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Effect of oxygen supply on *Monascus* pigments and citrinin production in submerged fermentation

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The influence of oxygen supply on *Monascus* pigments and citrinin production by *Monascus ruber* HS.4000 in submerged fermentation was studied. For *Monascus* cultivation with high pigments and low citrinin production, the initial growth phase, mid-stage phase, and later-stage production phase were separated by shifting oxygen supply. The optimal condition for the fermentation process in shake-flask fermentation was a three-stage rotating rate controlled strategy (0–48 h at 150 rpm, 48–108 h at 250 rpm, 108–120 h at 200 rpm) with medium volume of 100 mL added to 250 mL Erlenmeyer flasks at 30°C for 120 h cultivation. Compared to constant one-stage cultivation (medium volume of 100 mL, rotating rate of 250 rpm), the pigments were reduced by 40.4%, but citrinin was reduced by 64.2%. The most appropriate condition for the fermentation process in a 10 L fermentor is also a three-stage aeration process (0–48 h at 300 L/h, 48–96 h at 500 L/h, 96–120 h at 200 L/h) with agitation of 300 rpm at 30°C for 120 h cultivation, and 237.3 ± 5.7 U/mL pigments were produced in 120 h with 6.05 ± 0.19 mg/L citrinin in a 10 L fermentor. Compared to aeration-constant (500 L/h) cultivation, pigment production was increased by 29.6% and citrinin concentration was reduced by 79.5%.

[**Key words:** *Monascus*; Oxygen supply; Citrinin; Pigments; Submerged fermentation]

The filamentous fungus *Monascus* sp. has been widely used as an enzymatic agent to produce fermented food products in Asian countries. The main *Monascus* fermented products are *Monascus* pigments which have been used as natural food additives. *Monascus* pigments are also recognized as one of the potential food colorants in European Union and United States (1). *Monascus* pigments are reported to prevent cancer, decrease blood sugar levels, and exhibit anti-inflammatory and anti-tumor properties (2), which are also successfully employed as total or partial substitutes of nitrate and nitrite salts in coloration, flavoring and preservation of red meat (3). *Monascus* pigments were considered as safe food coloring agents until citrinin, a nephrotoxic mycotoxin, was detected in *Monascus* products (4). The creation of citrinin rules out the use of the *Monascus* pigments in food production and processing. It was reported that citrinin could cause 50% cell death to human embryonic kidney cell in a range of 1.8–4.7 mg/mL (5). In the field of food safety, citrinin concentration is limited officially to 0.2 mg/L in Japan, and the standard of specification on the citrinin concentration for functional food products should be less than 2 mg/L in Taiwan (6).

Citrinin and pigments biosynthetic enzymes are regulated independently (7), allowing the prevention of citrinin formation during pigments production by process control. Pigments and citrinin synthesis depend on the *Monascus* strain (8), carbon source, nitrogen sources (9), the ratio of nitrogen to carbon concentration (10), pH (11,12), and other nutritional factors and environmental factors (13–17). In many aerobic fermentation processes, there is often an increase of viscosity in broth with time due to increased cell concentration, or the accumulation of products during fermentation (18). These changes result in a series of problems, especially oxygen supply (18). In cultivation of *Cordyceps militaris*, Mao et al. (19) reported that improving oxygen supply increased cell growth, whereas the highest cordycepin production was obtained a relatively lower oxygen supply. Dou et al. (20) reported that suitable oxygen supply was helpful for improving the biomass and helvolic acid production in large-scale fermentation of *Cordyceps taii*. The oxygen level was reported to play a vital role in the synthesis of pigments and citrinin by *Monascus* sp. (15,16). Both Hajjaj et al. (15) and Pereira et al. (16) found that a high dissolved oxygen concentration enhanced the production of pigments and citrinin in liquid fermentation. Mohamed et al. (17) reported that the effect of aeration and agitation on red pigment production in a 2 L stirred-tank fermentor (STF), but the citrinin concentration wasn't considered. Further studies should be done to optimize the oxygen supply process to obtain high pigments and low citrinin yield in *Monascus* cultivation. The control of the oxygen supply environment is necessary in optimized process control strategies according to time course of cell growth and metabolite productions. Well-directed process parameters shift representing a valuable control strategy will be beneficial to the cell growth and metabolite biosynthesis. PH-shift strategy (12) or temperature-shift strategy (21) has been proven to be an efficient strategy for the production of bioactive metabolites in *Monascus* fermentation. Oxygen

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supply-shift has been proven to be an efficient strategy for the production of bioactive metabolites in many other microorganisms (22–26). However, to the best of our knowledge, little research has been reported about the effect of shift oxygen supply control strategy on pigments and citrinin production in the submerged fermentation of *Monascus*. Therefore, the article was to investigate the shift oxygen supply control strategy on *Monascus* pigments accumulation and citrinin formation by *Monascus ruber* HS.4000 in shake-flask and a 10 L fermentor. Oxygen supply was adjusted in flask cultivation by variation of medium volumes and rotating rate in 250 mL flasks. In a 10 L fermentor, aeration and agitation were adjusted to explore the best oxygen supply conditions for high pigments yields with low citrinin production.

MATERIALS AND METHODS

Equipment and reagents An Agilent1200 series high performance liquid chromatography (HPLC) (Agilent Technologies, USA) consisting of a quaternary pump and a fluorescence detector was used to determine citrinin content. The 10 L SG series stainless steel fermentation tank was purchased from Shanghai Lianhuan Bio-engineering Equipment Co. Ltd. The ZHWY-2102C series of shaking incubators was purchased from Shanghai Zhicheng Analytical Instrument Manufacturing Co. Ltd. The acetonitrile were of HPLC grade. All other reagents were of analytical grade.

Microorganism *M. ruber* HS.4000 was obtained from Key Laboratory of Fermentation Engineering (Ministry of Education), College of Bioengineering, Hubei University of Technology.

Medium The seed medium consisted of (g/L): rice flour, 30; $MgSO_4 \cdot 7H_2O$, 1; KH_2PO_4, 1.5; $NaNO_3$, 5. The liquid mycelia culture medium (g/L): glucose, 90; $MgSO_4 \cdot 7H_2O$, 1; KH_2PO_4, 1.5; $NaNO_3$, 5. The fermentation medium was composed of (g/L): rice flour, 60; glucose, 30; peptone, 20; $MgSO_4 \cdot 7H_2O$, 2; KH_2PO_4, 1.5; $NaNO_3$, 2.

Inoculum preparation 10 mL of cell suspension were inoculated into a 250 mL flask containing 100 mL of seed medium, which was adjusted to a final pH of 5.5, and then cultivated at 30°C on rotary shaker at 150 rpm for 48 h.

Fermentation process in shake flasks The effect of oxygen supply on pigments and citrinin was studied in shake flask containing various volumes and rotating rate of fermentation media to give different levels of oxygen supply. The 250 mL Erlenmeyer flasks contained 60 mL, 80 mL, 100 mL and 120 mL of fermentation medium. The medium pH was adjusted to 5.5 with lactic acid. Ten percent (v/v) of the seed culture was used to inoculate the flasks. The culture was incubated on a rotary shaker at 30°C and 200 rpm for 120 h.

During the entire fermentation process, the rotating rate was controlled at 150 rpm, 200 rpm, 250 rpm, two-stage strategy (0–48 h at 150 rpm, 48–120 h at 250 rpm), or three-stage strategy (0–48 h at 150 rpm, 48–108 h at 250 rpm and 108–120 h at 200 rpm), respectively, to investigate the effects of different rotating rate on pigments and citrinin of *M. ruber* HS.4000 with volume fixed at 100 mL in 250 mL Erlenmeyer flasks at 30°C for 120 h cultivation.

Batch operation in a 10 L bioreactor The effect of oxygen supply on the strain culture was also studied by batch fermentation in a 10 L bioreactor with a working volume of 6 L. The medium, pH and temperature used in this study were the same as that of the shake-flasks culture. The fermentor was incubated according to the culture condition for 120 h.

During the entire fermentation process, the agitation speed was constantly controlled at 300 rpm, 600 rpm, or two-stage agitation controlled strategy (0–60 h at 600 rpm, 60–120 h at 300 rpm), respectively, to investigate the effects of different agitation speeds on pigments and citrinin of *M. ruber* HS.4000 with aeration rate fixed at 200 L/h.

In the fermentation, the aeration was controlled at 500 L/h, 300 L/h, 200 L/h, two-stage aeration controlled strategy (0–48 h at 300 L/h, 48–120 h at 500 L/h), or three-stage aeration controlled strategy (0–48 h at 300 L/h, 48–96 h at 500 L/h and 96–120 h at 200 L/h) respectively, to investigate the effects of different aeration on pigments and citrinin of *M. ruber* HS.4000 with agitation rate fixed at 300 rpm.

Biomass estimation The insoluble substrate (rice flour) was used in the medium, so it was difficult to determine the biomass concentration directly. The method of biomass estimation was followed by Liu et al. (27). The intracellular nucleic acid was measured to represent the mycelia concentration.

Estimation of pigments concentrations The concentration of *Monascus* pigments was presented and estimated according to the similar method of Chinese National Standard, GB15961-2005 and some reported articles (8,28–32). A suspension (10 mL) of *M. ruber* HS.4000 cultivated on the fermentation medium was sprayed into 5 volumes of 70% (v/v) ethyl alcohol, and shaken for 2 h on a reciprocal shaking water bath at 200 rpm. Supernatants were obtained by centrifugation at 7000 rpm for 15 min (8). The supernatant was collected and the pigment production was analyzed by measuring the absorbance of the supernatant using UV–visible spectrophotometer (Unic-2000) (8,28–31). *Monascus* pigments mainly include yellow-colored, orange-colored and red-colored pigments. The highest absorbance (U) of these pigments was measured at 505 nm (red), 460 nm (orange), and 400 nm (yellow) with a spectrophotometer (32). The sum of the three values was used as an index of pigments production.

Citrinin analysis Citrinin was extracted by procedure of Pattanagul et al. (31). The HPLC conditions were as follows (33): Used a quaternary pump and a fluorescence detector. Column temperature 28°C, flow rate 1 mL/min. Citrinin was determined by HPLC on a C18 column (Zorbax Eclipse XDB C185 μm, 250 mm × 4.6 mm) using the mobile phase with the composition of water (pH was adjusted to 2.5 with H_3PO_4) to acetonitrile (50:50). Detection wavelength: λ_{ex} = 331 nm, λ_{em} = 500 nm. Aliquots of 20 μL standard or samples solutions were injected into the HPLC for the determination. All injections were repeated at least three times.

RESULTS AND DISCUSSION

Pigments and citrinin production in 250 mL flasks with rotating rate controlled at 200 rpm and working volume controlled at 80 mL To investigate the tendency of time course of cell growth and metabolite productions, a trial of rotating rate controlled at 200 rpm and working volume controlled at 80 mL on pigments and citrinin production was firstly examined respectively at different time points in 250 mL flasks. The flasks were incubated on a rotary shaker at 30°C, and duplicate flasks were withdrawn at intervals of 12 h. The comparative results are shown in Fig. 1. *Monascus* started to grow from 0 h to 48 h, but few pigments or little citrinin production was obtained. From 48 h to 108 h, *Monascus* showed the phenomenon of secondary growth and the biomass reached the maximum of 1.2 ± 0.02 g/100 mL at 108 h. The pigments concentration were mainly increased from 48 h to 96 h, and reached the maximum of 71.4 ± 1.05 U/mL at 120 h. Hu et al. (34) determined the time course of batch fermentation of *Monascus anka* and also found that there was nearly no pigment formation on the first day, but the pigments concentration increased dramatically from the second to the fourth day. Most of the metabolites were produced after the rapid microbial growth. From 48 h to 120 h, the content of citrinin in the process was constantly increased and it reached as high as 0.98 ± 0.018 mg/L at 120 h. The results indicated that *Monascus* pigments and citrinin were secondary metabolites, and when the pigments were obtained at the highest degree, citrinin was not largely produced. Although the biosynthesis pathways of pigments and citrinin are related, our results showed that among *Monascus*, the two pathways are independent under the conditions of these experiments. After Carvalho et al. (35) studied the relation between growth, respirometric analysis and pigments production from *Monascus* by solid-state fermentation,

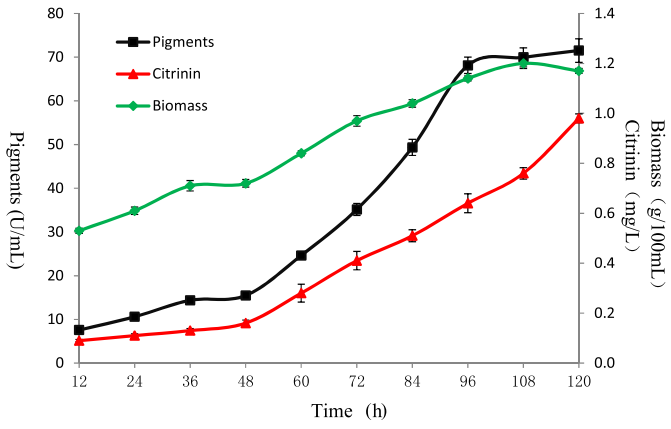

FIG. 1. Effects of constant working volumes (80 mL), rotating rate (200 rpm) on biomass content, pigments and citrinin production in 250 mL flasks. The error bars in the figure indicate the standard deviations from three independent samples.