Effect of Ammonium Salts on Pigments Production by *Monascus anka* Mutant in 5L Bioreactor

Bo Zhou* [a,b,c], Yan Wang [a,b,c], Huamin Lu [a,b,c] and Yangqing Zhou [a,b,c]

[a] School of Food Science and Engineering, Central South University of Forestry and Technology, Changsha, 410004, P. R China.
[b] National Engineering Laboratory for Rice and By-products Processing, Changsha, 410004, P. R China.
[c] Hunan Province Key Laboratory of Grain, Oil Processing and Quality Control, Changsha, 410004, P. R China.

*Author for correspondence; e-mail: zhbo78@hotmail.com

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ABSTRACT

This work characterized the fermentation and *Monascus* pigments production of *Monascus anka* mutant in a 5L bioreactor under the different ammonium salt conditions: ammonium chloride, ammonium sulfate or ammonium nitrate. All the experimental results obtained in this work suggest that not the pH environment established by ammonium salts but ammonium salts itself played a critical role on the production of *Monascus* pigments and the growth of *Monascus anka* mutant. ammonium ion could inhibits the activity of some key enzyme for *Monascus* pigments synthesis and the inhibition effect on some key enzyme for *Monascus* red pigments synthesis is stronger than on some key enzyme for *Monascus* yellow pigments synthesis. Key enzyme for inhibited *Monascus* pigments production would be researched clearly by experiments in our future researching.

Keywords: *Monascus anka* mutant, ammonium salts, pH, yellow pigments, red pigments

1. INTRODUCTION

In food industrial nowadays, yellow pigments production has two main approaches: chemical synthesis and plant extraction. The former, with products such as tartrazine [1], due to its safety risk, has withered in use. Meanwhile, the latter, with extracts such as gardenia yellow [2]*safflower yellow [3], is natural and low in toxicity, but is subject to the season change, resulting in an unstable industrial production. Compared with these two approaches, microbiological fermentation for yellow pigments production, because of its few safety hazards, low cost, and wide variety of resource choices [4-7], has received increasing attention as a promising alternative. As far as industrial production is concerned, *Monascus* is one of the microbial resources now in use to produce pigments.

Thousands years before 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 [8-10]. The genus *Monascus* produces a mixture of three categories of pigments, orange, red, and yellow pigments. These three pigments have two components of polyketide origin in common, which are secondary metabolites possessing an azaphilone skeleton [8, 11-13]. The red pigments produced by *Monascus* are applied widely due to their safety with the characteristics of high protein adhesion, thermal stability, and wide-pH stability [14]. But with citrinin production by *Monascus* was discovered [15], which is nephrotoxic (kidney damaging) and possibly carcinogenic [16-18] to human beings, the safety of *Monascus* products has been paid more and more attention to.

The production of *Monascus* red pigments has been researched a lot. Some parameter factors affecting the growth of *Monascus* and production of red pigments, such as gas environment [19], agitation and aeration [20], carbon and nitrogen sources [21] and pH [22-24] were reported, and the perstractive fermentastion of intracellular *Monascus* pigments in nonionic surfactant Triton X-100 micelle aqueous solution was also reported [25-27]. However, due to unavailability of microbial species, researches on production of *Monascus* yellow pigments (*Monascusone A and B*) is very limited [28, 29]. One *Monascus anka* mutant able to produce high yield of yellow pigments (*Ankaflavin and monascin*) screened by physical and chemical combination mutagenesis was obtained in our laboratory, the yellow pigments produced by *Monascus anka* mutant have maximum absorption at 410 nm [30]. After incubating for 3 days on MEA medium (Per Liter contains malt extract 20 g, peptone 1 g, glucose 20 g, and agar 15 g), the colonies of *Monascus anka* mutant showed yellowish in color with the size of 25-30 mm. After incubating for 5 days, the aerial mycelium was white; the colonies showed yellow orange in color with the size of 29-30 mm, and the air-borne mycelium was white. Both conidiospores and ascocarpes were colorless. The conidiospores were 40-50 μm in size and the ascospores were oval-shaped with the size of 30-35 μm.

As the use of available microbial source of nitrogen, it was key inhibition effect factors for antibiotics formation by high concentrations of nitrogen sources, such as 14-membered macrolide antibiotic erythromycin [31] and the 16-membered macrolide antibiotics leucomycin [32]. But in our previous study, ammonium salts played a critical role on *Monascus* yellow pigments production by *Monascus anka* mutant in the shaker cultivation [30].

In present study, we characterized the fermentation and *Monascus* pigments production of *Monascus anka* mutant in the bioreactor under different ammonium salt conditions, ammonium chloride, ammonium sulfate or ammonium nitrate, and then made an analysis about ammonium salts affects on *Monascus* yellow pigments production by *Monascus anka* mutant.

2. MATERIALS AND METHODS
2.1 Organism and Cultivation

The microorganism used in this work is *Monascus anka* mutant able to produce comparatively high-production of yellow pigments, which was screened by combination of physical and chemical mutagenesis in our laboratory. The original strain of *Monascus anka* obtained from China Center of Industrial Culture Collection (CICC 5013).

Stock cultures of the mutant were maintained and periodically subcultivated
on wort agar slants, which contains 15° wort (provided by Guangzhou Zhujiang Beer Co., Ltd, China) and 20g/L agar (Difco Labatory, Loveton Circle, USA). Cultures were reactivated by being transferred onto fresh wort agar slants. After cultivation of 2-3d at 31 °C, spores were collected with 5 mL of sterilized water, and the spore suspension corrected was used as inoculum preparation. 1 mL of spores suspension was inoculated in 100 mL of seed medium (30g/L corn powder, 3g/L NaNO₃, 0.01g/L FeSO₄·7H₂O, and 4g/L KH₂PO₄, pH6.0), and cultured at 31 °C, 200rpm for 2d in a rotatory shaker as the seeds cultures.

The seed cultures were inoculated in the submerged culture medium at volume ratio of 1:9, and fermented in a 5L bioreactor (BIOFLO 3000 Batch/Continuous Bioreactor, New Brunswick Scientific Edison, N.J., USA) at 300 rpm, 31 °C and aeration of 0.15 m³.h⁻¹ for 6-7d. Submerged culture medium contains ammonium salts, 12g/L corn steep liquor, 5g/L KH₂PO₄, 20g/L glucose, 40g/L starch and 0.1g/L CaCl₂, pH4.0. The type of ammonium salts was various in different experiments, and the dosage of ammonium salts was determined by NH₄⁺, 0.3mol/L of which was used for each fermentation. The 5L bioreactor loaded 3 L of submerged culture medium.

2.2 Measurement of Pigments

5 mL of the culture broth was extracted with 5 mL of 70% (v/v) ethanol at room temperature for 1h, and then centrifuged at 4000rpm for 20min. The supernatant obtained was filtered with filter paper. The filtrate contained two pigments: yellow pigments and red pigments, whose concentrations were determined by measuring the optical density of the filtrate using a 2802SUV/VIS spectrophotometer (Unicosh Scientific Instrument Co., Ltd, Shanghai, China) at 410nm and 510nm respectively (Yoshimura et al. 1975). Results were expressed with OD units per mL of fermented broth. The linearity equation between absorbance and diluting proportions is \( y = -0.0054x + 1.5462 \) (\( R^2 = 0.9907 \)). In where, \( y \) is absorbance and \( x \) is dilution proportions (in the range from 100 to 300).

2.3 Measurement of Dry Cell Weight (DCW)

5mL of cultures broth was filtered through fibre-glass membranes (Whatman GF/C), the retained biomass was rinsed twice with distilled water, vacuum dried completely at 60!, and weighed.

2.4 Data Calculation and Analysis

The specific growth rate, \( m(h^{-1}) \), was calculated with 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 yellow pigments, \( q_{Y} \) (OD units /h), was calculated with the equation: \( q_{Y} = \frac{1}{X} \frac{dY}{dt} \), where \( Y \) is the yellow pigments value (OD units) at time \( t \) (h). The specific production rate of red pigments, \( q_{R} \) (OD units /h), was calculated with the equation: \( q_{R} = \frac{1}{X} \frac{dR}{dt} \), where \( R \) is the red pigments value (OD units) at time \( t \) (h).

2.5 Data Analysis

All the data shown in Figures were expressed as mean ± standard deviation of the three replicates. The statistical evaluation of all data was done by OriginPro8.0.

3. RESULTS AND DISCUSSION

3.1 Effect of Ammonium Salts on pH of Cultures in Fermentation of Monascus anka Mutant

When using ammonium chloride or ammonium sulfate as nitrogen source for production of pigments, the pH
value of cultures changed in downtrend during the whole fermentation and the final pH value of cultures was 1.90 and 2.30, respectively (Figure 1). When using ammonium nitrate for production of pigments, pH value of the cultures changed in downtrend within first 54hrs and pH value reached 2.97 at 54h, however, afterwards pH value of the cultures increased gradually and got to 6.00 in the end of fermentation (Figure 1).

Three kinds of ammonium salts used in this work are all strong-acidity and weak-alkalescence salts. Ammonium chloride is the strongest in acidity, and followed by ammonium sulfate and ammonium nitrate successively. Along with $\text{NH}_4^+$ consumed as nitrogen sources for Monascus growth, the acidity of fermentation broths rises and the pH of cultures declines. The lowest pH (pH<2) environment was established because of strong acidity of ammonium chloride. When using ammonium sulfate for production of Monascus pigments, the final pH value could maintain within the range from 2 to 2.5. But the different situation occurred while using ammonium nitrate for production of Monascus pigments, because Monascus can not only use $\text{NH}_4^+$ for Monascus growth but also use $\text{NO}_3^-$ for production of Monascus pigments [21]. In the beginning, $\text{NH}_4^+$ was used for Monascus growth and the pH of cultures declined, but 54hrs after fermentation, the pH of cultures rose due to a great deal of $\text{NO}_3^-$ consumption for production of red pigments.

3.2 Effect of Ammonium Salts on Growth of Monascus anka Mutant

Figure 2 indicates that ammonium salts used in this work have no significant effects on the maximum dry cell weight (DCW), but bear significant relationship with the growth process. The trend of growth is similar between the fermentations using ammonium chloride and ammonium sulfate, but different from the fermentation using ammonium nitrate. The time to reach maximum DCW in the fermentation using ammonium nitrate is earlier than those using ammonium chloride and ammonium sulfate. The maximum DCW could arrive at 27.86 g/L, 27.46 g/L and 27.78 g/L at 84h, 102h and 102h,

![Figure 1. Effect of ammonium salts on culture pH by Monascus anka mutant fermentation in a 5L bioreactor.](image-url)
respectively (Figure 2).

However, ammonium salts have effects on specific growth rate ($\mu$) (Figure 2). The trend of $\mu$ is similar between the fermentations using ammonium chloride and ammonium sulfate. When using ammonium nitrate, however, the $\mu$ value is higher than that using ammonium chloride and ammonium sulfate before 40h. Their maximum value can get to 0.048 h$^{-1}$ at 24h, 0.041 h$^{-1}$ at 12h and 0.045 h$^{-1}$ at 12h, respectively.

Figure 2. Effect of ammonium salts on dry cell weight (DCW) and specific growth rate ($\mu$) of *Monascus anka* mutant.

In a way, ammonium salts little influence the yield but affect the specific growth rate of *Monascus anka* mutant.

Effect of ammonium salts on *Monascus* pigments production by *Monascus anka* mutant

As far as yield of yellow pigments, the maximum amounts of yellow pigments has no significant difference between ammonium chloride, ammonium sulfate and ammonium nitrate, the maximum amounts of yellow pigments got to 54.87 OD units at 96h, 51.12 OD units at 96h, 49.56 OD units at 120h, respectively (Figure 3). But it has obviously difference for yield of red pigments, the maximum amounts of red pigments got to 11.26 OD units at 80h, 34.08 OD units at 96h, 42.06 OD units at 132h, respectively (Figure 3).

When using ammonium chloride as nitrogen source for production of *Monascus* pigments, compared to yellow pigments, the production of red pigments was remaining at a very low level during the whole fermentation. (Figure 3). Before 48hrs, the trend of production of yellow pigments was similar to that of red pigments when using ammonium sulfate for production of pigments, but the production of yellow pigments was obviously higher than that of red pigments after 48hrs, and the pH value of cultures was 2.56 at this time (Figure 1). The pH of cultures declined to 2.97 after 54hrs of fermentation when using ammonium nitrate as nitrogen
source for production of *Monascus* pigments (Figure 1). Compared to yellow pigments, the production of red pigments was obviously retarded before 54hrs and then accelerated, and the yield of yellow pigments was 4.5 times as much as that of red pigments before 54hrs. In the end of fermentation, the yield was not of significant difference between yellow and red pigments (Figure 3). The maximum production of red pigments using ammonium chloride or ammonium sulfate is just 26.77% and 81.10% as much as that using ammonium nitrate, respectively (Figure 3). The maximum yield of yellow pigments using ammonium chloride, ammonium sulfate, ammonium nitrate, was 4.87 times, 1.50 times, 1.18 times, respectively, as much as that of red pigments (Figure 3).

![Graph](image_url)

**Figure 3.** Effect of ammonium salts on red and yellow pigments production by *Monascus anka* mutant in a 5L bioreactor.

The specific production rate of yellow pigments ($q_y$) can maintain at a low level (the maximum $q_y$ can get to 0.032 OD units /h at 72h) when using ammonium nitrate for fermentation of *Monascus* pigments. When using ammonium chloride and ammonium sulfate as nitrogen sources, from 36h to 60h, the value of $q_y$ can increase to 0.08 OD units /h from 0.05 OD units /h. The maximum value of $q_y$ can reach 0.076 OD units /h and 0.072 OD units /h at 54h and 36h, respectively (Figure 4).
Figure 4. Effect of ammonium salts on specific production rate of red pigments ($q_R$) and yellow pigments ($q_Y$) of Manascus anka mutant.

Compared with using ammonium nitrate and ammonium sulfate, the production of red pigments was inhibited more obviously when ammonium chloride was used, but the trend is similar to that when using ammonium sulfate and only the inhibitory degree is different, the specific production rate of red pigments ($q_R$) can be up to 0.037 OD units/h for ammonium chloride and 0.048 OD units/h for ammonium sulfate at 36h. But when using ammonium nitrate as nitrogen source, at 84h, the $q_R$ can get to 0.043 OD units/h, and the can be kept at this value or up for longer than that when using ammonium chloride and ammonium sulfate (Figure 4).

Despite the trend of production of red pigments using ammonium chloride as nitrogen source is similar to that using ammonium sulfate, the descending speed of using ammonium chloride is faster than that using ammonium sulfate. Thus, the yield of red pigments using ammonium sulfate is higher than that using ammonium chloride (Figure 3). When ammonium nitrate was used for production of red pigments, not only the can keep at a low value (low to 0.016 OD units/h) for a long time, but also the time to reach maximum is later, and the dropping range is narrower than those using ammonium chloride and ammonium sulfate (Figure 4). The trend of production of yellow pigments is similar to that using ammonium chloride or ammonium sulfate. In the case of ammonium chloride, both the rising and dropping slopes of are steep. As a result, the yield of yellow pigments was of no significant difference between using ammonium chloride and ammonium sulfate (Figure 3).

When using ammonium nitrate for production of yellow pigments, not only the can keep at a low level (from 0.01 OD units/h to 0.03 OD units/h) for a long time, but also the time to reach maximum is later than that using ammonium chloride and ammonium sulfate (Figure 4). So, the yield of yellow pigments using ammonium nitrate was of no significant difference from using ammonium chloride and ammonium sulfate (Figure 3).
Kurono et al. reported that the ammonium salts were bad for production of yellow pigments and also had no significant influence on production of red pigments [14], Chen et al. observed that low pH was good for production of yellow pigments [24]. Our results obtained in the present work indicate that although ammonium salts did obviously influence not only yellow pigments production but also red pigments production by Monascus anka mutant, they did influence the specific production rate of yellow pigments (Fig. 4). Ammonium salts influenced the yield as well as specific production rate of red pigments (Fig. 4). Taken together with the results that ammonium salts did not influence the yield but affected the specific growth rate of Monascus anka mutant and with the results that ammonium salts affected the pH of cultures (Fig. 4). Ammonium salts influenced the yield as well as specific production rate of red pigments (Fig. 4). Taken together with the results that ammonium salts did not influence the yield but affected the specific growth rate of Monascus anka mutant and with the results that ammonium salts affected the pH of cultures (Figs. 1-2). Three kinds of ammonium salts used in this work are all strong-acidity and weak-alkalescence salts. Ammonium chloride is the strongest, ammonium sulfate the second and ammonium nitrate the third in acidity. Along with NH\textsubscript{4}\textsuperscript{+} consumed as nitrogen sources for Monascus growth, the acidity of fermentation broths rises and the pH of cultures declines. There is still one different point, however, in the case of ammonium nitrate, it is clear as Pastrana et al. reported that NH\textsubscript{4}\textsuperscript{+} as one nitrogen source is mainly used for the growth of Monascus [21], NO\textsubscript{3}\textsuperscript{-} is a good nitrogen source able to be used not only for the growth of Monascus but also for production of red pigments. The pH of cultures using ammonium nitrate first declines then increases gradually along with the consumption of NO\textsubscript{3}\textsuperscript{-}. In the end of fermentation, the pH of cultures using ammonium chloride, ammonium sulfate or ammonium nitrate was 1.90, 2.3 and 6.0, respectively (Figure 1). Low pH of cultures (<2) like using ammonium chloride greatly inhibits the production of red pigments and high pH of cultures like using ammonium nitrate is beneficial for the production of red pigments. Lin et al. also reported the similar results [22]. The maximum production of red pigments using ammonium chloride is just 26.77% as much as that using ammonium nitrate in our experiments. Taken together with the results, we made an assumption that pH of cultures of Monascus anka mutant directly influenced the production of Monascus pigments, and pH of cultures influences the whole process of the production of Monascus pigments including yellow and red pigments.

In order to verify our assumption, a two-stage pH control strategy was carried out in an attempt to maximize yellow pigment formation in this paper. Firstly, an initial pH of 6.5 was kept with 0.1 mol/L NaOH at 31°C for 2 days in order to obtain maximum biomass. In the second stage, the pH was adjusted to 2.5 with 0.1 mol/L H\textsubscript{2}SO\textsubscript{4} to stimulate the production of yellow pigment for the next 4 days. However, yellow pigment yield was no greater than with the single stage fermentation (data not shown), the results was the same as described by Yongsmith [33]. Our results further demonstrated that not the pH environments established by ammonium salts but the ammonium ion itself played a critical role on the production of Monascus pigments and the growth of Monascus in the Monascus anka mutant fermentation.

As the use of available microbial source of nitrogen, it was key inhibition effect factors for antibiotics formation by high concentrations of nitrogen sources [34, 35], such as 14-membered macrolide antibiotic
erythromycin [31] and the 16-membered macrolide antibiotics leucomycin [32] and tylosin [36] is inhibited by high concentrations of ammonium. The production of polysaccharides by *Aureobasidium pullulans* was also suppressed under high nitrogen levels [37]. The inhibition effect was considered that the key enzyme activity for antibiotics synthesis has been inhibited, such as valine dehydrogenase [38] and threonine dehydratase [39] as key enzyme providing the precursors for tylosin formation are repressed by ammonium ion in batch culture.

According to the results in this paper and previously reported results, ammonium ion maybe inhibits the activity of some key enzyme for *Monascus* pigments synthesis, and the inhibition effect on some key enzyme for *Monascus* red pigments synthesis is stronger than on some key enzyme for *Monascus* yellow pigments synthesis. This hypothesis should be verified by experiments in our future researching.

The production of mycotoxin citrinin of *Monascus* is affected not only by the growth conditions [39, 40] but also by genus itself [15, 41]. Sandra *et al.* reported that the high pH (about 6) was not good for but low pH was good for the citrinin production by *Monascus* [42]. In this study, the citrinin was unable to be detected by HPLC method as described by Xu [43], namely, the *Monascus anka* mutant may produce no or just a little citrinin (the detection limit is 0.1mg/L).

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