Highly efficient improvement of *Monascus* pigment production by accelerating starch hydrolysis in *Monascus ruber* CICC41233

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Abstract

To investigate the relationship between starch hydrolysis and *Monascus* pigments (MPs) production, the α -amylase gene (*AOAmyA*) from *Aspergillus oryzae* was heterologously expressed in *Monascus ruber* CICC41233, and we obtained a positive transformant named *Monascus ruber* Amy9. In *M. ruber* Amy9, the α -amylase activities were 6.65- and 4.26-fold higher at 72 h and 144 h, respectively, than those in the parent strain with the glucose as solo carbon medium. Surprisingly, in the MPs fermentation medium with rice powder as solo material, *M. ruber* Amy9 completely degraded starch at 48 h, while 43.93 and 7.29 mg/mL starch remained at 48 and 144 h, respectively, in the parent strain. *Monascus ruber* Amy9 accelerated starch hydrolysis, which enhanced biomass and also increased total MPs by 132% after 144 h. Compared with *M. ruber* CICC41233, the relative gene expression levels, as determined by a quantitative real-time polymerase chain reaction analysis, of *acl2* encoding ATP-citrate lyase subunit 2, *pks* encoding polyketide synthase, and *fasB* encoding the fatty acid synthase beta subunit increased by 33.14, 145.18, and 32.15%, respectively, after 144 h in *M. ruber* Amy9. The up-regulated expression of these key genes in MPs synthesis contributed to the large increase in MPs production. This interesting work provided us with a new idea and a new target for the study of the MPs production.

Keywords α -Amylase · *AOAmyA* gene · *Monascus* pigment · *Monascus ruber* · Starch

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Introduction

HongQu, also called red mold rice, is usually produced by *Monascus* spp., which have been used in food, medicine, and industry for over 1000 years in Asian countries, especially in China (Patakova 2013; Chen et al. 2015). *Monascus* pigments (MPs), one of the most famous fermented products of red mold rice, possess a broad range of functions, such as anti-inflammatory, anti-atherosclerotic, anti-diabetic, and anti-cancer activities, and they are used commonly as food coloring agents (Feng et al. 2012; Vendruscolo et al. 2016). The MPs are a mixture of azaphilones, which usually include yellow, orange, and red constituents. Pigment biosynthesis in *Monascus* is believed to follow a polyketide pathway and fatty acid pathway (Hajjaj et al. 2000). Recently, a polyketide synthase (PKS)-fatty acid synthase (FAS) gene cluster involved in the synthesis of pigments was identified in *Monascus pilosus*, and a homolog of the key PKS gene was identified by mutagenesis in *Monascus purpureus* (Balakrishnan et al. 2013), and in *Monascus ruber* M7 based on *albino* mutants from a T-DNA insertion library (Feng

et al. 2012; Chen et al. 2015). It is very meaningful to help understand the MPs biosynthetic pathway. However, the biosynthetic pathway of MPs is still unclear and controversial.

Feng et al. (2012) and Vendruscolo et al. (2016) summarize several kinds of factors, such as strain selection, substrate, pH, nitrogen source, light intensity, temperature, broth rheology, and oxygen, which affect *Monascus* pigment production. Rice grains contain large starch are usually used to manufacture MPs. To investigate the relationship between starch degradation and MPs production, we hypothesized that enhancing the hydrolysis of starch would improve the yield of MPs. Nemoto et al. (2012) reported that *A. oryzae* RIB40 mainly produces largely amylase which hydrolyzes starch, and that the *AOamyA* gene is one of the primary contributors among the three α -amylase genes. Therefore, to test this hypothesis, the α -amylase gene *AOamyA* was cloned from *Aspergillus oryzae* NRRL3488 and transferred into the strain *M. ruber* CICC41233 via *Agrobacterium tumefaciens*-mediated transformation (ATMT) to obtain transformants. To our knowledge, this is the first report of an α -amylase gene application to MPs production in *Monascus* spp.

Materials and methods

Strains and culture conditions

Monascus ruber CICC41233 was purchased from the China Center of Industrial Culture Collection and grown on malt–peptone–starch (MPS) agar (10 g/L malt extract, 10 g/L peptone, 40 g/L soluble starch, and 2 g/L agar) at 30 °C for 8 days. For phenotypic characterization, two kinds of media including MPS agar medium with 0.2% acetate, and GM agar medium (20 g/L glucose, 10 g/L maltose, 3 g/L NaNO₃, 2 g/L KCL, 1 g/L KH₂PO₄, 0.5 g/L MgSO₄·7H₂O, 0.02 g/L FeSO₄·7H₂O, and 2 g/L agar) with 0.2% acetate were utilized. The strains *Aspergillus oryzae* NRRL3488, *Escherichia coli* DH5a, and *A. tumefaciens* EHA105 were also used.

Construction of heterologous gene expression vectors

The *AOamyA* gene (1500 bp, GenBank accession no. D00434.1) was cloned from *A. oryzae* using cDNA as template via a polymerase chain reaction (PCR) using the primers amy-*Hind* III-F and amy-*Sac* I-R (Supplementary Table S1). After sequencing, the gene fragment and the plasmid of pNeo0380 (Long et al. 2018) were digested by *Hind* III and *Sac* I, and then ligated to construct the binary expression vector pNeo0380-amy.

Obtaining positive transformants

The *A. tumefaciens* EHA105, containing a binary vector (pNeo0380-amy), mediated the *AOamyA* gene transfer into *M. ruber* CICC41233 with geneticin (G418, 80 µg/mL) as the screening marker (Long et al. 2018). Putative visible transformants were picked up initially and transferred to an MPS agar plate containing G418. Genomic DNA of the transformants was extracted from all available mycelia according to the method of Balakrishnan et al. (2016). Positive transformants were detected by PCR amplification using the primers amy-*Hind* III-F and amy-*Sac* I-R. Then, the positive transformants were cultured three times on MPS agar plates without G418. In addition, they were transferred to MPS plates containing G418 to determine the genetic stability of the transformants.

Fermentation culture

Experiments were conducted in 250-mL Erlenmeyer flasks that contained 50 mL of MPs fermentation culture medium (9.0% rice powder, 0.2% NaNO₃, 0.1% KH₂PO₄, 0.2% MgSO₄·7 H₂O, and 0.2% acetate). Three replicate flasks were examined at 40, 48, 72, 96, 120, and 144 h.

Simultaneously, glucose medium (9.0% glucose, 0.2% NaNO₃, 0.1% KH₂PO₄, 0.2% MgSO₄·7 H₂O, and 0.2% acetate) was used to identify the α -amylase activity of *M. ruber* CICC41233 and positive transformants. Three replicate flasks were examined at 72 and 144 h.

The final number of inoculated, freshly harvested spores was 10⁵ conidia/mL. The flasks were shaken at 30 °C with a rotation speed of 180 rpm. All the experiments were conducted independently in triplicate.

Analysis of MPs production

After fermentation, 25 mL of culture broth was centrifuged (16 °C, 30 min, 9000×g). The supernatant containing the extracellular pigments (also called water-soluble pigment) was removed. The precipitate was re-suspended in 25 mL of 70% (v/v) ethanol and incubated at 60 °C for 1 h with shaking at 90×g, and then centrifuged (16 °C, 15 min, 9000×g); the resulting supernatant contained the intracellular pigment (also called ethanol-soluble pigment). The mycelia precipitate that remained was dried to a constant weight at 60 °C to determine its biomass.

The absorbance spectrum of the pigments was adjusted to 0.1–1.0 and recorded by a spectrophotometer at 510 nm. The MPs results were expressed in units of absorbance (U) at a given wavelength, multiplied by the dilution factor (Lopes et al. 2013; Shi et al. 2015). The total MPs

contained water-soluble pigment and ethanol-soluble pigment.

Quantitative real-time PCR and RT-PCR analysis

Approximately 20 mL of the remaining broth was taken from the flask and centrifuged (4 °C, 30 min, 9000×g). The precipitate was frozen immediately or ground to a fine powder under liquid nitrogen to extract RNA using the TRIzol reagent. The quality of the extracted total RNA was assessed by agarose gel electrophoresis, and the concentration of mRNA was measured by a spectrophotometer (Nanodrop 2000, Thermo Fisher Scientific, Waltham, MA, USA). Reverse transcription was conducted using the PrimeScript™ RT reagent Kit with gDNA Eraser (TaKaRa, Dalian, China). Relative expression was determined using 10.0 µL of SYBR® Premix Ex Taq™ (Tli RNaseH Plus) (TaKaRa), 1.0 µL of 10.0 µM forward primer, 1.0 µL of 10.0 µM reverse primer, 6 µL of water, and 2.0 µL of template cDNA (tenfold dilution). Thermal cycling conditions comprised an initial denaturation at 95 °C for 3 min, followed by 40 amplification cycles at 95 °C for 15 s, 60 °C for 30 s, and 72 °C for 30 s, with a final extension step at 72 °C for 5 min by a real-time PCR (RT-PCR) system (CFX Connect, Bio-Rad, Hercules, CA, USA). The *actin* gene was used as the endogenous control gene. RT-PCR was used to assess *AOamyA* gene expression using the primers amy-YGF & amy-YGR. The *acl2* gene encodes ATP-citrate lyase subunit 2, which converts citric acid into acetyl coenzyme A (Hynes and Murray 2010; Chen et al. 2014). The *pks*, *fasA*, and *fasB* are the key genes in the biosynthetic gene cluster of MPs, which encode PKS and FAS alpha and beta subunits (Supplementary Figure S1), respectively (Feng et al. 2012; Balakrishnan et al. 2013; Chen et al. 2015). The primers used are listed in Supplementary Table S1.

Analysis of α-amylase activity and starch content

After the fermentation culture, 25 mL of the medium was centrifuged. The supernatant was collected and analyzed by an α-amylase assay kit (AMS, Nanjing Jiangcheng Bioengineering Institute, China) using I_2 -starch colorimetry. A starch concentration of 0.4 mg/mL was used as a control. The iodine solution (10 mM) was used as a chromogenic agent. The optical density at 660 nm (OD_{660}) was used to measure the reaction of AMS by a spectrophotometer. The starch concentration was determined by a standard curve using soluble starch with an iodine solution (2.6 g/L I_2 , 5.0 g/L KI). The OD_{660} was used to measure the reaction by a spectrophotometer.

Statistical analysis

Each experiment was repeated at least in triplicate. Numerical data are presented as the mean ± SD. The differences among different treatments were analyzed using one-way ANOVA. The $p < 0.05$ or $p < 0.01$ was considered statistically significant.

Results

Screening positive transformants and phenotypic characterization

The *AOamyA* gene (Supplementary Figure S2) was heterologously expressed in *M. ruber* CICC41233 via ATMT, and ten transformants were obtained. After PCR detection, seven transformants tested positive (Supplementary Figure S3). They were all genetic stability of these engineered strains. After MPs production analysis, and one of the transformants, named *M. ruber* Amy9, was selected and used in the following experiments.

Fig. 1 Growth phenotypes of *M. ruber* CICC41233 and *M. ruber* Amy9. (a: *M. ruber* CICC41233 and *M. ruber* Amy9 were grown on MPS and GM agar medium with 0.2% acetate after 8 days; b: *M. ruber* CICC41233 and *M. ruber* Amy9 were grown on MPs fermentation culture medium)

M. ruber CICC41233 and *M. ruber* Amy9 were grown on MPS and GM medium with 0.2% acetate after 8 days to observe their phenotypic characterization. Results showed that colony phenotypes of the two strains were almost identical, but the surface of mycelium for *M. ruber* Amy9 was more red than that *M. ruber* CICC41233 (Fig. 1a).

Comparison of MPs production in *M. ruber* CICC41233 and *M. ruber* Amy9

The growth phenotypes of *M. ruber* CICC41233 and *M. ruber* Amy9 after 40, 48, and 144 h of fermentation culture are shown in Fig. 1b. The results indicated that the pigment production of strain Amy9 was faster than that of the parent strain CICC41233.

As shown in Fig. 2a, the production of MPs by *M. ruber* CICC41233 and *M. ruber* Amy9 increased with increasing incubation times. Compared with the parent strain, the total MPs production for *M. ruber* Amy9 increased by 46.29 and 21.99% after 48 and 72 h, respectively. However, there was no difference for both strain by statistics analysis ($p > 0.05$). While the total MPs production for *M. ruber* Amy9 was increased by 45.68, 49.05, and 56.39% after 96, 120, and 144 h, respectively, it displayed a highly significant ($p < 0.01$).

The biomasses of both strains are shown in Fig. 2b. After 96 h of cultivation, the biomass of *M. ruber* Amy9 was significantly greater than that of the parent strain CICC41233 by statistics analysis ($p < 0.05$). While compared with 96 h, there was no greatly increase after 144 h, for both strains. However, the biomass of the parent strain CICC41233 was higher than that of *M. ruber* Amy9 for 48 h, but there was no difference by statistics analysis ($p > 0.05$).

Analysis of starch content and determination of the α -amylase activity of *M. ruber*

The supernatants of the culture after centrifugation are shown in Supplementary Figure S4 (a) The samples from *M. ruber* CICC41233 were turbid because of high levels of remaining starch which was 43.93, 17.45, and 7.29 mg/mL at 48, 96, and 144 h, respectively (Fig. 2c), while those from *M. ruber* Amy9 were clear, because the starch was degraded completely. These results can be explained by Supplementary Figure S4 (b) Compared with the control (0.4-mg/mL starch), according to the α -amylase assay kit, the absorbances were higher for *M. ruber* Amy9, and were lower for *M. ruber* CICC41233. The OD₆₆₀ values of AMS are shown in supplementary Table S2. The α -amylase activities were 66.67, 71.79, and 72.55 U/dL after 40, 48, and 144 h, respectively, for *M. ruber* Amy9. However, because of starch

Fig. 2 Analysis of *Monascus* pigment production and biomass with starch content for *M. ruber* CICC41233 and *M. ruber* Amy9. The significance of the difference from *M. ruber* CICC41233 is indicated by asterisks for *M. ruber* Amy9 in each group. * $p < 0.05$, ** $p < 0.01$. Error bars represent SD from triplicate replicates

left for parent strain, the α -amylase activities could not be determined accurately.

To measure the α -amylase activity accurately, the parent strain and *M. ruber* Amy9 were cultivated in glucose medium. In *M. ruber* Amy9, the α -amylase activities were 6.65- and 4.26-fold higher at 72 and 144 h, respectively, than those in the parent strain, which were only 8.40 and 16.05 U/dL (Fig. 3).

Fig. 3 α-amylase activity for *M. ruber* CICC41233 and *M. ruber* Amy9. The strains were cultivated in glucose medium. The significance of the difference from *M. ruber* CICC41233 is indicated by asterisks for *M. ruber* Amy9 in each group. * $p < 0.05$, ** $p < 0.01$. Error bars represent SD from triplicate replicates

Increased expression level of key genes correlates with increased MPs production in *M. ruber* Amy9

First, as determined by RT-PCR with the *actin* gene as an endogenous control, the *AOamyA* gene was expressed in *M. ruber* Amy9 throughout the entire cultivation period, while it was not expressed in the parent strain CICC41233 (Fig. 4a). The expression levels of *acl2*, *pks*, *fasA*, and *fasB* were up-regulated at 72 h and down-regulated at 144 h, compared with those at 48 h, in both *M. ruber* Amy9 and *M. ruber* CICC41233 (Fig. 4b). Compared with the parent strain CICC41233, the expression of *acl2* increased by 1.43-, 1.57-, and 1.33-fold at 48, 72, and 144 h, respectively, in *M. ruber* Amy9 ($p < 0.05$), while the expression of *pks* increased by 2.43-, 2.20-, and 2.4-fold ($p < 0.01$), and the expression of *fasB* increased by 1.39-, 1.80-, and 1.32-fold ($p < 0.01$) (Fig. 4c). The expression of *fasA* decreased by 15.49, 21.48, and 15.14% at 48, 72, and 144 h, respectively, in *M. ruber* Amy9 (Fig. 4c).

Discussion

Starch, the main ingredient in rice, is usually the major carbon source for *Monascus* growth (Patakova 2013; Chen et al. 2015). Although there was a breakthrough in researching the pigment gene cluster in *Monascus* (Feng et al. 2012; Balakrishnan et al. 2013; Chen et al. 2015), to date, there is no report about the relationship between starch degradation and MPs production. Therefore, the α-amylase gene *AOamyA* (Nemoto et al. 2012) was cloned from *A. oryzae* NRRL3488

Fig. 4 Gene expression analysis for *M. ruber* CICC41233 and *M. ruber* Amy9. **a** Lanes 1–8 show the product of the RT-PCRs of the endogenous *actin* gene using the primers *actin*-YGF and *actin*-YGR. Lanes 9–16 show the RT-PCR products of the *AOamyA* gene generated by the primers *amy*-YGF and *amy*-YGR. Lanes 1–4 and 9–12: the *actin* and *AOamyA* gene amplicons of *M. ruber* CICC41233 at 40, 48, 72, and 144 h, respectively; lanes 5–8 and 13–16: the *actin* and *AOamyA* gene amplicons of *M. ruber* Amy9 at 40, 48, 72, and 144 h, respectively. **b** The relative expression levels of *acl2*, *pks*, *fasA*, and *fasB* in *M. ruber* CICC41233 and *M. ruber* Amy9. **c** The relative fold increase in expression of *acl2*, *pks*, *fasA*, and *fasB* in *M. ruber* Amy9. The significance of the difference from *M. ruber* CICC41233 is indicated by asterisks for *M. ruber* Amy9 in each group. * $p < 0.05$, ** $p < 0.01$. Error bars represent SD from triplicate replicates

and transferred into the parent strain *M. ruber* CICC41233 via ATMT to obtain the transformant *M. ruber* Amy9.

RT-PCR showed that the *AOamyA* gene was expressed in *M. ruber* Amy9 (Fig. 4a). It produced a high level of α -amylase activity (Fig. 3), which accelerated starch degradation (Fig. 2c; Supplementary Figure S4a and 4b). It improved the biomass in *M. ruber* Amy9 for 96 h and 144 h (Fig. 2b). The data showed that the biomass of the parent strain CICC41233 was higher than that of *M. ruber* Amy9 for 48 h, because the starch was not consumed completely by the parent strain (43.93 mg/mL at 48 h, Fig. 2c, Supplementary Figure S4 a and 4 b). Therefore, apart from the strain cell, the residual starch was also attributed to the biomass.

Subsequently, it promoted MPs production as well in *M. ruber* Amy9 (Figs. 1a, 2a). However, Puttananjaiah and Dhale (2012) reported that increased amylase activity promoted glucose release from starch at pH 3.0, which led to reduced pigment synthesis in *Penicillium* sp. NIOM-02 because of carbon catabolite repression. In contrast, a transcriptomics analysis by Yang et al. (2015) showed that the expression of the regulatory factor *creA* that mediates carbon catabolite repression in *Monascus purpureus* YY-1 is down-regulated in rice medium.

In this study, we also observed increasing transcript levels of key genes involved in MPs production (Fig. 4b, c, Supplementary Figure S1). The gene *acl2* encoding a subunit of ATP-citrate lyase is required to produce acetyl coenzyme (Hynes and Murray 2010; Chen et al. 2014), which is used as a precursor for MPs synthesis (Hajjaj et al. 2000). The inactivation of the *pks* gene in *M. purpureus* and its deletion in *M. ruber* resulted in the abolishment of pigment production (Balakrishnan et al. 2013; Chen et al. 2015). The *fasA* and *fasB* genes are part of the MPs biosynthetic gene cluster in *M. purpureus* and *M. ruber* (Feng et al. 2012; Chen et al. 2015). The *fasA* involved in the formation of the short-chain fatty acyl thioester for MAZP biosynthesis, while *fasB* in *M. purpureus* is required to modify the azaphilone polyketides during pigment biosynthesis (Balakrishnan et al. 2013, 2014). Compared with the parent strain CICC41233, the expression of *acl2*, *pks*, and *fasA* was up-regulated in *M. ruber* Amy9 (Fig. 4c).

Therefore, the *AOamyA* gene was expressed heterologously in *M. ruber* CICC41233. The starch was fastly hydrolyzed by *M. ruber* Amy9, which meant that the carbohydrate metabolism was quickly contribute to supply metabolic flux for the acetyl-CoA formation, and then led to the up-regulation of expression of key genes in the MPs synthesis cluster and enhanced *Monascus* pigment production.

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Compliance with ethical standards

Conflict of interest The author declares that they have no conflict of interest.

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