The alpha-amylase MrAMY1 is better than MrAMY2 in rice starch degradation, which promotes *Monascus* pigments production in *Monascus ruber*

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Abstract

Previously, *AoamyA*, the alpha amylase-encoding gene from *Aspergillus oryzae*, was heterologously expressed in *Monascus ruber* CICC41233 to promote starch hydrolysis and increase the production of *Monascus* pigments. The target of this study is to screen the effective alpha-amylases from *M. ruber* for starch fast degradation and investigated for *Monascus* pigments production. The 13 types of predicted alpha-amylases in the *M. ruber* NRRL1597 genome were divided into four classes based on EC number and into five groups based on the glycoside hydrolase sub-family. The predicted alpha-amylases MrAMY1 (protein ID 440333) and MrAMY2 (protein ID 324551) showed the closest match with AOamyA by phylogenetic analysis. The genes encoding alpha-amylase, *Mramy1* and *Mramy2*, were cloned from *M. ruber* CICC41233. However, the gene sequence of *Mramy1* from *M. ruber* CICC41233 differed from that of *M. ruber* NRRL1597 in the length of the intron sequence. Furthermore, the *Mramy1*-overexpressed strain *M. ruber* 440333-6A completely degraded the starch of rice grain in 2 d; in contrast, starch (40.32 mg/mL) remained when rice grain was incubated with the *Mramy2*-overexpressed strain, *M. ruber* 324551-D even after 2 d, while 45.43 mg/mL and 10.48 mg/mL starch remained after 2 d and 6 d, respectively, in wild type *M. ruber* CICC41233. Compared to that of *M. ruber* CICC41233, the total *Monascus* pigments and ethanol-soluble pigments in *M. ruber* 440333-6A increased by 71.69% and 119.33% after 6d, respectively; however, it decreased by 21.40% and 26.58% after 6d, respectively, in *M. ruber* 324551-D. This study demonstrated that alpha-amylase MrAMY1 was superior to MrAMY2, as it effectively degraded the starch of rice grain and enhanced *Monascus* pigments production.

Keywords Alpha-amylase · *Monascus ruber* · *Monascus* pigments · Starch degradation

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Introduction

Natural pigments such as *Monascus* pigments (MPs), carotenoids, melanins, flavins, quinones, monascins, violacein, phycocyanin, and indigo are usually derived from microorganisms (Dufossé et al. 2005; Vendruscolo et al. 2016). The MPs manufactured by *Monascus* species have been used worldwide as natural food colorants for over 1000 years, especially in China (Patakova 2013; Chen et al. 2015), as they possess several advantages, such as ease of production from cheap substrates, high bioactive metabolite content, and good solubility in water and ethanol (Mostafa and Abbady 2014; Chen et al. 2015, 2017). Therefore, developing methods of increasing the production of MPs is important. Although improvement of MPs production can be affected by various factors, such as strains, fermentation substrate, nitrogen source, pH, dissolved oxygen, temperature,

and light intensity (Feng et al. 2012; Vendruscolo et al. 2016), genetic engineering can best improve MPs production (Shao et al. 2014; Chen et al. 2015).

Rice grains, which contain abundant starch, are often used to produce MPs (Chen et al. 2015). We have previously reported that the alpha-amylase-encoding gene *AoamyA* of *Aspergillus oryzae* can be heterologously expressed in *Monascus ruber* CICC41233 (Long et al. 2018a). Alpha-amylase activity, starch degradation, and MPs production increased significantly in the transformant (Long et al. 2018a). However, the complete genome sequence of *Monascus purpureus* YY-1 showed that it harbors 13 putative alpha-amylase-encoding genes (Yang et al. 2015). The *M. ruber* NRRL1597 genome database (<https://genome.jgi.doe.gov/Monru1/Monru1.home.html>) also predicted it to contain 13 types of alpha-amylases (protein ID P440333, P324551, P379161, P411620, P435885, P63242, P454978, P460054, P464710, P469192, P469571, P501041, and P472279) from the KOG functional classification for carbohydrate transport and metabolism. In this study, the aim was to screen the effective alpha-amylase from the above predicted proteins for starch degradation and evaluate the effect on MPs production. We introduced a valid method involving phylogenetic tree analysis and molecular experiments to screen an effective alpha-amylase. The alpha-amylase genes (*Mramy1* and *Mramy2*) from *Monascus ruber* CICC41233 were cloned and overexpressed to evaluate the relationship between starch hydrolysis and MPs production. To the best of our knowledge, this is the first study on alpha-amylases from *Monascus* species for the production of MPs.

Materials and methods

Strains

M. 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) medium (Long et al. 2018b). The strains *Escherichia coli* DH5a and *Agrobacterium tumefaciens* EHA105 were used for DNA manipulation.

Phylogenetic tree analyses

The phylogenetic tree was constructed based on the sequences of alpha-amylase *AoamyA* from *A. oryzae* (Long et al. 2018a) and the predicted 13 alpha-amylases from *M. ruber* NRRL1597 (Supplementary Sequence S1) using ClustalX and Mega 4 software.

Vector construction and creation of positive transformants

Mramy1 and *Mramy2* (encoding alpha-amylases) were amplified via polymerase chain reaction (PCR) from the cDNA and total DNA of *M. ruber* CICC41233 as the template. The primers 440333-*Hind* III-F and 440333-*Sac*I-R were used to amplify *Mramy1*, and 324551-*Hind* III-F and 324551-*Sac*I-R were used to amplify *Mramy2* (Supplementary Table S1). Then, *Mramy1* and *Mramy2* were sequenced by BGI, analyzed using BLAST on NCBI database, and aligned with the sequence from *M. ruber* NRRL1597. The plasmid pNeo0380 (Long et al. 2018b) and the gene fragments *Mramy1* and *Mramy2* were digested with *Hind*III and *Sac*I, respectively. Then, the digested gene fragments *Mramy1* and *Mramy2* were ligated to the digested plasmid pNeo0380 to construct the binary expression vector pNeo0380-440333 and pNeo0380-324551, respectively. The vectors pNeo0380-440333 and pNeo0380-324551 were transferred into *M. ruber* CICC41233 via *A. tumefaciens* EHA105. The positive strains were identified using PCR.

Analysis of *Monascus* pigments, enzyme activity, and starch concentration

Five million freshly harvested spores were inoculated into 50 mL medium for *Monascus* pigments fermentation (Long et al. 2018a). The medium component per liter was 90 g rice powder (78.5 g carbohydrate per 100 g rice, 7.8 g protein per 100 g rice, 0.6 g lipid per 100 g rice), 2 g NaNO₃, 1 g KH₂PO₄, 2 g MgSO₄·7H₂O, and 0.2% acetate. The fermentation samples of 2 d and 6 d were analyzed. The water-soluble pigments, ethanol-soluble pigments, and biomass were determined using a published method (Long et al. 2018a, b).

The α-amylase (AMS) assay kit (C016-1-1, Nanjing Jiangcheng Bioengineering Institute, China) was used to assay alpha-amylase activity, and the starch concentration was determined using iodine solution (2.6 g L⁻¹ I₂, 5.0 g L⁻¹ KI) as described previously (Long et al. 2018a).

Gene expression analysis

Quantitative reverse transcription-PCR was performed as described previously (Long et al. 2018a, b). The primer sequences of the control gene *Actin* and alpha-amylase genes *Mramy1* and *Mramy2*, and the key genes *pks* and *mppr1* of the MPs gene cluster are shown in Supplementary Table S1.

Results and discussion

Predicted information regarding alpha-amylases from the *M. ruber* NRRL1597 genome

M. ruber NRRL1597 was predicted to harbor 13 types of alpha-amylases from the KOG functional classification for carbohydrate transport and metabolism (<https://genome.jgi.doe.gov/cgi-bin/kogBrowser?class=G&model=s=1&type=KOG&db=Monru1>) (Table 1). Apart from the protein ID 435885, which belonged to KOG3625, the others belonged to KOG0471. They were divided into four classes, namely, EC:3.2.1.1 (P440333, P324551, P472279), EC:3.2.1.20 (P379161, P411620, P63242, P454978, P460054, P501041), EC:2.4.1.183 (P464710, P469192, P469571), and EC:3.2.1.33 (P435885) based on the EC number. In addition, they were all part of the glycoside hydrolase family 13, but belonged to different sub-families, such as subf1 (P440333, P324551), subf5 (P472279),

subf40 (P379161, P411620, P63242, P454978, P460054, P501041), subf22 (P464710, P469192, P469571), and subf25 (P435885).

Selection of alpha-amylase from *M. ruber* NRRL1597 based on the phylogenetic tree and cloning the target alpha-amylase gene from *M. ruber* CICC41233

Previous reports showed that heterologous expression of *AoamyA* in *M. ruber* CICC41233 accelerated starch hydrolysis and enhanced *Monascus* pigments production (Long et al. 2018a). This indicated that *AOamyA* from *A. oryzae* was an effective alpha-amylase associated with MPs production. We predicted that *M. ruber* NRRL1597 (Supplementary Sequence S1) contains 13 alpha amylases, which is consistent with that observed for *M. purpureus* (Yang et al. 2015). Phylogenetic analysis showed that the protein P440333 (named as MrAMY1) was closest to *AOamyA*

Table 1 The 13 predicted alpha-amylases of *M. ruber* NRRL1597 from the KOG functional classification

Protein ID	EC number	Best hit	CAZy database	Length of gene (bp)	Number of exons
440333	EC:3.2.1.1	gil212,541,000l, alpha-amylase, putative, <i>Penicillium marneffei</i> ATCC 18,224 Identity: 65%	GH13_1 ^a	1706	2
324551	EC:3.2.1.1	gil350,636,595l, alpha-amylase, <i>Aspergillus niger</i> ATCC 1015 Identity: 57%	GH13_1	2145	8
472279	EC:3.2.1.1	gil317,140,381l, alpha-amylase, <i>Aspergillus oryzae</i> RIB40 Identity: 68%	GH13_5	2125	8
379161	EC:3.2.1.20	aor:AO090026000034, alpha-amylase, <i>Aspergillus oryzae</i> Identity: 72%	GH13_40	1860	3
411620	EC:3.2.1.20	afm:Afu3g07380, oligo-1,6-glucosidase, <i>Aspergillus fumigatus</i> Af293 Identity: 79%	GH13_40	1834	2
63242	EC:3.2.1.20	gil242,806,039l, maltase, <i>Talaromyces stipitatus</i> ATCC 10,500 Identity: 65%	GH13_40	1907	1
454978	EC:3.2.1.20	afm:Afu2g11620, oligo-1,6-glucosidase, <i>Aspergillus fumigatus</i> Af293 Identity: 79%	GH13_40	2389	10
460054	EC:3.2.1.20	gil345,564,184l, hypothetical protein AOL_s00097g3, <i>Arthrobotrys oligospora</i> ATCC 24,927 Identity: 48%	GH13_40	1934	3
501041	EC:3.2.1.20	gil119,481,197l, alpha-glucosidase/alpha-amylase, putative, <i>Neosartorya fischeri</i> NRRL 181 Identity: 76%	GH13_40	2295	9
464710	EC:2.4.1.183	gil242,818,376, alpha-1,3-glucan synthase, putative, <i>Talaromyces stipitatus</i> ATCC 10,500 Identity: 57%	GH13_22	7859	11
469192	EC:2.4.1.183	afm:Afu2g11270, alpha-1,3-glucan synthase, <i>Aspergillus fumigatus</i> Af293 Identity: 71%	GH13_22	7595	6
469571	EC:2.4.1.183	aor:AO090010000106, glycogen synthase, <i>Aspergillus oryzae</i> Identity: 80%	GH13_22	7428	4
435885	EC:3.2.1.33	gil119,497,357l, amylo-alpha-1,6-glucosidase, putative, <i>Neosartorya fischeri</i> NRRL 181 Identity: 73%	GH13_25	4761	3

^aThe GH13_1 represented Glycoside Hydrolase Family 13 / Sub-family 1

(79% confidence level) (Fig. 1a). P440333 and AOamyA were closest to the protein P324551 (named as MrAMY2) (Fig. 1a). The results of phylogenetic tree analysis were consistent with the classification of predicted amylase based on glycoside hydrolase sub-family (Table 1).

To further investigate the effect of the protein on starch hydrolysis and MPs production, full length *Mramy1* (encoding protein P440333) and *Mramy2* (encoding protein P324551) were first amplified from *M. ruber* CICC41233 using PCR with DNA and cDNA as templates, respectively, followed by sequencing. *Mramy1* from *M. ruber* CICC41233 possessed an intron from 994 bp to 1050 bp (Fig. 1b, Supplementary Figure S1), while the intron of *M. ruber* NRRL1597 *Mramy1* was from 994 bp to 1037 bp (Fig. 1b, Supplementary Figure S1). The sequence of *Mramy2* from *M. ruber* CICC41233 was similar to the sequence from *M. ruber* NRRL1597. The deduced 548 amino acid sequence of MrAMY1 from *M. ruber* CICC41233 exhibited the closest match to the alpha-amylase (69% identity) of *Rasamsonia emersonii* CBS 393.64 (GenBank Accession No.

XP013323067.1), and also matched with the sequence of taka-amylase A (69% identity) of *A. oryzae* (GenBank accession no. AAA32708.1). The deduced 571 amino acid sequence of MrAMY2 from *M. ruber* CICC41233 exhibited the closest match to the alpha-amylase (64% identity) of *Rasamsonia emersonii* CBS 393.64 (GenBank accession no. XP013325801.1), and also matched with the sequence of taka-amylase A (48% identity) of *A. oryzae* (GenBank Accession No. AAA32708.1).

Alpha-amylase MrAMY1 was superior to MrAMY2 with respect to starch degradation and *Monascus* pigments production

Mramy1 and *Mramy2* were over-expressed in wild type strain *M. ruber* CICC41233, respectively. The transformants named *M. ruber* 440333-6A (*Mramy1* overexpression) and *M. ruber* 324551-D (*Mramy2* overexpression) were verified using PCR and MPs fermentation analysis (data not shown).

M. ruber CICC41233, *M. ruber* 440333-6A, and *M. ruber* 324551-D were simultaneously cultivated on fermentation medium for MPs production. The 2 d and 6 d samples were further analyzed. The alpha-amylase activity was detected using the alpha-amylase assay kit and the absorbance values are shown in Table 2. The alpha-amylase activities of *M. ruber* 440333-6A were 63.98 and 64.01 U/dL after 2 d and 6 d, respectively (Supplementary Figure S2). However, the alpha-amylase activities of *M. ruber* CICC41233 and *M. ruber* 324551-D could not be accurately measure owing to the presence of large starch residues (Supplementary Figure S3). After 2 d and 6 d, 45.43 mg/mL and 10.48 mg/mL starch was left in *M. ruber* CICC41233, respectively (Fig. 2a). In contrast, 0.06 mg/mL starch remained after 2 d and no starch was detected after 6 d in *M. ruber* 440333-6A (Fig. 2a). However, 40.32 mg/mL and 6.73 mg/mL starch remained in *M. ruber* 324551-D after 2 d and 6 d, respectively (Fig. 2a), indicating that the alpha-amylase MrAMY1 was superior to MrAMY2 in starch degradation.

Monascus pigments production did not differ significantly among the three strains after 2d (Fig. 2b). However the total MPs and ethanol soluble pigments for *M. ruber* CICC41233 were 42.34 U and 31.30 U after 6 d, respectively, whereas they were 72.69 U and 68.65 U after 6 d, respectively, for *M. ruber* 440333-6A and 33.28 U and

Fig. 1 Phylogenetic analysis of the amylase from *M. ruber* NRRL1597 (a) and comparative analysis of target alpha-amylase gene sequence from *M. ruber* NRRL1597 and *M. ruber* CICC41233 (b). (a) The amylase AOamyA was from *A. oryzae*. The 13 amylases were from *M. ruber* NRRL1597. The arrow indicates the target protein in this study. (b) The length of the intron of the *Mramy1* gene of *M. ruber* CICC41233 differed from that of *M. ruber* NRRL1597)

Table 2 The absorbance values of α -amylase at 660 nm

Samples	48 h	144 h
Control	0.53	0.498
<i>M. ruber</i> CICC41233	5.33 $\pm$ 0.34	1.29 $\pm$ 0.11
<i>M. ruber</i> 440333-6A	0.09 $\pm$ 0.01	0.03 $\pm$ 0.00
<i>M. ruber</i> 324551-D	4.73 $\pm$ 0.01	0.829 $\pm$ 0.22

Fig. 2 Analysis of starch content (a), *Monascus* pigments production (b), and gene expression fold (c) for *M. ruber* CICC41233, *M. ruber* 440333-6A, and *M. ruber* 324551-D. (The asterisks indicate the differences between *M. ruber* CICC41233, and *M. ruber* 440333-6A and *M. ruber* 324551-D in each group. Error bars represent SD from duplicate replicates. The differences among different treatments were analyzed using one-way ANOVA. * $p < 0.05$, ** $p < 0.01$)

22.98 U for *M. ruber* 324551-D, respectively, after 6 d. Compared to the wild type strain CICC41233, the total MPs and ethanol soluble pigments in strain 440333-6A increased significantly by 71.69% and 119.33%, respectively, after 6d (Fig. 2b), while the total MPs and ethanol soluble pigments in strain *M. ruber* 324551-D decreased by 21.40% and 26.58%, respectively, after 6d (Fig. 2b). This was consistent with the results of our previous study (Long et al. 2018a). Surprisingly, the proportion of ethanol-soluble pigments in total pigments increased from 73.93% in the parent strain CICC41233 to 94.44% in *M. ruber* 440333-6A. This showed that ethanol-soluble pigments account for a large proportion of total pigments. This phenomenon was common in *Monascus* spp. (Kang et al. 2014; Shi et al. 2015; Chen et al. 2017). It also showed that rapid degradation of starch can enhance MPs production and increase the yield of alcohol-soluble pigments. However, due to carbon catabolite repression (CCR), large amounts of glucose were obtained from starch because of increase in amylase activity, which reduced pigments production in *Penicillium* sp. NIOM-02 (Puttananjai and Dhale, 2012). In contrast, the transcription factor *creA* that negatively regulates CCR was down-regulated in *M. purpureus* YY-1 for MPs fermentation (Yang et al. 2015).

At the same time, *Mramy1* of *M. ruber* 440333-6A was up-regulated by 4.03- and 1.88-fold compared to that of the wild type strain after 2 d and 6 d, respectively (Fig. 2c). This showed that it mainly contributed to the alpha-amylase activity and starch degradation. Similar result was obtained in our previous report where *AoamyA* was expressed heterologously in *M. ruber* (Long et al. 2018a). Although *Mramy2* of *M. ruber* 324551-D was up-regulated by 43.84- and 90.53-fold compared to that of the wild type strain after 2 d and 6 d, respectively (Fig. 2c), *M. ruber* 324551-D degraded starch only slightly (Table 2, Supplementary Figure S3).

Compared to the wild type strain CICC41233, the expression levels of *pks* (encoding polyketide synthase) and *mppr1* (encoding positive regulatory factor in the MPs biosynthetic gene cluster) in *M. ruber* 440333-6A increased by 4.95- and 1.42 fold, respectively, after 2 d, and by 16.81- and 1.46 fold, respectively, after 6 d (Fig. 2c). The expression levels of *pks* and *mppr1* in *M. ruber* 324551-D decreased by 75.03% and 92.54%, respectively, after 2 d; *pks* expression increased by 38.12-fold and *mppr1* expression decreased by 64.46% after 6 d (Fig. 2c). The *pks* and *mppr1* deletions (Supplementary Figure S4) resulted incomplete loss in the ability to produce pigments (Balakrishnan et al. 2013; Xie et al. 2013; Chen et al. 2015).

Therefore, *Mramy1* was overexpressed in *M. ruber* CICC41233, which enhanced amylase activity and accelerated starch hydrolysis. This implied that the supply of metabolic flux contributed to MPs production.

Conclusions

In this study, 13 types of predicted alpha-amylases in the *Monascus ruber* NRRL1597 genome were divided into four classes based on the EC number and five groups based on the glycoside hydrolase sub-family. Phylogenetic analyses showed that the proteins MrAMY1 (P440333) and MrAMY2 (P324551) among these 13 types of alpha-amylases of *M. ruber* NRRL1597 were closest to AOamyA. Data showed that the alpha-amylase MrAMY1 of *M. ruber* CICC41233 was superior to MrAMY2 in terms of starch degradation and *Monascus* pigments production.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests.

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