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Effect of aeration mode on the red pigment production by *Monascus ruber* on rice during fermenting in the packed bed bioreactor

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Abstract

Solid state fermentation for a red pigment production of *Monascus ruber* NBRC 32318 on rice in an aerated packed bed bioreactor was studied. The constant and the inconstant aeration rates were studied in this research. The constant rates were performed using 0.05 and 0.10 vvm during fermentation. The inconstant ones were conducted by changing the aeration rate when the growth of fungi entered the stationary phase. The inconstant aeration were studied in three different modes composed of 1) starting with 0.10 vvm aeration and then changing to 0.05 vvm one, 2) starting with 0.10 vvm aeration and then increasing to 0.15 vvm one, and 3) starting with 0.05 vvm aeration and then increasing to 0.10 vvm one. The growth and the density of red pigment were analyzed along the height of packed bed. The results show the effect of changing aeration rate during fermentation on the red pigment production. The appropriated aeration rate seemed to be relative with the height of bed.

Keywords : *Solid state fermentation, aeration, packed bed bioreactor.*

1. Introduction

Color is one of important factors to enhance the consumers' acceptability of foods. Currently, customers' awareness about the synthetic food ingredients leads to increase the use of natural ones. Most natural colorants are from plant and animal extracts that are exempt from certification are utilized worldwide. However, they are and their applications are limited. The interest in microbial productions of natural food colorants have been growing as alternative ways. A famous microorganism producing red pigment for food is a filamentous fungus Monascus. The Monascus red pigment has traditionally been produced by solid state fermentation (SSF) of Monascus on steamed rice in a multi-tray bioreactor. Since the thickness of bed is limited to prevent accumulated heat and to provide an aerobic condition[1], a big production area is required. This

research interests to use a packed bed bioreactor for the SSF due to the aeration which supply oxygen to fungi and remove an accumulated heat in the bed; hence, the thickness of bed can be increased.

The previous work[2] conducting on the Monascus red pigment production by submerged fermentation showed the effect of volumetric liquid–phase mass transfer coefficient ($k_L a$) of oxygen on both cell mass concentration and red pigment production. Another work[3] indicated that the red pigment production was highly influenced by the morphology of filamentous fungal M. ruber affected by a level of oxygen supply[4].

Some researches conducting SSF on the Monascus red pigment production indicated that higher aeration in packed bed bioreactor improved the red pigment production because of more supply of oxygen that was essential for polyketides synthesis which related to pigmentation[5], but biomass and red pigment productions were reduced due to an evaporation[6]. Although the red pigment of Monascus is the secondary metabolite that is produced when the growth of fungi becomes a stationary phase or after the log phase, there are a few researches studying on a difference of oxygen requirement or optimum aeration between primary and secondary metabolisms of Monascus. This research aimed to determine the effect of aeration change in stationary phase on the growth and the red pigment production of M. ruber on rice using the packed bed bioreactor.

2. Materials and Methods

2.1 Spore production

Dehydrated M. ruber NBRC 32318 was obtained from the Department of Biotechnology, National Institute of Technology and Evaluation, Chiba, Japan. It was revived in a sterilized solution containing yeast extract 2 g/L, peptone 10 g/L, and $MgSO_4 \cdot 7H_2O$ 1 g/L. Inoculum suspension of M. ruber spores was prepared by incubating the strain for 10 days at 37°C on the growth medium (2% agar) containing glucose 10 g/L, yeast extract 3 g/L, K_2HPO_4 2 g/L, and $MgSO_4 \cdot 7H_2O$ 0.2 g/L in 86 mm diameter plastic disposable Petri dishes. Then, the sporulated culture of M. ruber was scrapped and suspended in 5 mL sterilized water and the suspension was filtrated through Whatman No.1 filter paper under strict aseptic conditions. The number of spores in the filtrate was counted using a hemocytometer (Kayagaki, Tokyo, Japan). The density of spore in the suspension was adjusted to 1×10^6 spores/mL by adding sterilized water.

2.2 Substrate preparation and inoculation

The amount of 40 g of rice (Oryza sativa) was mixed with distilled water to obtain the moisture content 50% (w/w), and then it was sterilized at 121°C for 20 min. After the sterilized rice substrate was cooled to ambient temperature under aseptic condition, it was inoculated with the spore suspension 2×10^5 spores/g wet substrate.

2.3 Solid state fermentation

The inoculated rice 80 g was packed in the glass column which was connected to the sterilized air system as shown in Fig. 1. The height of bed was set at 18 cm for all experiments. Ten sets of rice packed column with aeration device were used for one test run which conducted the SSF for 10 d. Each fermented column was taken at 1 d interval to analyze the fungal growth and the pigment production until 10 d. The aeration rates of humidified air were studied at constant and inconstant rates. The constant rates were 0.05 and 0.10 vvm (mL of air/mL of bed/min.). The inconstant ones were studied by changing the aeration rate when the fungal growth had become the stationary phase. The three conditions of inconstant aeration in this research consisted of 1) starting with 0.10 vvm aeration and then changing to 0.05 vvm one, A010B005; 2) starting with 0.10 vvm aeration and then increasing to 0.15 vvm one, A010B015; and 3) starting with 0.05 vvm aeration and then increasing to 0.10 vvm one, A005B010. The incubation temperature was controlled at 30°C. Each experiment was conducted in duplicate.

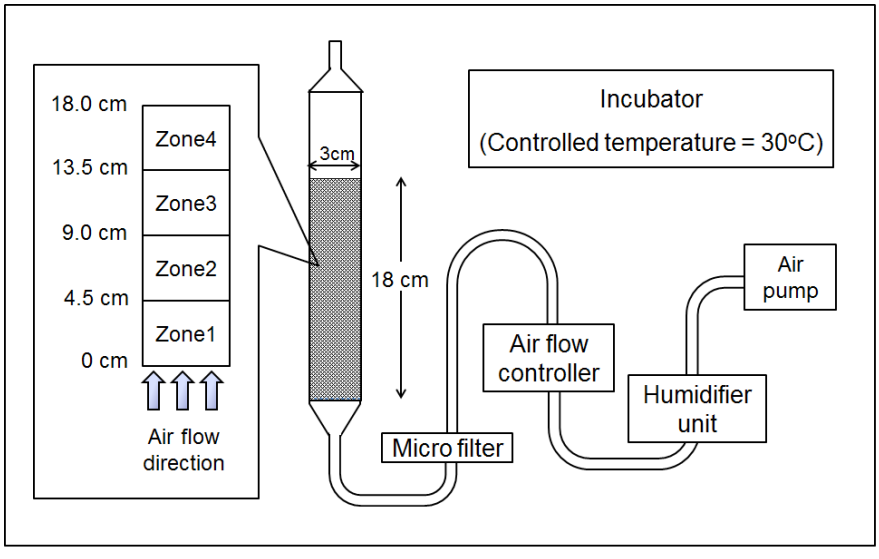

Figure 1. Packed bed column used in this study

2.4 Growth analysis

The amount of 1 g of fermented rice was hydrolyzed by 60% sulfuric acid solution and analyzed the glucosamine content using a colorimetric method[7]. The growth of *M. ruber* was reported in a unit of glucosamine content in dry fermented rice (mg/g). The analyses were performed in triplicate.

Data of glucosamine content in fermented rice in the log phase were used to calculate the specific growth rate using equation (1).

$$m = \frac{1}{x}\frac{dx}{dt} \quad (1)$$

in which x is glucosamine content (mg/g biomass), t is incubation time (d), and μ is specific growth rate (1/d).

2.5 Red pigment determination

Red pigment was extracted from fermented rice by soaking 0.50 g of dry fermented rice in 5.0 mL 95% of ethanol solution for 1 d, and then centrifuging at 1,500 × *g* for 5 min. The red pigment was determined by measuring an absorbance at the 500 nm wavelength of the supernatant by spectrophotometer.

3. Results

3.1 Growth of *M. ruber* NBRC 32318

The growth of *M. ruber* was presented in glucosamine content in dry fermented rice. The fungal growth in the packed bed column with aeration rate at 0.10 and 0.05 vvm are shown in Fig. 2. The initial glucosamine content, which was 8.3 ± 0.2 mg/g dry substrate, was from the initial inoculum of *M. ruber* spores. The maximum contents of glucosamine were determined as 23.6 ± 2.3 and 17.9 ± 1.2 mg/g dry fermented rice for the SSF with 0.10 and 0.05 vvm aeration, respectively. The glucosamine content in fermented rice increased until 3 days of SSF with both aerations 0.10 (S010) and 0.05 (S005) vvm, and then they became slightly lower for the run with 0.10 vvm and kept constant for the run with 0.05 vvm. The specific growth rates were calculated using eq.(1) as 0.35 and 0.25 d^{-1} for the SSF with the aeration at 0.10 and 0.05 vvm, respectively.

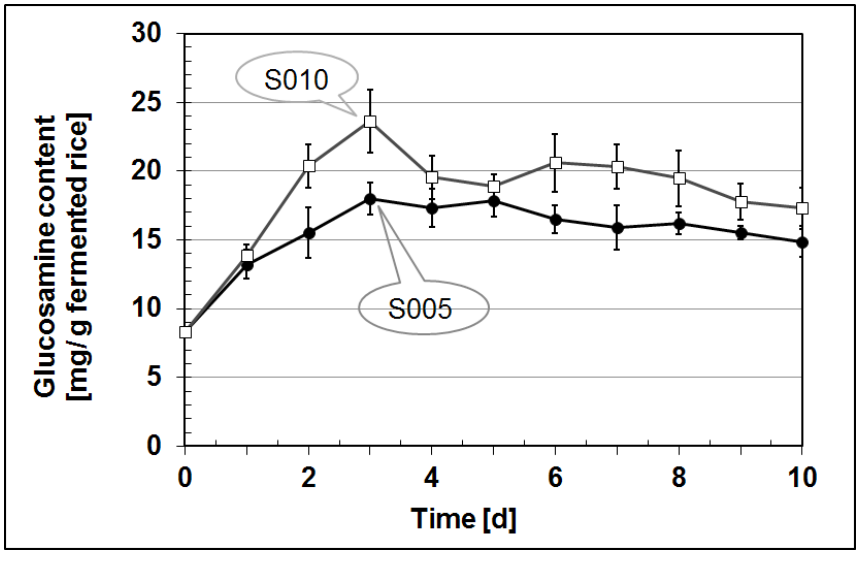

Figure 2. Average contents of glucosamine analyzed from fermented rice in four zones of bed during SSF with aeration at 0.05 and 0.10 vvm. Circle, 0.05 vvm aeration rate. Square, 0.10 vvm aeration rate. Error bars represent the standard deviations of the triplicate measurements.

The growths were monitored until 10 days by measuring the glucosamine contents in fermented rice collected from four zones along the height of packed bed fermented with different conditions of aeration. Fig. 3 shows the measured glucosamine contents in fermented rice after terminating SSF. The maximum values found on the third day of fermentation presented in the bed with 0.10 vvm aeration (S010) were 23.0 ± 1.3, 24.7 ± 0.5, 23.6 ± 1.9 and 23.0 ± 0.7 mg/g dry fermented

rice from zone 1 to 4, respectively. The maximum ones in the bed with 0.05 vvm aeration (S005) were 17.6 ± 0.7, 18.3 ± 1.2, 18.5 ± 1.0 and 17.5 ± 0.9 mg/g dry fermented rice obtained from zone 1 to 4, respectively.

The slightly increasing glucosamine content in every zone along the bed in stationary phase were found in both runs with the constant aeration at 0.05 vvm (CON005) and another run increasing aeration to 0.10 vvm in stationary phase (A005B010). In contrast, the analyzed glucosamine contents obtained from the SSF with starting aeration 0.10 vvm kept constant values since starting a stationary phase (S010), even if the aeration were decreased to 0.05 vvm (A010B005) or increased to 0.15 vvm (A010B015).

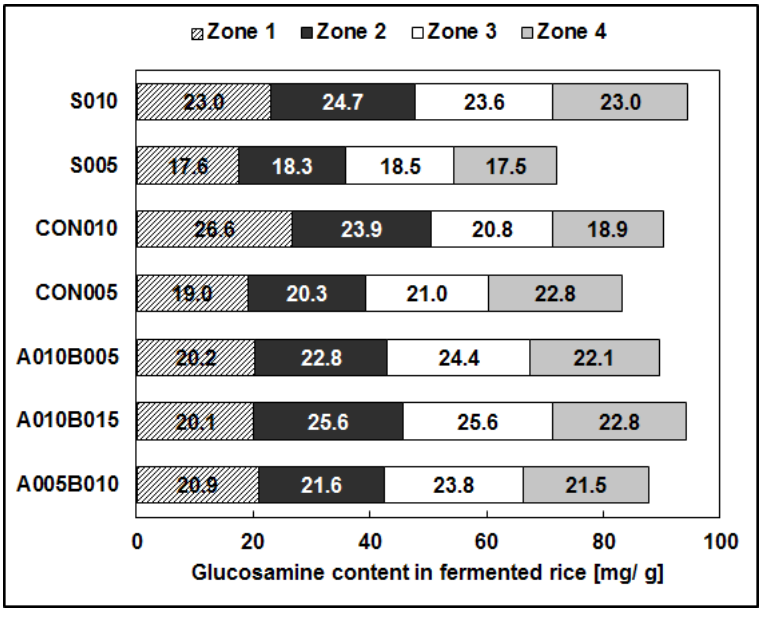

Figure 3. Glucosamine content in fermented rice in different zones of column. S010 and S005 were the conditions of 3 d SSF under the constant aeration rate at 0.10 and 0.05 vvm respectively. CON010 and CON005 were the conditions under the constant aeration rates at 0.10 and 0.05 vvm respectively. A010B005 was the conditions under which the initial aeration rate was 0.10 vvm then changed to 0.05 vvm after the fungus entered the stationary phase. A010B015 was the conditions under which the initial aeration rate was 0.10 vvm then changed to 0.15 vvm after the fungus entered the stationary phase. A005B010 was the conditions under which the initial aeration rate was 0.05 vvm then changed to 0.10 vvm after the fungus entered the stationary phase.

3.2 Red pigment production

The red pigment was detected in the column at the third day of SSF or at the time when the fungus entered the stationary phase (S010 and S005 in Fig. 1). Fig. 4 shows the red pigment densities of the extracts of red rice fermented in the different aeration. The highest average red pigment production was found in the SSF with A005B010 as 316 ± 24/g dry fermented

rice, followed by the SSF with CON005 (236 ± 15/g dry fermented rice), which was not significant different with CON010 (226 ± 11/ g dry fermented rice), and A010B005 (212 ± 9/g dry fermented rice, which was not significant different with A010B015 (202 ± 8/ g dry fermented rice). The results show that there was no difference in the average red pigment production by SSF with the constant aeration at 0.10 and 0.05 vvm. However, the lowest red pigment production was found as 191 ± 11/g dry fermented rice at the top of column in CON010 while it was found as 166 ± 18/g dry fermented rice at the bottom of column in CON005. Comparing with the constant aeration 0.10 vvm (CON010), both decreasing (A010B005) and increasing (A010B015) aeration after stationary phase slightly decreased the average red pigment production from 226 ± 14 to 212 ± 12 and to 202 ± 19 /g dry fermented rice, respectively.

It was evident that the red pigment density of fermented rice obtained from the SSF with increasing aeration from 0.05 to 0.10 vvm (A005B010) was higher that the SSF with constant aeration at 0.05 vvm (CON005) in all zones of column. The highest red pigment density was found in the SSF with the aeration of A005B010 at the position near the top of the column as 349 ± 7 /g dry fermented rice. The lowest one was found from the SSF with the aeration of A010B015 at the bottom of column as 135 ± 11 /g dry fermented rice.

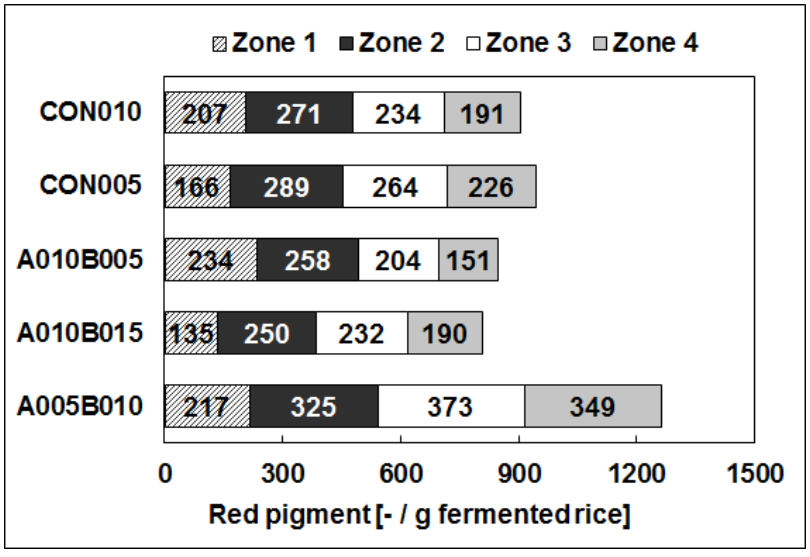

Figure 4. Red pigment production of M. ruber cultivated for 10 days in the packed bed column under different aerating conditions. CON010 and CON005 were the conditions under the constant aeration rates at 0.10 and 0.05 vvm respectively. A010B005 was the conditions under which the initial aeration rate was 0.10 v vm then changed to 0.05 vvm after the fungus entered the stationary phase. A010B015 was the conditions under which the initial aeration rate was 0.10 vvm then changed to 0.15 vvm after the fungus entered the stationary phase. A005B010 was the conditions under which the initial aeration rate was 0.05 vvm then changed to 0.10 vvm after the fungus entered the stationary phase.

3.3 Moisture content of bed

The moisture contents of fermented rice in the packed bed column after fermentation are summarized in Table 1. All analyzed moisture contents along the height of bed, which was obtained from all conditions of aeration, show higher values than that of the initial substrate (50% wet basis). The results showed the lowest moisture contents at the bottoms of the bed (zone 1) in the SSF with the aeration of CON010, CON005, A010B005 and A010B015 as 61.8 ± 1.7, 62.8 ± 0.8, 64.3 ± 0.8 and 60.5 ± 0.7 %, respectively. There were no significantly different among the moisture content of fermented rice along the height of bed in the SSF with A005B010 aeration. The measured moisture content obtained from the run of A010B005 was higher than that with the other conditions in all zones of packed bed. Among all experiments, the lowest and highest moisture contents of fermented rice were found as 60.5 ± 0.7 % at the bottom of bed (zone 1) in the run of A010B005 and as 69.0 ± 2.1% at the middle of bed (zone 2) in the run of A010B005, respectively.

Table 1. Moisture contents of fermented rice along the height of packed bed after 10 days SSF with different aerating conditions

Zone	CON010	CON005	A010B005	A010B015	A005B010
4	65.0 ± 2.0^{a}	64.0 ± 2.2^{a}	65.0 ± 1.1^{ac}	64.0 ± 1.3^{a}	62.8 ± 2.4^{a}
3	65.8 ± 2.5^{a}	66.1 ± 2.1^{a}	67.2 ± 1.3^{ab}	65.8 ± 1.7^{ac}	64.9 ± 1.2^{a}
2	65.4 ± 1.2^{a}	65.6 ± 1.3^{a}	69.0 ± 2.1^{b}	62.3 ± 0.8^{ad}	63.2 ± 1.9^{a}
1	61.8 ± 1.7^{b}	62.8 ± 0.8^{b}	64.3 ± 0.8^{c}	60.5 ± 0.7^{b}	62.3 ± 1.6^{a}

Remark: - S010 and S005, when stationary phase begin in the SSF with aeration 0.10 and 0.05 vvm, respectively. CON010 and CON005, after SSF with constant aeration at 0.10 and 0.05 vvm, respectively. A010B005, after SSF with inconstant aeration rate starting at 0.10 vvm and then decreasing to 0.05 vvm. A010B015, after SSF with inconstant aeration rate starting at 0.10 vvm and then increasing to 0.10 vvm. A005B010, after SSF with inconstant aeration rate starting at 0.05 vvm and then increasing to 0.10 vvm.

- Different letters indicate significant differences in mean values in each column. Mean values with the same superscript letters (a, b, c or d) were similar and no statistically significant differences were observed for these samples.

4. Discussions

4.1 Growth of *M. ruber*

The growths evaluated by the glucosamine content in fermented rice showed the changes of growth phase from a log to a stationary phase at the same time which was the third day of fermentation in both SSF with aeration 0.10 and 0.05 vvm. Therefore, the aeration did not affect the time starting stationary phase. From this finding, the aeration rate in the experiments of inconstant aeration was changed at the third day of fermentation. To explain the effect of aeration change on the fungal growth, the experiment was divided into two groups; 1) the constant aeration 0.10 vvm (CON010) comparing with the inconstant ones consisting of reducing aeration to 0.05 vvm (A010B005) and

increasing to 0.15 vvm (A010B015) in stationary phase and 2) the constant aeration 0.05 vvm (CON005) comparing with the inconstant one increasing aeration to 0.10 vvm in stationary phase (A005B010). The results of both groups indicated that there was no effect of changing aeration on the growth phases because the measured glucosamine contents in dry fermented rice after finishing SSF obtained from the run with constant and inconstant aeration were not significantly different. It was because that the aerations changes were conducted after the fungal growths entered the stationary phase. Nevertheless, the aeration rate affected the value of specific growth rate which that of the SSF with 0.10 vvm aeration was greater than that with 0.05 vvm one. It could be implied that the amount of fungi for red pigment production from CON010, A010B005 and A010B015 were higher than that from CON005 and A005B010.

4.2 The relation between the growth and the red pigment production of *M. ruber*

The red pigment production in the SSF with CON010 and CON005 were not significantly different even if the growth rate of CON010 was higher than that of CON005. Therefore, other than a fungal growth rate, a cultivating condition during spore germination and growth phase; for example, an amount of oxygen supply and a heat accumulation problem should be considered to increase the red pigment production in SSF using a packed bed bioreactor. The measured temperature at the center of packed bed during fermentation confirmed that there was no effect from heat accumulation because the measured temperature was constant at 30°C throughout the fermentation.

Changing aeration affect not only red pigment production, but also the distribution within the bed. The bed with aeration A005B010 showed the most uniform moisture distribution. However, the bed with the aeration of A005B010 in different height of column showed the different amount of red pigment production.

Increasing aeration in stationary phase from 0.10 to 0.15 vvm (A010B015) reduced the red pigment production, especially at the top (zone 4) of the column but the red pigment production extremely raised when the aeration increased from 0.05 to 0.10 vvm (A005B010), even if the moisture contents of their beds in the same zone were quite close. Oxygen (O_2) or/and carbon dioxide (CO_2) partial pressure in the packed bed column, besides moisture content of substrate, seemed to be an important factor affecting the red pigment production because the productions of red pigment in different zones in the SFF with A005B010 aeration were different even if the moisture contents of bed was not statistically different. The red pigment in the SSF with A010B005 aeration was lower than that with CON010 aeration because of 1) high moisture contents in the beds due to too low aeration resulting in the low moisture transfer from center to the top of the bed and 2) low oxygen supply resulting in lower red pigment production in the higher zone. Also, the red pigment in the SSF with A010B015 aeration was lower than that with CON010 aeration, especially at the bottom of column (zone 1) because increasing aeration caused the high evaporation. In contrast to the SSF with A005B010 aeration, the red pigment production increased when the aeration increased from 0.05 to 0.10 vvm in stationary phase. It may be due to the

optimum oxygen partial pressure, in particular, near the top of column (zone 3). The results indicated that the aeration appropriated to the growth and the pre-culturing was 0.05 vvm but it required higher aeration in the period of red pigment production.

5. Conclusion

The red pigment production of *M. ruber* was clearly affected by how aeration was applied. Changing aeration rate in stationary phase influenced the red pigment production but it did not affect the growth. It was believed that the important factors affecting the red pigment production were the oxygen supply along the height of packed bed and moisture distribute besides heat accumulation.

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