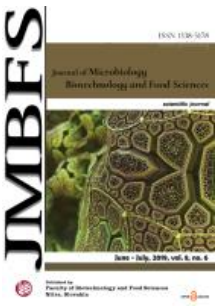

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APPLICATION OF A TWO-STAGE AGITATION SPEED CONTROL STRATEGY TO ENHANCE YELLOW PIGMENTS PRODUCTION BY *MONASCUS ANKA* MUTANT

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ABSTRACT

Monascus anka mutant strain was isolated using conventional mutation techniques in our laboratory. In our previous study, strain *Monascus anka* mutant showed high yield of yellow pigments with the characteristics of high protein adhesion, thermal stability, and wide-pH stability. This work focused on the agitation optimization in a 5 L fermentor of strain *Monascus anka* mutant and two stage agitation speed control strategy was operated as follows: the agitation speed was 400 r/min to improve the *Monascus* growth and yellow pigments production before culturing 96 hour and then 300 r/min was carried out to extend the time of yellow pigment production with efficient using substrate. The yield of monascus yellow pigments reached 149.43 *OD*, which was 49.37%, 49.25%, 35.56%, 18.73%, and 41.01% higher than that of 250 r/min, 300 r/min, 350 r/min, 400 r/min, and 450 r/min fermentation, respectively. The maximum specific growth rate (μ_{max}) and the maximum specific production rate of yellow pigments (q_y) reached 0.0528 h^{-1} and 0.2177 $OD.g^{-1}.h^{-1}$, respectively. Specific growth rate (μ) maintained higher than 0.01 h^{-1} from 6 hours to 54 hours, and q_y could maintain at 0.10 $OD.g^{-1}.h^{-1}$ from 24 hour to 66 hour, which could not be obtained by single agitation control. The data of this work could contribute to making the industrial production of *Monascus* yellow pigments feasible.

Keywords: *Monascus anka* mutant, Yellow pigments, Specific growth rate, specific production rate of yellow pigments, Agitation

INTRODUCTION

Food colorants can be classified into synthetic colorants such as quinoline yellow **(Zhang *et al*., 2015)** and tartrazine **(Xu *et al*., 2015)**, as well as natural ones, such as lycopene **(Xu *et al*., 2016)**, and curcumin **(Upadhyaya, *et al*., 2015)**. At present, natural pigments comprise 31% of the food pigments market (reach up to US$27.5 billion in 2018 **(Mapari *et al*., 2010)**. However, due to a few safety hazards, natural yellow pigments from animals, plants, or microorganisms have become more attractive in recent years **(Vendruscolo *et al*., 2016)**. Among these pigments, microbiology pigments have a good quality for harvest, scale-up of production is easier and they are not subject to the vagaries of nature **(Gomes *et al*., 2016)**. However, prior to food use, toxicological assessments must be conducted because some fungal species producing pigments are also myco-toxigenic producers **(Mapari *et al*., 2009b)**. Only *Monascus* is an important microbial resource now in use to produce pigments in an industrial level.
Thousands years ago, ancient Chinese had been using Monascus-fermented red rice as a food colorant to make red rice wine, red soybean cheese, meat and fish products and so on **(Blance *et al*., 1994; Ma *et al*., 2000; Wild *et al*., 2002)**. Currently, more than 50 patents regarding to utilization of *Monascus* pigments for food have been issued in Japan, the United States, France, and Germany **(Lin *et al*., 1992; Wang *et al*, 2007)**. The pigments produced by original *Monascus* contains three categories of pigments, yellow (monascin and ankaflavin), orange (monascorubrin and rubropunctatin), and red (monascorubramine and rubropunctamine) pigments **(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)**. Because of their excellent resistance to photodegradation and their pH and thermal stability **(Mapari *et al*., 2009a)**, *Monascus* yellow pigments have been receiving much attention. Except for uses as colorants, *Monascus* yellow pigments have been reported to possess health benefits, such as in reducing diabetes and obesity **(Hsu *et al*., 2014)**, hypolipidemic **(Lee *et al*., 2010)**, anti-obesity **(Lee *et al*., 2013)**, anti-inflammation **(Hsu *et al*., 2012)**, antitumor **(Su *et al*., 2005; Lee *et al*., 2013)** and antioxidative stress **(Shi *et al*., 2012)**, and have wider applications than its in the food industry **(Klinsupa *et al*., 2016)**. Research continues in the development of improved *Monascus* yellow pigments yields, as well as in identifying new Monascus yellow pigments **(Krairak *et al*., 2000; Chen *et al*., 2015)**. *Monascus* yellow pigments have been widely researched due to, which are related to the molecular structures of yellow pigments **(Su *et al*., 2005)**. In the last few decades, 35 *Monascus* yellow pigments and its derivatives have been identified and characterized **(Gong and Zhengqiang, 2016)**. However, the yellow pigments are still not suitable for industrial production, due to their relatively low production and purity, unavailability of microbial species. Not only microbial genus, but also the environmental conditions play a key influence on monascus yellow pigments production in submerged culture. Environmental conditions include chemical conditions like the type and content of carbon, nitrogen, phosphate, and metals, and physical conditions like mechanical stress, temperature, agitation and pH **(Shi *et al*., 2015; Bo *et al*., 2009; Hu *et al*., 2012; Tao *et al*., 2017).** Meantime, fungal morphology, influenced by genotypes of strains and environmental conditions **(Kaup et *al*., 2008; Krull *et al*., 2010)**, is also considered as a key bioprocess parameter for submerged, which not only has a significant impact on mixing and mass transfer, but also determines the overall process productivities and subsequent economics **(Wucherpfennig *et al*., 2011; Hyun *et al*., 2002)**.
In our preliminary experiments **(Bo *et al*., 2009; 2012; 2014)**, the pH, aeration and temperature influenced the *Monascus* yellow pigments production by *Monascus anka* mutant in 5 L fermenter. Therefore, the aim of this work was to systematically investigate the effect of agitation on the production of the yellow pigments in *Monascus anka* mutant.

MATERIALS AND METHODS

Organism and cultivation

Microorganism used in this study is *Monascus anka* mutant, which was screened from physical and chemical combination mutagenesis in our laboratory **(Bo *et al*., 2009)**. Stock cultures of the mutant were maintained on wort agar slants, which contains 15º wort (provided by Guangzhou Zhujiang Beer Co., Ltd, China) and 20 g/L agar (Difco Labatory, Loveton Circle, USA), and subcultivated periodically. Cultures were reactivated by being transferred onto fresh wort agar

slants. After cultivation for 2-3 days at 31 ºC, spores were collected with 5 mL sterilized water, and the corrected spore suspension was used as inoculum preparation. Spores suspension (0.3 mL) was inoculated in 250 mL Erlenmeyer flasks containing 30 mL of seed culture medium which was composed of 30 g/L of corn flour, 3 g/L of $NaNO_3$, 4 g/L of KH_2PO_4, and 0.01 g/L of $FeSO_4.7H_2O$. The seed culture medium (initial pH 6.0) was cultivated at 31 ºC and 200 rpm for 1-2 days and then transferred into a 5 L fermenter (BIOFLO 3000 Batch/Continuous Bioreactor, New Brunswick Scientific Edison, NJ, USA). The submerge fermentation medium ingredients included 10 g/L corn steep liquor, 15 g/L NH_4Cl, 5 g/L KH_2PO_4, 20 g/L glucose and 70 g/L starch. Temperature of 31 ºC and aeration of 1.5 m^3/h were maintained during fermentation in fermenter. Various agitation rates from 250 r/min to 450 r/min were applied.

Determination of pigments

According to the similar method of Chinese National Standard, GB15961-2005 and some reported articles **(Bo *et al*., 2009)**, absorbance was applied to represent the pigments concentration. Five mL of culture broth was mixed with 5 mL of 70% (v/v) ethanol for 1 hour, and then centrifuged at 4,000 rpm for 20 min. The obtained supernatant was filtered through filter paper (45 μm, Xinhua Paper Industry Co., Ltd, Hangzhou, China). The filtrate contained two pigments: yellow pigment and red pigment, whose concentrations were determined by measuring the optical density of the supernatant using a 2802SUV/VIS spectrophotometer (Unicosh Scientific Instrument Co., Ltd, Shanghai, China) at 410 and 510 nm, respectively. Results were expressed as *OD* units per mL of fermented broth. The linearity equation between absorbance and diluting proportions is $y = -0.0054x+1.5462$ (R^2= 0.9907), where y is absorbance and x is dilution proportions (in the range from 100 to 300).

Determination of Dried Cell Weight (DCW)

Fungal biomass was determined by gravimetric analysis after filtration of cell samples through preweighed nylon filters (45 mm diameter, 0.8 μm porosity) and dried to constant weight at 60 ◦C under partial vacuum (200 mm Hg).

Determination of Residual Sugar Concentration (RSC)

The residual glucose in the fermentation broth was determined with a spectrophotometer by the standard 3,5-dinitrosalicylic acid (DNS) method **(Miller, 1959)**, and the calibration curve was prepared using glucose.

Determination of NH_4^+ Concentration

The Berthelot reaction **(Weatherburn, 1967)** was used for determination of ammonium ion.

Determination of Soluble Starch Concentration

The soluble starch was measured by the modified method of Teng **(Teng and Feldheim, 2001)**. Briefly, sample (0.5 mL) was hydrolyzed by using 0.5 mL of thermostable α-amylase (EC 3.2.1.1, Novo, Denmark) at 100 ◦C for 60 min and then 0.25 mL of amyloglucosidase (EC 3.2.1.3, Novo, Denmark) at 60 ◦C overnight. The amount of glucose was determined by the DNS method. The starch content was calculated as the amount of glucose×0.9 and expressed as g/L (g starch content/L cultures)

Imaging and Morphological Analysis

Sample preparation was carried out following the method described by Haack et al. **(Martin *et al*., 2006)**. For image analysis, 2 mL of the sample was taken from the culture broth, and one or two drops of lactophenol blue was added to stop growing and increase the contrast of the images. Image capture wassig accomplished on a Zeiss light microscope. The pellets were distinguished from clumps and dispersed mycelia by the differences in the greyness levels, an approach that has been used to provide a definition for a pellet **(Thomas 1992)**. Morphological measurements were carried out using a CMOS camera (IXUS115; Canon, Japan) and the Image-Pro PLUS software (Media Cybernetics Inc., USA). The average clump diameter was calculated on images obtained using a 4×objective. Data, reported as the mean±SD, were obtained from a population size of approximately 100 events per sample.

Data analysis

The specific growth rate, μ (h^{-1}), was calculated following the equation: $\mu = \left(\frac{1}{X}\right)\left(\frac{dX}{dt}\right)$, where X is the cell concentration (g/L) at time t (h). The specific production rate of yellow pigments, q_y ($OD.g^{-1}.h^{-1}$), was calculated following the equation: $q_Y = \left(\frac{1}{X}\right)\left(\frac{dY}{dt}\right)$, where Y is the yellow pigments value (*OD*) at time t (h). All the data shown in Tables and Figures were expressed as mean of triplicates. The statistical evaluation of all data was performed by Origin 8.0.

RESULTS AND DISCUSSION

Effect of agitation speed on yellow pigments production and *Monascus anka* mutant growth

Significant influence of agitation on monascus yellow pigments production and *Monascus anka* mutant growth has been noticed in this working (Figure 1). When agitation speed was 250, 300, 350, 400, and 450 r/min, the maximum monascus yellow pigments yield was 100.04, 100.12, 110.23, 125.85, and 105.97 *OD* units at 144, 126, 126, 96, and 96 hour, respectively (Figure 1A). Maximum of q_y was 0.1659, 0.2264, 0.1741, 0.1792, and 0.2177 $OD.g^{-1}.h^{-1}$ at 66, 54, 54, 42, and 48 hour, respectively (Figure 1B). μ_{max} was 0.026, 0.047, 0.034, 0.078, and 0.064 h^{-1} at 18, 24, 18, 6, and 24 hour , respectively (Figure 1C).

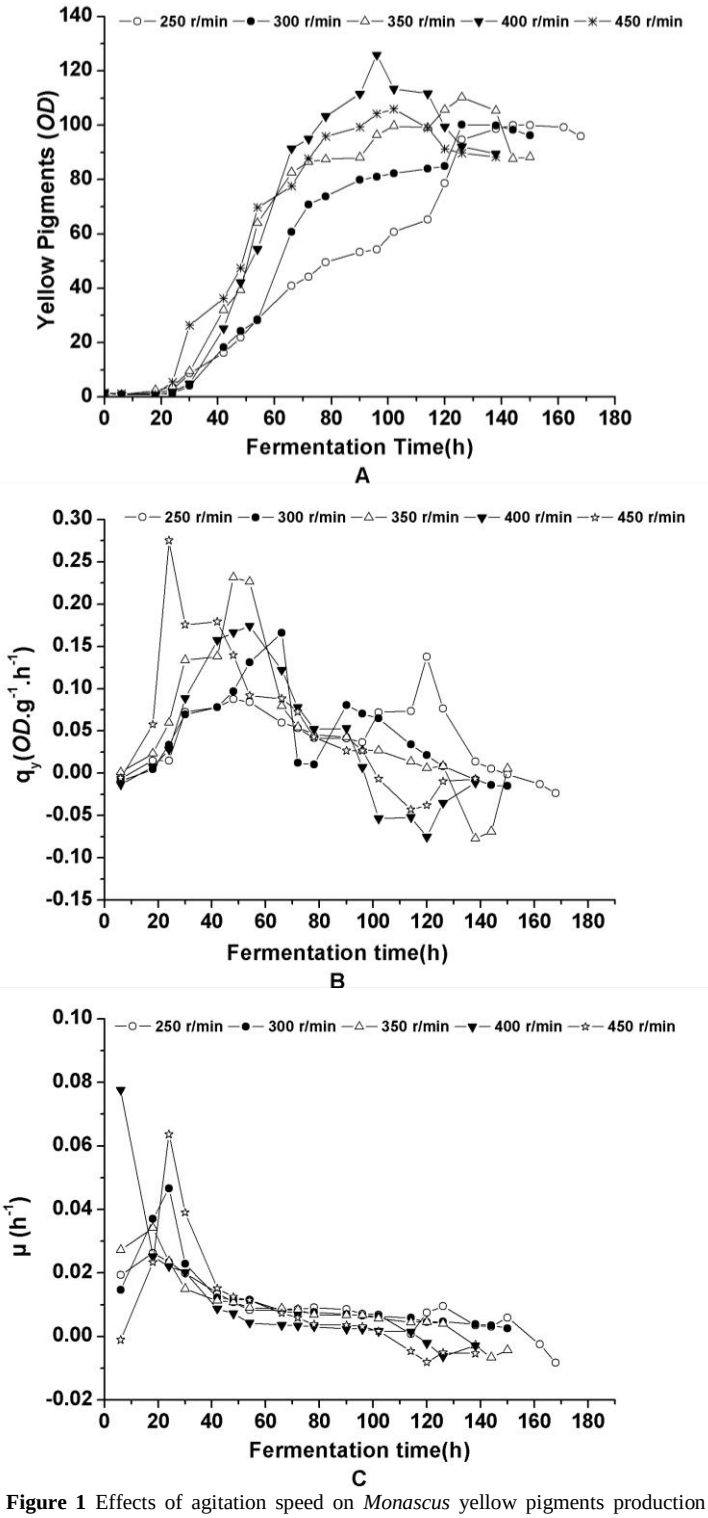

Figure 1 Effects of agitation speed on *Monascus* yellow pigments production (A), q_y(B) and μ(C)

These results demonstrated that time for yellow pigments production has been in advanced with high maximum specific production rate by high agitation speed, but higher agitation speed led to faster decrease of q_y at fermentation anaphase. Meanwhile, high agitation speed can improve specific growth rate for *Monascus anka* mutant at fermentation prophase, but negative for *Monascus anka* mutant growth at fermentation anaphase.

Effect of agitation on substrates utilization by *Monascus anka* mutant

With the increase of agitation speed, the maximum residual sugar content in culture appeared at 18 hour. Thereafter, the residual sugar content showed a downward trend with increased agitation (Figure 2A). The soluble starch content almost reached stable stage at 18 hours as shown in Figure 2B. The possible reason for this phenomenon is that hydrolysis of soluble starch was faster than the consumption of glucose at the initial stage **(Teng *et al*., 2001)**. Prolonged fermentation time did not digest starch very much. The soluble starch in the culture with higher agitation speed was lower at the end of the fermentation. (Figure 2B). NH_4^+ was used as nitrogen source in the present work. Sharp decrease of NH_4^+ was observed in the first 48 h beside of culture at 250 r/min (Figure 2C).

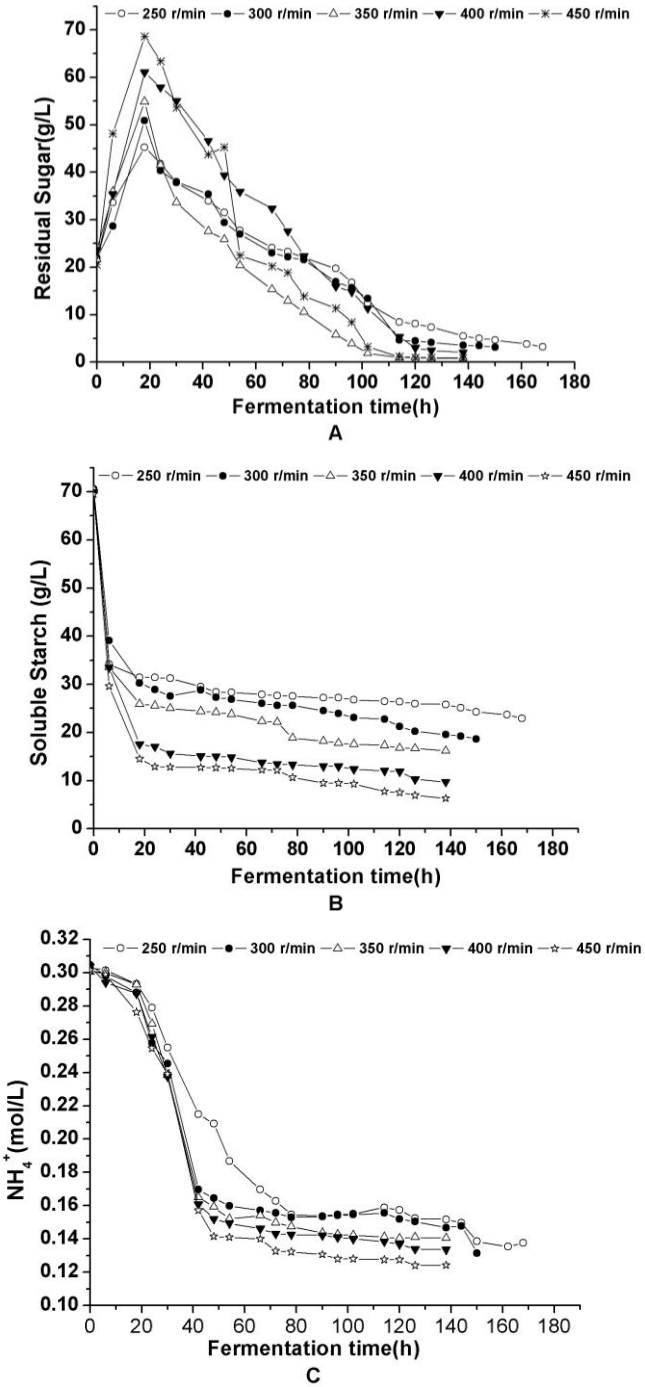

Figure 2 Effects of agitation on residual sugar (A), soluble starch (B) and NH_4^+ (C) utilization by *Monascus anka* mutant

The above results indicated that 400 r/min at fermentation metaphase while 300 r/min at fermentation anaphase was beneficial for substrates utilization. In general, higher agitation speed improved the hydrolysis of soluble starch **(Teng *et al*., 2001)** and NH^{4+} consumption **(Bo *et al*., 2014)**. From Figure 1A, it seemed that residual glucose in fermentation broth with agitation speed of 450 r/min was higher than that with the speed of 350 r/min. However, it should be noted that the residual soluble starch in fermentation broth with agitation speed of 250 r/min was much higher than that of the fermentation with the speed of 450 r/min. Therefore, fermentation with higher agitation speed metabolized more glucose.

Effect of agitation-shift on yellow pigments production by *Monascus anka* mutant

From the above experimental results concerning different agitation speed on the yellow pigments production by *Monascus anka* mutant, stage-divided strategy may improve yellow pigments production and enhance *Monascus anka* mutant growth with efficient utilization of substrate theoretically. The stage-divided strategy was as follows: 400 r/min was carried out to improve the *Monascus* mutant growth and yellow pigments production before fermentation of 96 hour, and then 300 r/min was applied to increase yellow pigment production with efficient substrate conversion.

Under agitation-shift strategy in batch monascus yellow pigments fermentation, the maximum yellow pigments yield reached 149.43 *OD* at 102 hour, which was 49.37%, 49.25%, 35.56%, 18.73%, and 41.01% higher than that of 250, 300, 350, 400 , and 450 r/min, respectively (Figure. 3A). Maximum q_y and μ_{max}, 0.2177 $OD.g^{-1}.h^{-1}$ and 0.0528 h^{-1}, respectively, was achieved at fermentation time of 48 hours and 6 hours (Figure 3B). μ and q_y could be maintained at 0.01 h^{-1} and 0.1 $OD.g^{-1}.h^{-1}$ from 6 hours to 54 hours and 24 hours to 66 hours, respectively. It was impossible to achieve using single temperature-shift strategy (Figure 3B). It showed that the yield of yellow pigments was higher than those of reports **(Shi *et al*., 2015; Klinsupa *et al*., 2016; Krairak *et al*., 2000; Bo *et al*., 2009; Hu *et al*., 2012; Tao *et al*., 2017)**.

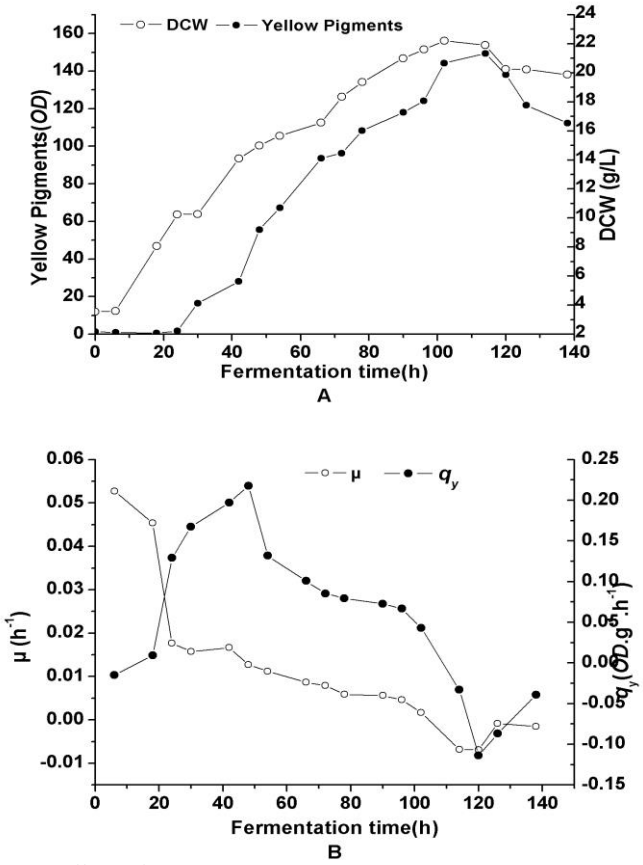

Figure 3 Effects of two stage agitation control on *Monascus* yellow pigments production (A) and *Monascus anka* mutant growth (B)

Under two stage agitation control, soluble starch was 19.79 and 10.37 g/L at 18 and 96hour, respectively. In the first 18 h, the content of soluble starch was decreased significantly. Meanwhile, reducing sugar content showed the highest at 8 h. The reducing sugar content, soluble starch content and NH_4^+ content was 8.24 g/L, 7.37 g/L and 0.135 mol/L, respectively at the end of fermentation. The yield of amylase produced by *Monascus*, which can hydrolyze soluble starch into sugar **(Teng *et al*., 2001)**, could be increased by the higher agitation speed and therefor the reducing sugar utilization was accelerated. Higher agitation speed significantly enhanced the carbon source (reducing sugar and soluble starch)

consumption by *Monascus anka* mutant (Figure.2). However, NH_4^+ may be only used for *Monascus anka* mutant growth because agitation has no obviously influence on change of NH_4^+ contents in post-fermentation but only in early-fermentation in culture (Figure1-2). All the above results indicated two stage agitation control could obviously improve substrate metabolism (Figure 4) , the yield of product relative to soluble starch and NH_4^+, and accelerate the *Monascus* growth and yellow pigments production, leading to a short fermentation time eventually (Table 1) compared with one-stage agitation strategy.

Table 1 Important parameters of yellow pigments production under different agitation condition

agitation (r/min)	Maxium μ(h^{-1})	Maxium q_Y (*OD* .g^{-1}.h^{-1})	yield of yellow pigments relative to ammonium ion(*OD* .mol^{-1})	yield of yellow pigments relative to starch(*OD*.g^{-1})	Culture colour change(h)
250	0.026(18 hr)	0.0877(48 hr)	652193.8	2202.67	23
300	0.047 (24 hr)	0.1659(66 hr)	649244.5	1990.17	22
350	0.034 (18 hr)	0.2264(54 hr)	691400.6	2051.62	21
400	0.078(6 hr)	0.1741(54 hr)	774223.3	2211.38	21
450	0.064(24 hr)	0.1792(42 hr)	610813.3	1760.16	15
Two-stage	0.053(6 hr)	0.2177(48 hr)	915568.9	2458.67	20

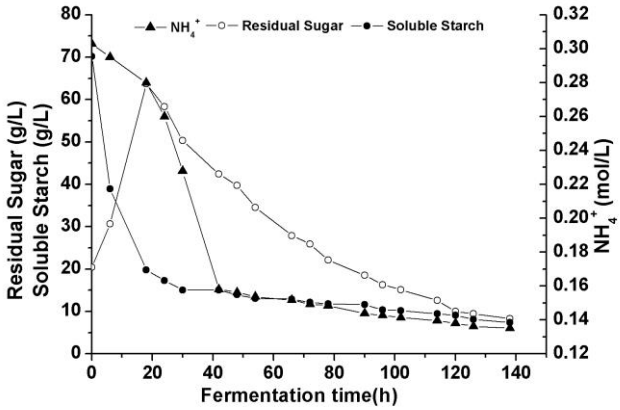

Figure 4 Effect of two stage agitation control on substrate utilization by *Monascus anka* mutant

Effect of agitation on morphological changes of *Monascus anka* mutant

Compared with single cell microorganism, there is morphological changes in filamentous fungi lifecycle by liquid and solid fermentation, especially more obvious and complex for submerge culture, then the morphological changes lead to influence on the target metabolites production. Three morphologies of filamentous fungi, such as free filaments, clump and pellet, appear in liquid culture **(Thomas 1992)**. There are two reasons of morphological changes to influence metabolite: 1) secretion mechanisms changes, 2) rheological properties changes of culture, including oxygen mass transfer **(Wucherpfennig *et al*., 2010)**. Besides incubation, pH, metal, substrate, temperature and oxygen, the agitation is the major factor on morphological changes **(Kaup *et al*., 2008; Krull *et al*., 2010).**

The mycelia morphology appeared free filaments and clump when agitation was 250 r/min and 300 r/min, respectively. Mycelia morphology was between free pellet and clump under 350 r/min. Mycelia morphology appeared well-distributed pellet with 0.52 mm in diameter when agitation was 400 r/min. But pellet was not well-distributed with diameter of 0.57 mm and some filaments appear in culture because of high agitation for 450 r/min. Mycelia morphology appeared to be pellet with diameter of 0.41 mm and yield of yellow pigments arrived 149.43 *OD*, which was 49.37% and 18.74% higher than that from 250 r/min and 400 r/min, respectively (Table 2). In the meantime, we also found seed age for incubation had an influence on mycelia morphology in this study, when old seed (beginning synthesis yellow pigments) was inoculated into fermenter, the mycelia morphology appeared between free filaments and pellet, or clump with low yellow pigments production. However, when inoculated with fresh seed, mycelia morphology appeared to be pellet with high yellow pigments production (data not shown). The mycelial morphology of *Monascus*, including the pellet size and hyphal diameter, was significantly influenced by the culture conditions such as the initial pH and shaking speed, which further exerted great impact on the production of yellow pigments. The relationship between the agitation and the fungal morphology has also been revealed in this study for enhanced production of natural yellow pigments **(Jun *et al*., 2017)**. All result demonstrated pellet of mycelia morphology was benefit for monascus yellow pigments production.

Meantimes, the citrinin was unable to be detected by HPLC method **(Bo *et al*., 2009)** in this study namely, the *Monascus anka* mutant may produce no or just a little citrinin (the detection limit is 0.1mg/L).

Table 2 Effect of agitation on the mycelia morphology and yellow pigments production

Agitation (r/min)	Mycelia Morphology	Mycelia diameter (mm)	Yellow pigments (*OD*)
250	Free filaments	1.39±0.07^a	100.04±8.76c
300	Free filaments and clump	0.98±0.03^b	100.12±9.54c
350	Clump and pellet	0.69±0.05^c	110.23±9.8^b
400	pellet	0.52±0.06^c	125.85±10.73^b
450	Pellet and Free filaments	0.57±0.03^c	105.97±8.79^c
Two-stage	pellet	0.41±0.04^c	149.43±11.34^a

Different letters in superscript within the same row indicate significant differences among the oil sample test (Tukey's test, $p<0.05$).

Previously, the yield of *Monascus* yellow pigment could be improved by controlling pH and nitrogen sources **(Shi *et al*., 2015)** or a novel approach of two-stage microbial fermentation in nonionic surfactant micelle aqueous solution **(Hu *et al*., 2012)**. However, due to its pH-dependent property, relatively low production and purity, it is impossible to scale-up yellow pigment production and application for all kinds of foods. The *Monascus* genus, which can produce yellow pigments owning pH-dependent property with high yield, is the key factor, such as *Monascusr* mutant strain KB **(Yongsmith *et al*., 1993; 1994)** and our specific strain of *Monascus anka* mutant **(Bo *et al*., 2009; 2012; 2014)**. In the current study, *Monascus* growth and yellow pigments production with high speed and longtime could be achieved by two stage agitation control strategy and short fermentation time to improve the yield of yellow pigments, which can make it possible to scale-up yellow pigment production.

CONCLUSION

To our knowledge, this is the first report focusing on the agitation optimization of *Monascus* yellow pigments production in submerge culture in fermenter as a source of natural yellow pigments. In the current study, *Monascus anka* mutant, which could produce yellow pigments with pH-independent property **(Bo *et al*., 2009; 2012; 2014)**, was cultured in a fermenter using two-stage agitation controlling strategy. Maximum yellow pigments of more than 149.43 *OD* were found in submerge culture under optimal agitation conditions: the agitation was 400 r/min to improve the *Monascus* growth and yellow pigments production before culture 96 hour and then 300 r/min was carried out to continue yellow pigment production with efficient substrate utilization. Future development on yellow pigments should focus on strain improvement for higher production of yellow pigments as well as process scaling up. Hopefully, the results in this paper have very important theoretical and realistic significance for realizing the industrial production of monascus yellow pigments by submerged culture. The data of this work could contribute to making the industrial production of *Monascus* yellow pigments feasible.

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