbonate solution with 0.45mL was added to the mixture for 10min in the darkness. The mixture was centrifuged and the supernatant was analyzed at 735nm. Gallic acid was used as a positive control to calculate a standard curve.

2.4. DPPH radical scavenging assay of M. ruber BCRC31535 in rice solid cultures
The M. ruber BCRC31535 extract with 0.4mL was mixed with 0.8mL of 1mM DPPH (dissolved in methanol). The mixture was stood in the darkness at room temperature for 30min. Distilled water and
ascorbic acid were used as the background, and positive control, respectively. The absorbance of the remaining DPPH radical was measured at 517nm by a spectrophotometer.

2.5. Reducing ability of M. ruber BCRC31535 in rice solid cultures
The 0.2 M sodium phosphate buffer (pH 6.6) and 1% K$_3$FeCN$_6$ were mixed with M. ruber BCRC31535 extract at 50°C for 20min. Then, 10% trichloroacetic acid was added, and the mixture was centrifuged at 10,000×g for 10min. The supernatant with 0.5mL was added into 0.5mL H$_2$O and 0.1% FeCl$_3$ of 0.1mL. The absorbance was measured at 700nm by a spectrophotometer. The ascorbic acid was used as a positive control to calculate a standard curve.

2.6. Monacolin K of M. ruber BCRC31535 in rice solid cultures
The extract of M. ruber BCRC31535 was filtered through a 0.22µm filter. The supernatant was conducted by high performance liquid chromatography (HPLC) with a Hitachi L-2130 system (Hitachi Seisakusho Co., Tokyo, Japan) fitted with a reverse-phase C18 column. The HPLC parameters were as follows: solvent A, 0.1% trifluoroacetic acid in water; solvent B, methanol; 10% A and 90% B; flow rate, 0.8 L min$^{-1}$; and detection by UV spectroscopy. A standard monacolin K was used to verify the HPLC analysis.

3. Results and discussion
Monascus species have been used in Chinese solid-state fermented foods. According to the previous study, the pigment production by solid-state fermentation was higher than that of submerged culture [10]. Therefore, the rice solid culture of M. ruber BCRC31535 was performed for the analysis of secondary metabolites in this study (Fig. 1). The result revealed that the mycelium of M. ruber BCRC31535 was full of the rice solid media on the 7th day. When Monascus was cultured to the 21st day, a red pigment can be clearly observed on the rice solid media.

![Figure 1](image-url)

Figure 1. Secondary metabolic analysis of M. ruber BCRC31535 in rice solid cultures. Solid cultures of M. ruber BCRC31535 were incubated at 20°C and 30°C under darkness, red light and blue light for 21 days. The red pigment, total polyphenolics, DPPH radical scavenging assay, reducing ability and monacolin K in rice solid cultures were determined.

The red pigments produced by various species of Monascus have enormous commercial value for food applications. Response to blue light has been documented in the inhibition of pigments in Monascus because of increased oxidative stress [8]. A similar result can be also observed in mycotoxin production of Aspergillus species, such as A. fumigatus, and A. flavus [11, 12]. Our result was consistent with the repression of red pigment by the induction of blue light as well as red light (Fig. 2). However, the result was the opposite at the low temperature. The red pigment under blue light was higher than those of red light and darkness on the 21st day. Meanwhile, the red pigments under darkness, red light, and blue light at 30°C were 27.6-, 6.6-, and 1.4-fold higher than the corresponding values at 20°C. This phenomenon may result from the decline of oxidative stress at the low temperature [13]. The proteins, VeA and FluG, involved in coordinate sexual development and secondary metabolite production were further regulated by light [14].
Figure 2. Red pigment determination of M. ruber BCRC31535 in rice solid cultures. Solid cultures of M. ruber BCRC31535 were incubated at (A) 20°C and (B) 30°C under darkness, red light and blue light for 21 days. The absorbance was measured at 500 nm by a spectrophotometer.

Total polyphenols are responsible for antioxidant activity and recognized as the factor to alleviate oxidative stress [15]. The total polyphenol content of the M. ruber BCRC31535 extract was determined using Folin–Ciocalteu method. The result showed that the total polyphenols at 30°C were higher than that of 20°C (Fig. 3). Besides, the total polyphenols were similar to the result of red pigment production, indicating high polyphenols under darkness at 30°C. It was increasing along with the cultivation of M. ruber BCRC31535, and did not be metabolized into other bioactive compounds. Nevertheless, no obvious difference was observed at 20°C under darkness, red light, and blue light.

Figure 3. Total polyphenols determination of M. ruber BCRC31535 in rice solid cultures. Solid cultures of M. ruber BCRC31535 were incubated at (A) 20°C and (B) 30°C under darkness, red light and blue light for 21 days. The total polyphenolic content was showed by Folin–Ciocalteu method corresponding to gallic acid.

To comprehend the antioxidant activity of M. ruber BCRC31535 extract, the DPPH radical scavenging assay and reducing ability were determined. The result revealed that no remarkable inhibition rate of DPPH radicals was observed no matter what kind of condition was applied. However, the high reducing ability was perceived with the cultivation of M. ruber BCRC31535 at 30°C (Fig. 4). This was in agreement with the result of red pigment and total polyphenols. At the low temperature, the reducing ability corresponding to ascorbic acid under red light and blue light was slightly higher than that in the darkness on the 21st day.
Figure 4. Reducing ability determination of M. ruber BCRC31535 in rice solid cultures. Solid cultures of M. ruber BCRC31535 were incubated at (A) 20°C and (B) 30°C under darkness, red light and blue light for 21 days. The reducing ability corresponding to ascorbic acid was detected by the Prussian blue reaction.

Monacolin K, a kind of polyketide of secondary metabolite, can be produced in M. ruber BCRC31535 which was lacking citrinin, a hepatotoxic compound to humans [2, 6]. Therefore, M. ruber BCRC31535 extract harvested from the different cultural conditions was subjected to HPLC for monacolin K analysis. Comparing the HPLC profile of M. ruber BCRC31535 extract, the metabolic abundance at 30°C was more than that at 20°C (Fig. 5). This was reflected in the production of monacolin K which followed the order, darkness (1098mg/L) > red light (762mg/L) > blue light (600mg/L) at 30°C, and blue light (99mg/L) > red light (11mg/L) > darkness (non-detectable) at 20°C. In addition to the effect of VeA and FluG on secondary metabolites, recent research indicated that long non-coding RNA (lncRNA), a kind of non-coding RNA, could be regulated by blue light and led to change the polyketide production [8]. In this study, the temperature was also involved in the regulation of light on monacolin K production.

Figure 5. HPLC profile of M. ruber BCRC31535 in rice solid cultures. Solid cultures of M. ruber BCRC31535 were incubated at (A) 20°C and (B) 30°C. The black triangle indicates the monacolin K.

4. Conclusion

Monascus species produced several secondary metabolites including bioactive substances for reducing hypertension, anti-putrefaction bacteria, anti-cancer, lowering blood sugar, antioxidants, and inhibitors of cholesterol synthesis. Thus, it was important to understand how to regulate the production of bioactive substances. Several studies have demonstrated that blue light can inhibit polyketide production such as red pigment and monacolin K, resulting from an increase of oxidative stress. This
was in accordance with our results. Moreover, total polyphenols and reducing ability were also influenced by blue light and red light at 30°C. By contrast, blue light and red light can improve the production of secondary metabolites as well as the antioxidant capacity at the low temperature. These results were helpful to explore the regulation of secondary metabolites in *Monascus* species.

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