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การผลิตสีจาก *Monascus* sp. U6V1 สายพันธุ์ไม่ผลิตซิตรินิน ในถังหมักแบบกวนขนาด 5 ลิตร

The Pigment Production from *Monascus* sp. U6V1, a Non-citrinin Producing Strain, in 5-L Stirred Tank Fermentor

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บทคัดย่อ

Monascus sp. U6V1 เจริญและให้การผลิตสีได้ดีที่สุดในอาหาร Cassava starch peptone medium 19.2±1.08 กรัมต่อลิตร และ 39.48±2.42 หน่วย ตามลำดับ อย่างมีนัยสำคัญ โดยเลี้ยงเชื้อบนเครื่องเขย่าความเร็ว 200 รอบต่อนาที 30 องศาเซลเซียส นาน 7 วัน เมื่อนำมาเลี้ยงเชื้อด้วยถังหมักแบบกวนขนาด 5 ลิตร เพื่อศึกษาอัตราการกวน และการให้อากาศที่เหมาะสมต่อการผลิตสี พบว่าการเลี้ยงเชื้อที่ความเร็วการกวน 500 รอบต่อนาที และอัตราการให้อากาศ 1.00ลิตรอากาศต่อลิตรอาหารต่อนาที ให้การสร้างสี ผลได้สี และอัตราการผลิตผลิตภัณฑ์จำเพาะสูงสุดอย่างมีนัยสำคัญ เท่ากับ 40.17±1.45 หน่วย 0.686 หน่วยต่อกรัม และ 0.143 หน่วยต่อกรัมต่อชั่วโมง ตามลำดับ เมื่อนำมาศึกษาอัตราการให้อากาศที่เหมาะสม พบว่าการให้อากาศ 0.75 และ 1.00 ลิตรอากาศต่อลิตรอาหารต่อนาที ให้การผลิตสีและอัตราการผลิตผลิตภัณฑ์จำเพาะสูงสุดไม่แตกต่างอย่างมีนัยสำคัญ เท่ากับ 36.04±1.04 40.17±1.45 หน่วย และ 0.145 และ 0.143 หน่วยต่อกรัมต่อชั่วโมง ตามลำดับ แรงเฉือน และสภาวะขาดออกซิเจนในระหว่างการเจริญเพิ่มจำนวนส่งผลกระทบต่อการผลิตสีในระยะคงที่ นำทุกตัวอย่างที่ได้จากการเลี้ยงเชื้อเมื่อสิ้นสุดการทดลอง มาวิเคราะห์ปริมาณซิตรินิน ผลการตรวจสอบไม่พบปริมาณซิตรินินในตัวอย่าง

คำสำคัญ : *Monascus* sp. ; สีผสมอาหาร; แป้งมันสำปะหลัง; การเลี้ยงเชื้อในถังหมักแบบกวน

Abstract

The significant maximum cell growth and pigment production of *Monascus* sp. U6V1 was observed in cassava starch peptone medium at 19.2±1.08 g/L and 39.48±2.42 UA_{500nm}, respectively, by the cultivation at 200 rpm of shaking speed, 30°C for 7 days. Then, the experimentation was carried in 5-L stirred tank fermentor for the optimal agitation speed and aeration rate. At 500 rpm of agitation speed and 1.00 VVM of aeration rate, *Monascus* sp. U6V1 gave significant pigment production, pigment yield and specific pigment production rate at 40.17±1.45 UA_{500nm}, 0.686 UA_{500nm}/g and 0.143 UA_{500nm}/g/h, respectively. When the cultivation was observed for the optimal aeration rate. The aeration rate at 0.75 VVM and 1.00 VVM gave the insignificant pigment concentration and specific pigment production rate at 36.04±1.04 and 40.17±1.45 UA_{500nm} and 0.145 and 0.143 UA_{500nm}/g/h, respectively. The shear rate and oxygen shortage during exponential phase showed some effects on pigment production in stationary phase. At the end of cultivation, all samples were analyzed for the citrinin content. The result showed that citrinin content was not detected.

Keywords : *Monascus* sp.; food color; cassava starch; stirred tank cultivation

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Introduction

Recently, natural food pigments comprise 31% of the food industrial colorant market share up to US$27.5 billion in 2018 (Zhou *et al.*, 2019). However, the chemical colorant shows the low safety assurance. Therefore, the natural food colorant pigments from animals, plants, or microorganisms have become more attractive in currently years (Vendruscolo *et al.*, 2016). Among these pigments, the pigments from microbial presents a high quality (such as heat stable, pH steady, UV resistant and low odor) (Yongsmith *et al.*, 1994, Krairak *et al.*, 2000) and reasonable for scale-up to economic production (Gomes & Takahashi, 2016). Meanwhile, the application of these microbial food colorants must be previously approved for the toxicological assessments because of some microbial pigment producer could synthesize hazardous compounds (such as mycotoxin) (Mapari *et al*., 2009). The *Monascus* food color has been wildly used for a thousand years in China named as Chinese Red Rice or Ang-kak for making red soybean cheese, red rice wine, meat and fish products (Blance *et al*., 1994; Yongsmith *et al*., 1994; Krairak *et al*., 2000). Nowadays, more than 50 patents have been issued for the application of *Monascus* pigments in the foods manufacture such as Japan, the United States, France, and Germany (Lin *et al.*, 1992; Wang & Lin, 2007). *Monascus* pigments typically comprise six major azaphilone pigments, those were categorized in 3 groups 1) yellow pigments (monascin and ankaflavin), 2) orange pigments (monascorubrin and rubropunctatin), and 3) red pigments (monascorubramine and rubropunctamine) (Feng *et al.*, 2012; Xiong *et al.*, 2015). Among these, the red pigments have been widely used in Asia for centuries as food colorant and now have been successfully produced by fermentation (Feng *et al*., 2012). However, the application of *Monascus* pigments is limited by the possibility of coproduction of the mycotoxin citrinin during the cultivation of *Monascus* sp. Citrinin, a secondary metabolite, has toxic effects on kidneys, such as teratogenicity, carcinogenicity and mutagenicity (Lui *et al*., 2021). Since a citrinin producing ability is related to the strain, the cultivation medium and environmental conditions (Feng *et al*., 2012), therefore, citrinin analysis should be conducted during pigment production by *Monascus* sp. Recent researches have focused on identification of non-citrinin producing *Monascus* strains (Wang *et al.*, 2013; Krairak *et al.*, 2016), lowering the production of citrinin in *Monascus* products (Shen *et al*., 2014a), and removal of citrinin by various physical and chemical methods (Shen *et al*., 2014b).

Traditional pigment production by *Monascus* is usually performed using solid-state fermentation (Kang *et al.*, 2014); however, there are number of reports of the advantages of submerged fermentation (Yongsmith *et al.*, 1994; Krairak *et al.*, 2000; Grimm *et al.*, 2005). The factors affecting cultivation of *Monascus* are formulation of the cultivation medium, temperature (Zhang *et al*., 2007), pH, agitation speed (Park *et al*., 2002), aeration conditions (Cho *et al.*, 2002) and the relation between specific growth rate and specific pigment production rate (Krairak *at al*., 2000).

Monascus sp. U6V1, the mutant strain was isolated using conventional mutation techniques, UV-irradiation on *Monascus* sp. SS14, the wild type (Krairak and Thongsuknok, 2015). In our previous study, *Monascus* sp. SS14 produced pigment and citrinin at 92.4±2.47 UA_{500nm} and 3.88±1.04 μg/g, respectively. However, the mutant, *Monascus* sp. U6V1, could not detect citrinin production in shake flask experiment (Krairak *et al*., 2016). This might be the high potential strain for the source of food pigment producer. This work focused on the optimization of *Monascus* sp. U6V1 cultivation in a 5-L fermentor at various agitation speed and aeration rate to maximize pigment production and prevent the citrinin synthesis.

Methods

Monascus strain and cultivation

Monascus sp. U6V1, the mutant, was received by UV mutation of *Monascus* sp. SS14, the wild type (Krairak *et al*., 2016), was examined throughout this study. *Monascus* sp. U6V1 was maintained on malt yeast starch agar (MYS) plate for 7 days at 30°C. The inoculum medium (glucose peptone medium (GP) consisted of glucose (30 g/L) and peptone (40 g/L) (Krairak *et al.*, 1999). Four pieces of 4.0 mm mycelial blocks of *Monascus* sp. U6V1 were inoculated to the 100-mL of GP broth which contained in 250 mL flask. The cultivation was incubated on 200 rpm of rotary shaker at 37 °C for 4 days. 75.0 mL of inoculum was transferred to 5-L stirred tank fermentor that contained 2.5-L of cassava peptone (CP) medium (consisted of cassava starch (30 g/L) and peptone (40 g/L) (Krairak *et al*., 1999)) for the further experimentation.

The cultivation in 250-mL shaking flask

Four pieces of 4.0 mm mycelium blocks of *Monascus* sp. U6V1 was transferred to 100-mL of investigative media. Four types of investigative media were glucose peptone (GP) medium, cassava starch peptone (CP) medium, glucose soybean flour (GS) medium (consisted of glucose (30 g/L) and soybean flour (40 g/L)), and cassava starch soybean flour (CS) medium (consisted of cassava starch (30 g/L) and soybean flour (40 g/L)) (Krairak *et al*., 1999). The cultivation was carried in 200-rpm of rotary shaker, 28-30°C for 7 days. The triplicate analysis of sample was examined on cell concentration, pigment concentration and glucose concentration, respectively.

The cultivation in 5-L stirred tank fermentor

The 5-L stirred tank fermentor (FS 01, FS V B Series, Winpact, USA) with a working volume of 2.5-L was performed in this study. The pre-culture of *Monascus* sp. U6V1 was used as an inoculum (Krairak *et al.*, 2016). 75.0 mL of inoculum was transferred to 5-L stirred tank fermentor containing 2.5-L of cultivation medium. At first, the aeration rate was set at 1.0 VVM with various agitation speed of 300 rpm to 600 rpm, respectively. During the cultivation, pH and dissolved oxygen were measured simultaneously until the end of cultivation. The cultivation was incubated at 30°C with initial pH medium of 6.8-7.2 for 168 h. During the cultivation, a silicone

antifoam agent was added when necessary. Finally, the experiment was examined on aeration rate at 0.50, 0.75 and 1.00 VVM, respectively, by using the optimal agitation speed. The triplicate analysis of sample was examined on cell concentration, pigment concentration and glucose concentration, respectively.

Analytical methods

The cell concentration (g/L) was determined by dry cell weight. About 5-mL of culture was centrifuged at 8000x g for 10 min and then the collected pellet was washed twice with distilled water and dried at 70°C overnight to a constant weight. The supernatant was analyzed for pigment concentration, glucose concentration and citrinin production, respectively. Glucose concentration in the culture broth was examined by reducing sugar analysis, the Somogyi method (Somogyi, 1952). The appropriated dilution was done for the reducing sugar determination. The reducing sugar content was determined using standard curve of glucose concentration (0-180 μg/mL). For pigment analysis, the retrieved supernatant was determined for the pigment value by spectrophotometric analysis on λ500 nm of maximum adsorption using 50% ethanol as a diluent and blank solution. Absorbance units at λ500 nm value was expressed as the concentration of pigment (Krairak *et al.*, 1999). Pigment yield was expressed as absorbance unit at λ500 nm (UA_{500nm}) per gram of dried cell weight (Zhou *et al.*, 2019). In case of citrinin content, the sample analysis was determined at the end of cultivation. The sample supernatant was filtered with 0.45 μm membrane and then applied to the HPLC system consisted of a reversed phase C-18 μBondapak column (10 μ, 3.9 mm.·x 3,000 mm., Waters, USA) under a 45% acetonitrile pH 2.5 as mobile phase. Flow rate was kept at 1.0 mL/min and absorbance was measured at λ283 nm (Xu *et al.*, 2003). Citrinin standard solutions (product C1017, Sigma Chemical Co., USA) was used for a calibration curve (0.2 μg/mL to 10.0 μg/mL).

Data analysis

The specific growth rate, μ_{max} (/h), was calculated following the equation: $\mu = \frac{1}{X}\frac{dX}{dt}$, where X is the cell concentration (g/L) at time t (h). The specific production rate of pigments, Q_p (UA_{500nm}/g/h), was calculated following the equation: $Q_p = \frac{1}{X}\frac{dP}{dt}$, where P is the pigments value (UA_{500nm}) at time t (h). The pigment product yield from biomass, (UA_{500nm}/g), $Y_p = \frac{dP}{dX}$ (Zhou *et al.*, 2019). For the statistics analysis, ANOVA and multiple comparison test were applied to evaluate the effect of independent variables on *Monascus* cell growth and pigment production. The significant results were identified by a p-value < 0.05. Each experiment represents the mean of three independent experiments, and the results were presented as mean ± SD.

Results

Cultivation medium

The cell growth and pigment production of *Monascus* sp. U6V1 were examined in glucose peptone medium (GP), cassava starch peptone (CP) medium, glucose soybean flour (GS) medium and cassava starch soybean flour (CS) medium, respectively. The cultivation was performed in 250-mL flask containing 100 mL of medium and incubated at 30°C on 200 rpm of rotary shaker for 7 days. The results are shown in table 1.

Table 1 The *Monascus* sp. U6V1 cell growth and pigment production for 7 days. The significance of differences between the values are illustrated with letters. Different letters indicate significant differences between the experimental values.

Medium	pH	Cell concentration (g/L)	Glucose concentration (g/L)	Pigment concentration (UA_{500nm})
GP	5.82	14.5 ± 1.64^{b}	0.98 ± 0.073	31.44 ± 3.07^{b}
CP	5.77	19.2 ± 1.08^{a}	0.32 ± 0.081	39.48 ± 2.42^{a}
GS	7.24	12.9 ± 2.41^{b}	1.57 ± 0.13	5.36 ± 1.14^{c}
CS	7.87	10.7 ± 2.89^{c}	1.94 ± 0.26	3.24 ± 1.08^{c}

At 7 days of cultivation, the cell growth and pigment production of *Monascus* sp. U6V1 were maximized at 19.2 ± 1.08 g/L and 39.48 ± 2.42 UA_{500nm}, respectively, by cultivating in CP medium. For the cultivation in GP medium, the cell growth and pigment production of *Monascus* sp. U6V1 were low at 14.5 ± 1.64 g/L and 31.44 ± 3.07 UA_{500nm}, respectively. The cultivation in GS medium and CS medium presented the minimal cell growth and pigment production. ANOVA was applied to the results in order to identify if medium types influenced the production of pigments and cell concentration. For the multiple comparison test was conducted under 5% error. It was indicated that *Monascus* sp. U6V1 cultivation in CP medium gave significantly higher cell growth and pigment production than GP, GS and CS medium. Therefore, CP medium was used for *Monascus* sp. U6V1 cultivation in 5-L fermentor.

Effect of agitation speed on the cultivation of Monascus sp. U6V1 in 5-L fermentor

The *Monascus* sp. U6V1 cultivation was showed at different agitation speeds (Figure 1A-1D). All experiments, cell growth was increased exponentially with time when the agitation speeds were various from 300 rpm to 600 rpm. In case of agitation speed of 300 rpm and 400 rpm, the maximal cell growth was observed at 17.18 ± 2.32 g/L and 20.19 ± 1.86 g/L within 98 h of cultivation and remained constant until the end of the cultivation. For the cultivation with the agitation speed of 500 rpm, the cell growth increased with time until it reached 26.26 ± 1.67 g/L after 96 h and gradually reduced until the end of the cultivation. In case of the

cultivation at agitation speed of 600 rpm, the maximum cell concentration was the highest at 30.91±1.73 g/L. After 132 h of cultivation, the cell concentration was rapidly reduced due to the cell autolysis by highly shear rate of agitation speed. The results showed that the higher agitation speed, the more cell growth. The maximum cell concentration was increased from 17.18±2.32 g/L to 30.91±1.73 g/L by using agitation speed of 300 rpm to 600 rpm, respectively. This means that the agitation speed showed the strongly effect on cell growth due to the high oxygen transfer. At the end of each cultivation, all samples were examined for the citrinin concentration. The results showed that the citrinin was not detected in the all samples.

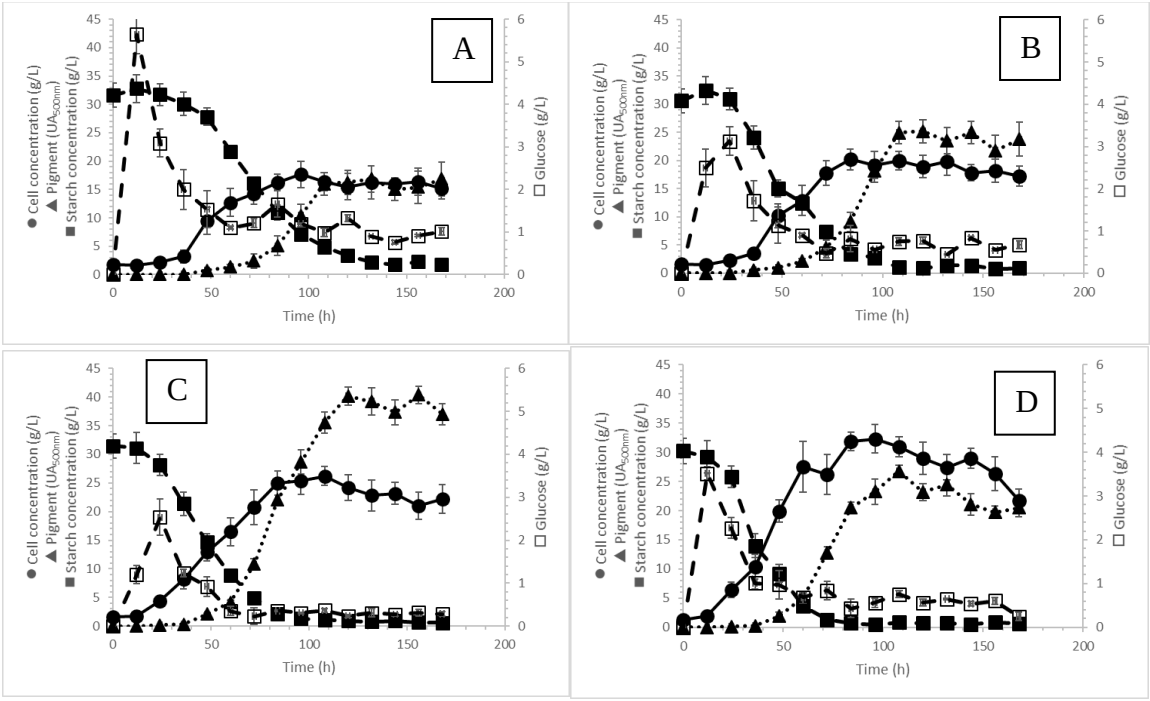

Figure 1 The fermentation time-courses of *Monascus* sp. U6V1 in 5-L stirred tank fermentor that was cultivated at A) 300 rpm, B) 400 rpm, C) 500 rpm and D) 600 rpm with aeration rate of 1.0 VVM, respectively. The incubation was carried at 28°C for 168 h.

In Figure 1A-1D, the pigment production was started after 48-60 h of cultivation depending on agitation speed. The maximum pigment production was increased from 16.23±2.10 UA_{500nm}, 25.19±1.92 UA_{500nm} and 40.17±1.45 UA_{500nm} when the agitation speed was increased from 300 rpm to 500 rpm, respectively. When the agitation speed reached 600 rpm, the pigment production was low (26.73±1.09 UA_{500nm}). In case of the cultivation at 300 rpm of agitation speed, the absent of dissolved oxygen was found during 18-48 h (data not shown) because the *Monascus* cell morphology was long and dispersed mycelium. Moreover, the cultivation

at 600 rpm of agitation speed showed the lower pigment production than one with 500 rpm of agitation speed. This would be some effects of shear rate on cell growth and pigment production. At 600 rpm of agitation speed, the morphology of *Monascus* sp. U6V1 was presented in compact and dense pellet. For the residual glucose profile is shown in figure 1A- 1D. The result showed that the cultivation at 400, and 500 rpm of agitation speed presented lower residual glucose (0.20 – 0.35 g/L) during stationary phase than one with the agitation speed of 300 rpm. However, at 600 rpm of agitation speed, the glucose concentration during stationary phase was about 0.42-0.65 g/L.

The growth kinetic parameters of *Monascus* sp. U6V1 cultivation on CP medium at different agitation speed in 5-L stirred tank fermentor are illustrated in Table 2.

Table 2 The performance and kinetic parameter values of *Monascus* sp. U6V1 cultivation in 5-L stirred tank fermentor with 1.0 VVM at 300, 400, 500 and 600 rpm of agitation speed for 168 h, respectively. The significance of differences between the values are illustrated with letters. Different letters indicate significant differences between the experimental values.

Culture condition	cell concentration (g/L)	Pigment concentration (UA_{500nm})	Specific growth rate (μ_{max}, /h)	Pigment yield ($Y_{p/x}$, UA_{500nm}/g)	Specific pigment production rate (Q_p, UA_{500nm}/g/h)
300 rpm	17.18 ± 2.32^{d}	16.23 ± 2.10^{c}	0.0460	0.411	0.083
400 rpm	20.19 ± 1.86^{c}	25.19 ± 1.92^{b}	0.0479	0.589	0.106
500 rpm	26.21 ± 1.67^{b}	40.17 ± 1.45^{a}	0.0491	0.686	0.143
600 rpm	30.91 ± 1.73^{a}	26.73 ± 1.09^{b}	0.0533	0.329	0.065

All experiments, the *Monascus* sp. U6V1 growth at 300 rpm, 400 rpm, 500 rpm and 600 rpm of agitation speed was increased exponentially with time. The specific growth rate (μ_{max}) was increased by 0.0460 /h, 0.0479 /h, 0.0491 /h and 0.0533 /h, respectively, during 12 h to 84 h of cultivation time. In case of the cultivation at agitation speed of 600 rpm, the significant maximum cell concentration and specific growth rate were the highest at 30.91 ± 1.73 g/L and 0.0533 /h, respectively. For the pigment production, When the cultivation was carried in 300 rpm of agitation speed, the pigment concentration, pigment yield and specific pigment production rate were the lowest at 16.23 ± 2.10 UA_{500nm}, 0.411UA_{500nm}/g and 0.083 UA_{500nm}/g/h, respectively. Then, the agitation speed for the cultivation was increased to 400 rpm and 500 rpm. The pigment concentration, pigment yield and specific pigment production rate were raised to 25.19 ± 1.92 and 40.17 ± 1.45 UA_{500nm}, 0.589 and 0.686 UA_{500nm}/g and 0.106 and 0.143 UA_{500nm}/g/h, respectively. However, the cultivation at 600 rpm of agitation speed showed the pigment concentration, pigment yield and specific pigment production rate at

26.73±1.09 UA_{500nm}, 0.329 UA_{500nm}/g and 0.065 UA_{500nm}/g/h, respectively. These results showed that the significant pigment concentration was maximized at 40.17±1.45 UA_{500nm} by cultivation with 500 rpm of agitation speed. At the same time, specific pigment production rate was 0.143 UA_{500nm}/g/h.

Effect of aeration rate on the cultivation of Monascus sp. U6V1 in 5-L fermentor

In this experiment, the cultivation was examined at 500 rpm of agitation speed using various aeration rate of 0.5 VVM, 0.75 VVM and 1.0 VVM, respectively. The fermentation time-courses of *Monascus* sp. U6V1 on cell growth, pigment production, glucose concentration and starch utilization are shown in Figure 2A-2C.

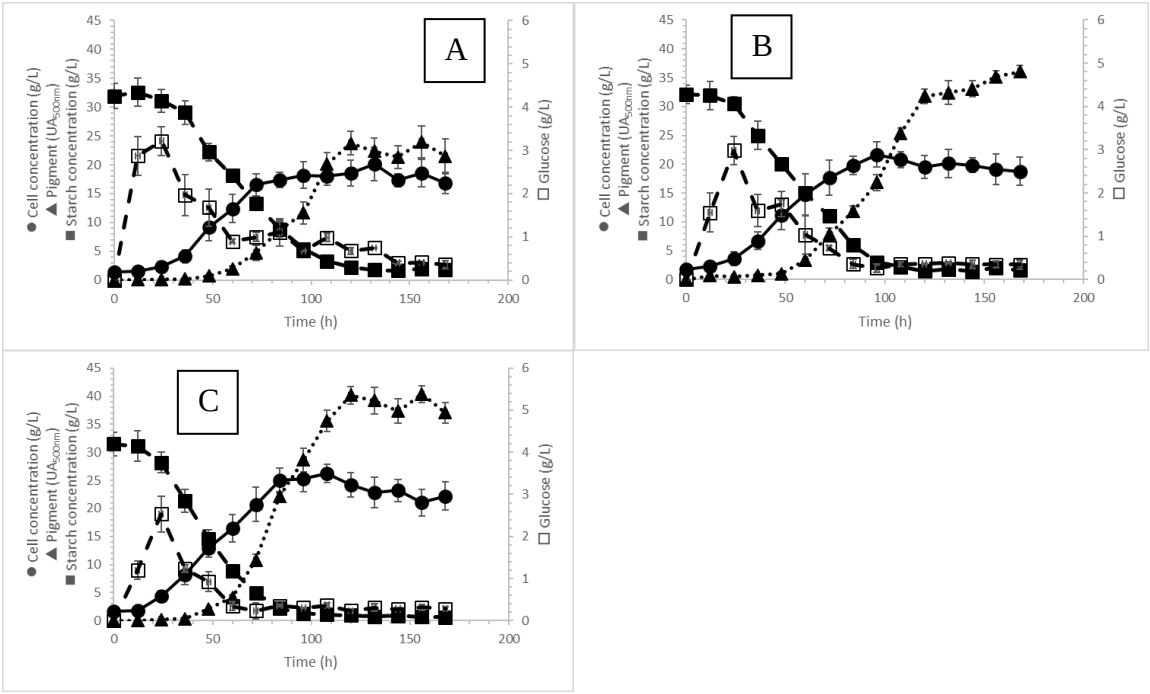

Figure 2 Cultivation time-course of *Monascus* sp. U6V1 on cell growth and pigment production was investigated in 5-L stirred tank fermentor at 28°C, 500 rpm of agitation speed and various aeration rate of A) 0.50 VVM, B) 0.75 VVM and C) 1.0 VVM, respectively, for 168 h.

For the cultivation of *Monascus* sp. U6V1 at the aeration rate of 0.5 VVM, 0.75 VVM and 1.0 VVM (Figure 2A-2C), the maximum cell concentration and pigment production were 18.19±2.15 g/L, 21.70±2.09 g/L and 26.21±1.67 g/L and 23.75±2.79 UA_{500nm}, 36.04±1.04 UA_{500nm}, and 40.17±1.45 UA_{500nm}, respectively. In this experiment, the aeration rate was proportional to cell growth and pigment production. By using aeration rate of 0.50 VVM, the dissolved oxygen value was zero during 30-40 h of cultivation (data not shown). However,

this situation was not found when the cultivation was examined with 0.75 and 1.0 VVM of aeration rate. This means that oxygen supply was enough for cell growth in aerobic condition.

At the beginning of cultivation, the cassava starch was simultaneous digested by the *Monascus* sp. U6V1 enzymatic activity. During 0-30 h, the accumulation of residual glucose was increased. After the cell growth reached the exponential phase, the residual glucose was reduced gradually. For the cultivation at 0.5 VVM of aeration rate, the residual glucose was high (about 0.59-0.98 g/L) during 96-132 h of cultivation period. When the cultivation was carried in 0.75 VVM and 1.0 VVM of aeration rate, the residual glucose was minimized about 0.21-0.35 g/L during stationary phase. The result showed that the pigment production by *Monascus* sp. U6V1 was secondary metabolite then the specific pigment production rate would be calculated during this period.

The growth kinetic parameters of *Monascus* sp. U6V1 cultivation on CP medium at different aeration rates in 5-L stirred tank fermentor are illustrated in Table 3.

Table 3 The performance and kinetic parameter values of *Monascus* sp. U6V1 cultivation in 5-L stirred tank fermentor at 500 rpm of agitation speed with 0.5 VVM, 0.75 VVM and 1.0 VVM of aeration rate for 168 h, respectively. The significance of differences between the values are illustrated with letters. Different letters indicate significant differences between the experimental values.

Culture condition	cell concentration (g/L)	Pigment concentration (UA_{500nm})	Specific growth rate (μ_{max}, /h)	Pigment yield ($Y_{p/x}$, UA_{500nm}/g)	Specific pigment production rate (Q_p, UA_{500nm}/g/h)
0.5 VVM	18.19 ± 2.15^{c}	23.75 ± 2.79^{b}	0.0457	0.496	0.101
0.75 VVM	21.70 ± 2.09^{b}	36.04 ± 1.04^{a}	0.0485	0.694	0.145
1.0 VVM	26.21 ± 1.67^{a}	40.17 ± 1.45^{a}	0.0491	0.686	0.143

As shown in Table 2, when the cultivation was carried in the aeration rate of 0.5, 0.75 and 1.0 VVM, the specific growth rate was 0.0457, 0.0485 and 0.0491 /h, respectively. For the cultivation at 0.5 VVM of aeration rate, the pigment concentration, pigment yield, and specific pigment production rate were the lowest at 23.75 ± 2.79 UA_{500nm}, 0.496 UA_{500nm}/g and 0.101 UA_{500nm}/g/h, respectively. This result might relate to the oxygen starvation during 30-40 h of cultivation. For the aeration rate of 0.75 and 1.0 VVM, the pigment yield and specific pigment production rate were 0.694 UA_{500nm}/g and 0.145 UA_{500nm}/g/h and 686 UA_{500nm}/g and 0.143 UA_{500nm}/g/h, respectively. These conditions, the absent of oxygen was not found during the whole period of cultivation. However, the more wall growth and high foam formation was observed during the *Monascus* cultivation in 1.0 VVM of aeration rate. From these results, the cultivation at 1.0 VVM of aeration rate gave the significant maximum

cell concentration at 26.21±1.67 g/L. In case of pigment production, the cultivation at 0.75 VVM and 1.0 VVM presented the insignificant pigment concentration and specific pigment production rate. Therefore, the cultivation at 0.75 VVM of aeration rate was preferred to minimize the effect of wall growth and foam formation during pigment production.

Discussion

The *Monascus* sp. U6V1 cultivation in CP medium gave the significant pigment production higher than the cultivation in GP, GS and CS medium. The simultaneous digestion of cassava starch enhanced the pigment production. As mention by Yongsmith *et al.* (1994) and Krairak et al. (1999) that the maximum yellow pigment production at 620 U/mL was observed when *Monascus* sp. KB20M10.2 was cultivated by using cassava starch and peptone as C-source and N-source, respectively. The slow releasing of glucose by the enzymatic digestion prevented the product inhibition by the induction of respiro-fermentative metabolism (Crabtree effect) in the aerobic cultivation of *M. purpureus* by high glucose levels (Juzlova *et al.*, 1996). In case of N-source, the growth of *Monascus* sp. U6V1 was higher in the medium composed of peptone than one with soybean flour. However, the pigment production was maximized by using peptone as N-source. It seemed like peptone promoted not only growth but also pigment production. On the other hand, soybean flour was support for growth. Normally, *Monascus* produces three kinds of described polyketides: citrinin, pigments, and monacolin K. In case of *Monascus* sp. U6V1, the citrinin formation did not detected. Therefore, the utilization of soybean flour might induce the other products formation such as other types of pigment and monacolin.

The fermentation of *Monascus* sp. U6V1 was investigated in 5-L stirred tank fermentor. The cultivation at 300 rpm of agitation speed and 1.0 VVM of aeration rate gave the lowest cell concentration and pigment production. The oxygen starvation was observed at 18-48 h during this cultivation. This situation should relate to the cell morphology in dispersed mycelium resulting in the high viscosity of culture. During the anaerobic condition in the exponential phase, cell might produce some unknown metabolites that inhibited the pigment production in stationary phase (Hajjaj *et al.*, 2000). By the cultivation at 400 rpm and 500 rpm of agitation speed, cell morphology was developed to the pellet form that reduced the culture viscosity. The situation of zero dissolved oxygen was not found during the cultivation at 400, 500 and 600 rpm of agitation speed. Therefore, the internal mass transfer of the pellet might support the pigment production. Grimm *et al.* (2005) proposed relationship between biomass density and pellet porosity might lead to limited accessibility of nutrients and secretion of product. At 500 rpm of agitation speed, the *Monascus* sp. U6V1 presented the significant maximum pigment concentration, pigment yield and specific pigment production rate, respectively. In this study, the level of glucose concentration was between 0.20-0.35 g/L during pigment production in stationary phase. This condition might support cell growth and promote pigment production. Therefore, Yongsmith *et al.* (1994) and

Krairak *et al*. (1999) suggested that the level of glucose concentration should be suitable during pigment production period. If glucose level was high, the cell growth might occur. On the other hands, if glucose level was low, the cell dead should observe previously. When the cultivation was carried in 600 rpm of agitation speed and 1.0 VVM of aeration rate, the specific pigment production rate was the lowest among other agitation speed. This indicated that the agitation speed showed the strongly effect on specific growth rate due to the high oxygen transfer. Therefore, the oxygen transfer might enhance the activity of cell metabolism to promote the cell growth and specific growth rate. The high agitation speed provided high shear rates that would cause the mycelium of the fungus to split, which in turn, reduced pigment production (Yoshimura *et al.*, 1975). It was reported that the pigment production in a stirred tank fermentor was low at aggressive agitation rates (Krairak *et al.*, 2000). In this study, 500 rpm of agitation rate presented the suitable condition for pigment production from *Monascus* sp. U6V1. Although mycelial damage at high stirrer speeds or power inputs can limit the acceptable range of speeds and subsequently, the oxygen transfers capability and the volumetric productivity of the fermentor (Amanullah *et al*., 1998).

The cultivation of *Monascus* sp. U6V1 was carried in 500 rpm of agitation speed and various aeration rate at 0.50, 0.75 and 1.00 VVM. The aeration rate of 0.5 VVM gave significant low pigment yield and specific pigment production rate. Because of the absent of oxygen during cultivation between 30 h - 40 h and the high residual glucose during stationary phase, presented the strong inhibition effect on pigment production. Hajjaj *et al*. (2000) reported that during the oxygen limiting conditions, the pigment production might be inhibited by the unknown product. When the aeration rate was increased to 0.75 VVM, the specific pigment production rate was increased. The sufficient oxygen supply during exponential phase might improve the pigment production in stationary phase. Smith & Calam (1980) observed the relation between the level of enzymes synthesis at the beginning of *Penicillium urticae* cultivation and the final level of secondary metabolites production.

The cultivation at aeration rate 0.75 VVM and 1.0 VVM showed the insignificant pigment concentration and specific pigment production rate. In case of glucose concentration, both aeration rate showed the low level during pigment production phase. In this study, the level of glucose concentration was between 0.20-0.35 g/L might support cell growth and promote pigment production. If glucose concentration was higher than 0.35 g/L, the catabolite repression might inhibit pigment production. On the other hands, if glucose concentration was lower than 0.20 g/L, the cell maintenance energy was not sufficient. However, the cultivation with 1.0 VVM showed higher wall growth and foam formation than one with 0.75 VVM. Wall growth and foam formation are the serious problems of *Monascus* cultivation in the stirred tank fermentor. The severity of foam formation depends on the rate of aeration, composition of the medium, viscosity of the culture, physical structure of the bioreactor vessel and biomass concentration (Taticek *et al*., 1991). *Monascus* cells entrapped in a stable foam might form a crust, stick to the internal wall of reactor vessel, agitation impeller, baffle, around probes and

sample ports affecting the operation and creating problems (such as a stagnant zone). The accumulating necrotic cells might secrete by-products such as proteases that could inhibit cell growth and product formation (Piehl *et al.*,1988). The control of the oxygen supply environment is necessary in optimized process control strategies according to time course of cell growth and metabolite productions (Yang *et al.*, 2015). Krairak *et al.* (2000) found that maximal pigment production of *Monascus* sp. 20M10.2 was related to the pellet size and the biomass ratio between pellet and mycelium. Meanwhile, the collected sample at the end of cultivation was analyzed for the citrinin content. The result showed that the citrinin content was not detected in all sample. The further experiment would be emphasized on the genetic analysis of *Monascus* sp. U6V1.

Conclusions

Monascus sp. U6V1 gave the significant maximum pigment production when cultivated in CP medium and incubated on 200 rpm of shaker speed at 28°C for 7 days. In case of C-source, the cultivation in cassava starch showed the maximum pigment production because residual glucose was low resulting in the minimum catabolite repression. When using soybean flour as N-source, the *Monascus* sp. U6V1 showed the low ability of soybean flour assimilation for pigment production. The cultivation of *Monascus* sp. U6V1 was further investigated in 5-L stirred tank fermentor for the optimal agitation speed and aeration rate. It was found that 600 rpm of agitation speed and 1.0 VVM of aeration rate gave the maximum cell concentration and specific growth rate of 30.91±1.37 g/L and 0.0533 /h, respectively, but the specific pigment production rate was the lowest at 0.065 UA_{500nm}/g/h among other agitation speeds. It seemed like the shear rate had a strong effect on cell morphology and pigment production. At 300 rpm of agitation speed, the specific pigment production rate was low at 0.083 UA_{500nm}/g/h due to the oxygen deficiency was observed during the cultivation. The result showed that shear rate and oxygen insufficiency during cultivation caused the low pigment production. When agitation speed was increased to 500 rpm, the significant pigment concentration and specific pigment production rate were detected at 40.17±1.45 UA_{500nm} and 0.143 UA_{500nm}/g/h, respectively. At 500 rpm of agitation speed provided the suitable shear rate and oxygen mass transfer for the *Monascus* sp. U6V1 cultivation. Later, the aeration rate was observed at 0.5 VVM, 0.75 VVM and 1.0 VVM by using agitation speed of 500 rpm. The aeration rate of 0.5 VVM presented the oxygen starvation during the cultivation of *Monascus* sp. U6V1 caused the low specific pigment production rate at 0.101 UA_{500nm}/g/h. The oxygen shortage during exponential phase gave some inhibition effects on pigment production in stationary phase. By using the aeration rate of 0.75 VVM and 1.00 VVM, the *Monascus* sp. U6V1 cultivation gave insignificant pigment concentration and specific pigment production rate at 36.04±1.04 and 40.17±1.45 UA_{500nm} and 0.145 and 0.143 UA_{500nm}/g/h, respectively. Therefore, the cultivation at 0.75 VVM of aeration rate presented lower wall growth and foam formation than one

with 1.00 VVM of aeration rate. It was concluded that the optimal culture condition for *Monascus* sp. U6V1 for pigment production was 500 rpm of agitation speed and 0.75 VVM of aeration rate. Moreover, the citrinin detection of all collecting samples was not detected.

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