Biotechnol. Bioprocess Eng. **2002**, 7: 21-25

Production of Red Pigments by *Monascus purpureus* in Solid-state Culture

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Abstract To maximize and sustain the productivity of *Monascus* pigments, various environmental and nutritional parameters, such as the initial moisture content, pH, inoculum size, sample size, and nutrient supplement, that influence pigment production were evaluated in solid-state cultures as follows: initial moisture content, 50%; pH, 6.0; inoculum size 1×10^4 spore cells (grams of dry solid substrate)$^{-1}$; sample size, 300 g. All supplementary nutrients (carbon, nitrogen, and mineral sources) added has inhibitory effects on the cell growth and red pigment production. In open tray culture the maximum biomass yield and specific productivity of red pigments were 223 mg DCW (grams of initial dry substrate)$^{-1}$ and, 47.6 OD_{500} (DCW grams)$^{-1}$ h^{-1}, respectively.

Keywords: environmental factors, *Monascus purpureus*, red pigments, solid-states culture

INTRODUCTION

Fungi from the *Monascus* species are known as sources of various secondary metabolites. Pigments synthesized by the fungi *Monascus* spp. have been traditionally used in Asia for coloring and securing a number of fermented foods, such as alcoholic beverage, red soybean curd, meat, and vegetables [1]. Furthermore, their therapeutic properties and relatively high stability with respect to pH and temperature are interesting features that promote their use as substitutes for synthetic colorants [2].

It has been reported that *Monascus* spp. can produce six related pigments, which can be divided into three groups: Two are orange (rubropunctatin and monascorubin), two are yellow (monascin and ankaflavin) and two are red (Rubropunctamine and monascorubramine). Among these, the red pigments are of particular interest, because red is the most popular food coloring and true red natural pigments suitable for food use are difficult to obtain [3].

The production of *Monascus* pigments can be obtained in both solid-state and submerged culture. Extensive submerged culture studies of red pigment synthesis by *M. purpureus* strains have revealed that the yield is markedly affected by many factors, including the medium composition, pH and agitation [4-6]. In addition, the pigments produced in submerged culture are mainly retained intracellularly, causing inhibition of further production [1,7]. For these reasons, the red pigment yield of solid culture is superior to that of submerged cultures.

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In general, the solid-state culture technique is preferred to the submerged culture technique because the solid-state technique is simple, requires less capital investment, features lower levels of catabolite repression and end-product inhibition, produces lower amount of waste water output, allows for better product recovery, and yield a high-quality product [8]. Therefore, a solid-state culture is considered to be a very attractive process for the production of primary and secondary metabolites.

To maximize and sustain the productivity of *Monascus* pigments optimization of the culture conditions is critical. Accordingly, the study sought to evaluate the effects of various environmental and nutritional factors - including the initial moisture content, pH, inoculum size, air rate, sample size, and nutrient supplements - to determine their influence on pigment production in solid-state cultures.

MATERALS AND METHODS

Microorganism and Medium

The strain used in this study was *Monascus purpureus* (ATCC 16362). The stock culture was maintained on a YM agar slant [9] containing: glucose, 20 g; malt extract, 3 g; peptone, 5 g; yeast extract, 3 g; agar, 1.5 g; and distilled water, 1 L. Long grain rice (from Thailand) was used as the substrate for the solid-state cultures.

Cultivation

Agar plates containing the YM medium were inoculated by *M. purpureus* from colonies on 5-day old agar plate and then incubated at 30°C for 5 days. Ten mL of

sterile distilled water was added to each agar plate, which had been gently scraped with flamed slide glass. This suspension of mycelia and spores was filtered through a syringe containing cotton to remove the mycelia and debris. A 10% volume of the spore suspension was then inoculated into rice and incubated in growth chamber with 95% relative humidity at 30°C for 7 days. To investigate the effect of the initial moisture content, pH, inoculum size, and nutrient supplement on red pigment production, solid-state flask cultures were performed in 100-mL flask containing 7 g of rice that was presoaked in distilled water at room temperature for 6 hours and sterilized at 121°C for 20 min. Moderate mixing with spoon (2 times/day) served to separate the rice particles and ensure a uniform growth of mycelia on the surface. The sample size and aeration rate were investigated in open tray (28 × 22 × 6 cm) cultures. Air was supplied through the porous tube fixed to the bottom of each tray. The humidification of the air was provided by a series of three 250 mL of bottles each containing 100 mL of sterilized water.

Assays

The red pigment estimation was performed using a HP 8890 UV/VIS spectrophotometer at 500 nm. The culture samples were freeze-dried and stored at –70°C. The dried samples were then ground into a powder using a mortar and pestle. Twenty milliliters of 95% ethanol was added to 0.1 g of the powdered sample in a 100 ml flask, then the pigment was extracted in a darkened room at 25°C after 24 h with occasional shaking. The resultant ethanol extracts were centrifuged at 5,000 rpm for 10 min to remove the suspended solids, and then diluted with 95% ethanol as necessary. After dilution, the optical density (OD) of extract was measured at 500 nm as the red pigment. The weight of the extracted pigments was also measured after evaporation. The water-soluble pigments were extracted in a water bath at 70°C for 20 min, and measured by method described above. A portion of the freeze-dried sample was also used for a biomass estimation. As an indirect measurement of cell growth, glucosamine was measured according to the method proposed by Adidoo *et al.* [10]. Glucosamine measurements of the dried solids were made in the course of fermentation, as calibrated by submerged culture measurements of the mycelial biomass. The moisture content of the samples was determined by drying the rice in dry oven at 100°C for 12 h and measured the weight loss.

RESULTS AND DISCUSSION

Effect of Various Factors on Cell Growth and Red Pigment Production

Initial Moisture Content (IMC)

The water content of substrate is one of the most important factors determining the yield of secondary metabolites in solid-state culture. The effect of IMC of the rice on the pigment production was examined by placing approximately 7 g of humidified autoclaved rice with various IMCs in a flask. The results are recording in Fig. 1. The IMC of the rice significantly affected the production of the red pigment. The mycelium of *Monascus purpureus* should permeate inside the rice and then use the substrates to manufacture enzyme and red pigments. With an IMC of up to 50%, the production of red pigments by *M. purpureus* increased in proportion to the increase in the IMC. The maximum production of biomass and pigments was obtained with a 50% IMC. The moisture content varied from an initial value of approximately 50% to a final value of about 77%. To a certain extent, the physicochemical effect of moisture is only relevant to primary metabolism. At an IMC of 60%, the red pigment production decreased drastically. Further move separating particles of fermenting rice with a moisture content greater than about 55% became very difficult. This aggregation of substrate particles resulted in oxygen starvation and a poor distribution of mycelia, thereby resulting in poor pigment production.

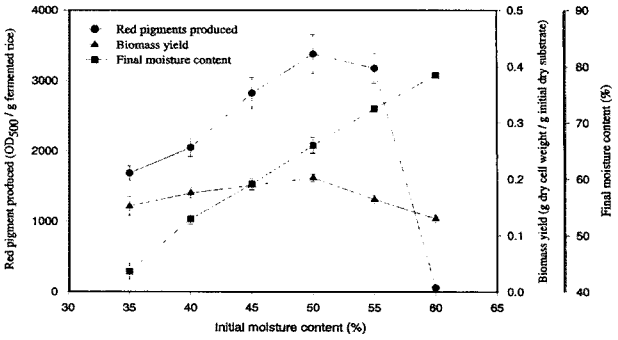

Fig. 1. Effect of initial moisture content on cell growth and red pigment production in solid-state flask cultures. Values represent means of three independent experiments and error bars indicate ± S.D.

pH

The results from the solid-state flask cultures performed at various pHs are presented in Fig. 2. The initial pH of the steeping water clearly influenced the red pigment formation. A pH range of 5.5 to 6.5 favored red pigment production, while pH higher than 6.5 and lower than 5.5 resulted in a decreased of red pigments. The optimum pH for growth was not the same as that for pigment production. The maximum biomass was obtained at pH of 4.5, while the highest levels of red pigment were obtained at a pH of 6.0, a pH level close to the natural pH of the rice samples soaked in distilled water. This phenomenon was attributed to inhibited rice reddening due to drop in the pH level during cultivation. The effect of the initial pH on the pigment production in the solid rice cultures was similar to that observed by Carels and Shepherd [4] and Lin [1] in sub-

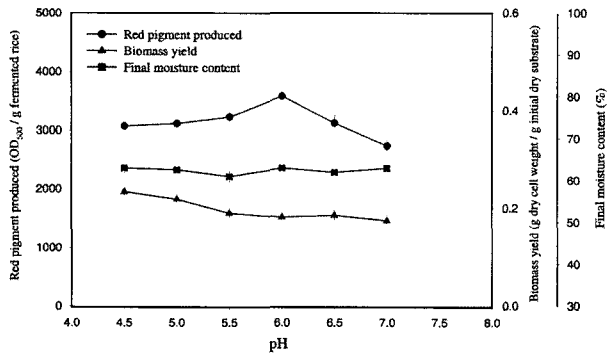

Fig. 2. Effect of pH on cell growth and production of red pigments in solid-state culture. Values represent means of three independent experiments and error bars indicate± S.D.

submerged cultures of *Monascus* species grown on carbohydrate media. They found that the initial pH of the culture determined the pigment yield, regardless of subsequent changes in the pH, and suggested that this was due to the effect of the pH on the nutrient absorption.

Nutrient Supplement

The effect of nutrient supplements on red pigment production was investigated using different sources of carbon, nitrogen, and mineral supplements added to solid-state flask cultures. The results are displayed in Fig. 3A. All the carbon and nitrogen supplement had an inhibitory effect on the biomass growth and red pigment production. Lotong and Suwanarit [11] also clearly demonstrated the inhibitory effect of added or enzymetically produced glucose on pigment production. Of the three nitrogen-based supplements that were studied, monosodium glutamic acid (MSG) had the most pronounced inhibitory effect on the red pigment production across a range of concentrations. Higher concentrations of carbon- and nitrogen-based supplements were found to limit the biomass and red pigment production more severely than lower concentrations. Generally speaking, nutritional factors are typically the limiting factors in the growth of most fungi found in nature. In the case of solid-state cultures, this limitation was likely much more pronounced due to a decrease in the diffusion rates of the substrate. Therefore, although effective use of a nutrient supplement can enhance fungal growth and red pigment production, in the current study, all the nitrogen- and carbon-based supplement tested inhibited cell growth and red pigment production. This phenomenon was attributed to rich nutrients already present in the rice.

Five different mineral supplements (Zn^{2+}, Mn^{2+}, Cu^{2+}, Mn^{2+} and Fe^{2+}) were also tested to examine their effects on growth and red pigment production. Fig. 3B shows that the addition of each mineral at different concentrations had a similar effect on the red pigment production to those observed with carbon- and nitrogen based supplements. Previous research has indicated that low concentrations of zinc are essential to fungal growth, yet

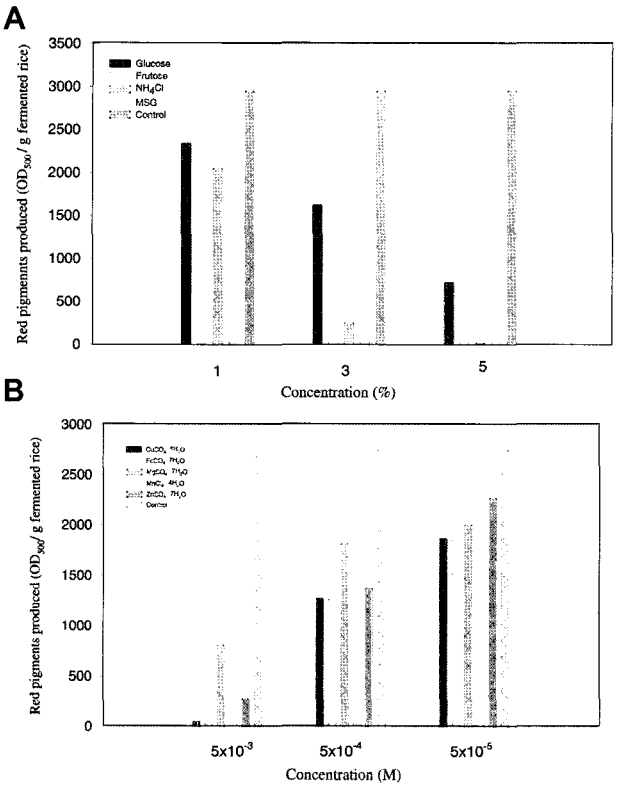

Fig. 3. Effect of nutrient supplement on red pigment production in solid-state flask cultures; (A) carbon and nitrogen sources, (B) mineral sources.

toxic at high concentrations [12]. However, in the present study the addition of zinc over a range of concentrations reduced the growth and red pigment production. This result was attributed to strain-specificity. In a study using both liquid and agar plate cultures, Bau and Wong reported that low levels of zinc inhibited growth and pigmentation in wild strains of *M. Purpureus*, while a mutant strain, N11S, showed an increased pigment yield at a zinc concentration of $1x10^{-3}$ M [13]. Zhang also reported that the growth and pigment production of *Monascus* R09 were markedly suppressed in the presence of iron [14]. Since the other three micronutrients had an inhibitory effect on the fungal growth and red pigment production, if was concluded that the rice medium did not benefit from the addition of supplementary micronutrients.

Inoculum Size

Determining the optimum inoculum size is another factor crucial to red pigment production. On the one hand, if the inoculum size is too small, this can slow growth of the microorganism and lead the contamination by undesirable organisms. Conversely, if the inoculum size is too large this can produce too much biomass and deplete the substrate, which is necessary for product formation [15]. Fig. 4 shows the result of the red pigment production relative to the inoculum size. The

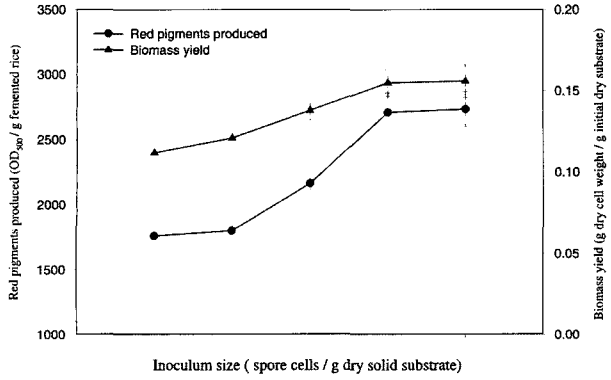

Fig. 4. Effect of inoculum size on cell growth and red pigment production in solid-state culture. Values represent means of three independent experiments and error bars indicate ± S.D.

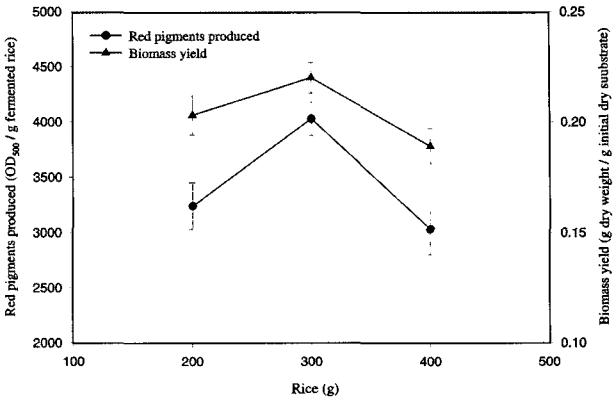

Fig. 5. Effect of sample size on cell growth and red pigment production in open tray culture. Values represent means of three independent experiments and error bars indicate ± S.D.

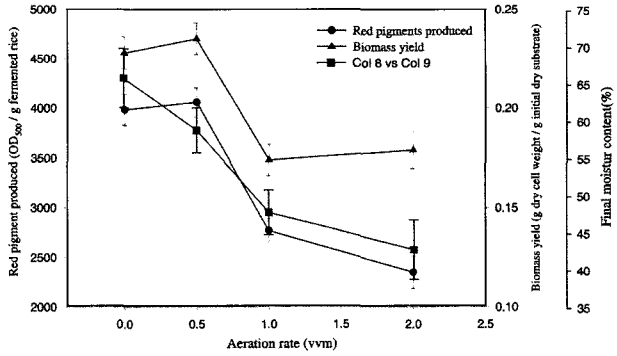

Fig. 6. Effect of aeration rate on cell growth and red pigment production in open tray culture. Values represent means of three independent experiments and error bars indicate ± S.D.

red pigment production saturated at a level of about $1x10^4$ spore cell (grams of dry solid substrate)$^{-1}$. At level lower than this, the value of the red pigment and biomass decreased in proportion to decrease in the inoculum size.

Sample Size and Aeration Rate

The effects of the sample size and aeration rate on the red pigment production were investigated using trays. The results are presented in Fig. 5 and Fig. 6, respectively. As seen in Fig. 5, a sample of 300 g produced the greatest amount of red pigments. In samples using 200 grams of rice, the production of the red pigments decreased, thereby suggesting that a smaller size would seem to contribute to a faster loss of substrate moisture than larger sample size. In samples using 400 grams of rice, the red pigment production was a slightly inhibited, suggesting that an increase in the depth of the culture bed would seem to limit the diffusion of oxygen and carbon dioxide. Figure 6 shows the effect of the aeration rate on the red pigment production. An aeration rate of 0.5 vvm produced the maximum yield, while the pigment production decreased at higher than 0.5 vvm. Generally speaking, aeration is considered to be important in solid-state cultures because it affect the gas, heat, and moisture transfer between the fermenting solids and the gas environment. In addition, *Monascus* pigments are polyketides, and oxygen is an essential substrate for their biosynthesis [16]. The current results suggest that aeration contributes to a significant loss of substrate moisture, especially in the initial growth stage, a loss that would appear to inhibit cell growth and pigment formation. The control (0 vvm) produced a yield of red pigment approximating the maximum yield. A possible explanation for this phenomenon result is that an open tray environment supplies oxygen for cell growth and pigment formation. In addition, daily mixing not only improved the circulation of oxygen and carbon dioxide, but also helped in providing homogeneity throughout the fermentation period; preventing the aggregation of solid particles and guaranteeing high interfacial surface area-to-liquid phase volume ratios; promoting gas transfer and heat exchanging; preventing localized changes; and effectively distributing the spore inoculum. Accordingly, these test results suggest that there is no need to supply additional oxygen to open tray cultures.

Tray Cultures

Tests involving open tray cultures were performed under optimum culture conditions specified for flask and tray cultures. The time course of the cultivation of *Monascus purpureus* by solid-state culture using rice as the raw material is shown in Fig. 7. The production of red pigments started at the beginning of the exponential growth phase, increased sharply after 3 day, and reached to 4,228 OD_{500} in the stationary phase. The pathways of secondary metabolism do not usually function continuously throughout the fungal life cycle and are only active after the growth rate slows. However, the production of red pigment in the current study showed a growth-associated trend. Other researchers have also reported such growth-associated pigment production by *Monascus* [17]. The maximum biomass

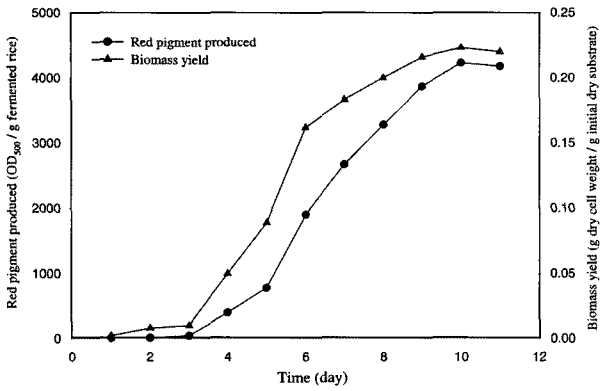

Fig. 7. The time course of solid-state cultivation of *Monascus purpureus* in optimum conditions.

yield and specific productivity of red pigment were 223 mg DCW (grams of initial dry substrate)$^{-1}$ and, 47.6 OD_{500} grams DCW^{-1} h^{-1}, respectively. The pigments produced were mainly water insoluble. One g of fermented rice contained 0.39 g of pigments. About 10% of them were found to be water-soluble. Thus to widen the application for food coloring, methods should be introduced to increase the water solubility.

CONCLUSION

Various environmental and nutritional parameters, such as the initial moisture content, pH, inoculum size, sample size, air rate, and nutrient supplement, that influence pigment production were evaluated in solid-state cultures to maximize and sustain the productivity of *Monascus* pigments. The optimum IMC and pH were determined to be 50% and 6.0, respectively. All supplemental nutrients (carbon, nitrogen, and mineral sources) added had an inhibitory effect on cell growth and red pigment production. The red pigment production saturated above 1 × 10^4 spore cell / grams of dry solid substrate. A sample of 300 g yielded the greatest amount of red pigment. It would appear that there is no need to supply additional oxygen to open tray cultures. In an open tray culture, the maximum biomass yield and specific productivity of red pigments were 223 mg DCW (grams of initial dry substrate)$^{-1}$, and 47.6 OD_{500} grams DCW^{-1} h^{-1}, respectively. The pigments produced were mainly water insoluble. One gram of fermented rice contained 0.39 g of pigments, and about 10% of them were found to be water-soluble.

Acknowledgements This work was supported by the Korean Ministry of Commerce, Industry and Energy; and the Korea Institute of Industrial Technology Evaluation and Planning (Grant No. A00-A04-5411-04).

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[Received December 6, 2001; accepted February 6, 2002]