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NOTE

Monascus Red Pigment Overproduction by Coculture with Recombinant *Saccharomyces cerevisiae* Secreting Glucoamylase

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In liquid cultures using sucrose media, the coculture of *Monascus* with recombinant *Saccharomyces cerevisiae* expressing the glucoamylase gene from *Aspergillus niger* enhanced red pigment production by approx. 19%, compared with the coculture of wild type *S. cerevisiae*. Coculture with recombinant *S. cerevisiae* was more effective than with wild type *S. cerevisiae* for *Monascus* red pigment production. Cocultures of *Monascus* with commercial amylases of *Aspergillus* also induced high production of pigment and morphological changes in a solid culture using sucrose media.

Key words: Coculture, *Monascus*, glucoamylase, *Saccharomyces cerevisiae*

The filamentous fungus *Monascus* belongs to the class *Ascomycetes* and the family *Monascaceae*, and reproduces both sexually and asexually (5). This fungus has traditionally been utilized in east Asia for making red rice wine, red soy bean cheese and anka (red rice) (16). The pigments produced by *Monascus* have been used as food colorants and meat disinfectants (16), and also as medicinal agents to relieve several diseases including indigestion, dysentery, muscle bruises, pain, and anthrax (17). Recently, some synthetic colorants have been identified as carcinogenic, teratogenic and allergenic agents and efforts to replace them with natural *Monascus* pigments have attracted worldwide attention.

Monascus produces at least six major pigments. The red pigments are monascorubranmin (2) and rubropunctamine (4), yellow pigments are ankaflavin and monascin (12, 13), orange pigments are monascorubin (2) and rubropunctatin (4). Moreover, two yellow pigments have been discovered recently. These recently discovered yellow pigments are yellow and xanthomonascin (14, 18). These pigment compounds are heat-stable and can be used in a wide range of pH (11). And they also have different solubility to various solvents. The composition ratios and productivity of pigment can be influenced by the *Monascus* strain, medium composition, and fermentation conditions in which they are produced (9). Therefore, many studies have attempted to enhance the production of *Monascus* pigments through strain improvement, carbon source change, and improvement of culture conditions.

In this study, *Monascus* sp. J101 (8) in which pigment productivity was enhanced by UV mutagenesis, was used to enhance red pigment production to levels sufficient for industrial application. Ju *et al.* (7) reported that when *Monascus* sp. J101 was cocultured with *Saccharomyces cerevisiae* (*S. cerevisiae*), an increase in the cell mass of *Monascus* occurred together with changes in cell morphology, and pigment production had significantly increased. In addition, Kim (10) and Shin *et al.* (15) showed that the effects caused by coculture were also accomplished by the addition of a *S. cerevisiae* culture filtrate, and the factors which stimulated pigment production were found to be chitinase and glucoamylase which were secreted outside of *S. cerevisiae* cells. These hydrolytic enzymes were thought to cause a change in cell morphology and enhancement of pigment production by degrading *Monascus* cell walls. An explanation of why hydrolysis of the cell walls causes these effects in *Monascus* can be considered as a defense mechanism. When the walls of *Monascus* cells suffer from hydrolysis, *Monascus* cells may overproduce hydrophobic substances, such as pigments for blocking these enzymatic attacks (15).

Based on previous results, we investigated whether red pigment production can be increased by coculturing *Monascus* with recombinant *S. cerevisiae* expressing the

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glucoamylase gene from *Asp. niger*, which enhances *Monascus* pigment production.

For this study, *Monascus* sp. J101 (8) was used to produce *Monascus* pigments. *S. cerevisiae* EY957 (MATa, *ura*3-1, *leu*2-3,112 *trp*1-1 *his*3-11) was used as a recombinant host in the expression and secretion of glucoamylase from *Aspergillus niger var. macrosporus*. The 2μ yeast-based recombinant plasmid containing the glucoamylase gene fused with the TP1 promoter was transformed into the host by the LiAc/ss-DNA/PEG procedure (1). This transformant was used as the coculture strain.

Monascus pigment production and cell morphology changes on plates treated with commercial amylases

In order to investigate the effect of commercial amylases (Sigma) on *Monascus* pigment production and cell morphology, solid cultures of *Monascus* sp. J101 using commercial amylases were performed with Hiroi agar medium also called sucrose agar medium (6). The medium components were as follows: 10% sucrose, 0.3% yeast extract, 0.5% casamino acid, 0.2% $NaNO_3$, 0.1% KH_2PO_4, 0.05% $MgSO_4 \cdot 7H_2O$, 0.05% KCl, 0.001% $FeSO_4$, and 2% agar. *Monascus* sp. cells grown on sucrose agar medium plates (10 cm diameter) for 2 days at 30°C, were further cultivated for 5 more days at 30°C after treatment with 1.5 ml of *Bacillus* sp. α-amylase, *A. oryzae* α-amylase and *A. niger* glucoamylase, respectively (Fig. 1). *Monascus* cells grew in thin layers where no other amylase was added (control), as seen in Fig. 1A. The pigment production and morphology of *Monascus* cells grown after the addition of α-amylase from *Bacillus* sp. varied slightly compared with the control (Fig. 1B). However, significant changes in pigment production and *Monascus* cell morphology occurred when the α-amylase and glucoamylase of *Aspergillus* were applied to *Monascus* cells (Fig. 1C and 1D). Enhanced cell growth and pigment production occurred together with changes in cell morphology.

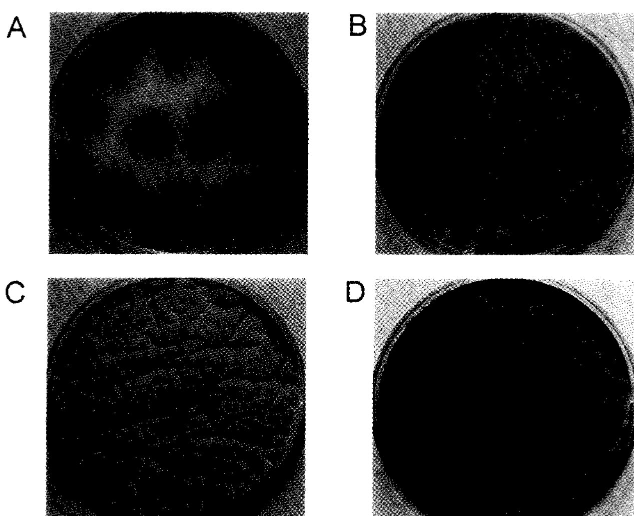

Fig. 1. *Monascus* colonies in cultures with amylases from different microorganisms. A: *Monascus* only, B: *Monascus* with the α-amylase of *Bacillus* sp., C: *Monascus* with the α-amylase of *A. oryzae*, D: *Monascus* with the glucoamylase of *A. niger*.

Measurement of glucoamylase activity

To measure glucoamylase activity, wild type *S. cerevisiae* and recombinant *S. cerevisiae* were grown in YPD (2% dextrose, 1% yeast extract, and 2% peptone; pH 6.6) and uracil-free liquid medium (2.67% minimal SD base, 0.077% ura dropout (DO) supplement; pH 5.8), respectively for 72 h. Each culture broth including the same number of cells was centrifuged, and the glucoamylase activity of the supernatant (0.5 ml) was examined as follows. The culture supernatant (0.5 ml) was incubated in 9.5 ml of 1.5% soluble starch in 0.2 M acetate buffer (pH 5.5) at 40°C for 40 min, then boiled at 100°C for 10 min to inactivate glucoamylase. Glucose produced by the action of glucoamylase on soluble starch was assayed using the Somogyi-Nelson method (19). Four hundred microliter of alkaline copper reagent (blue) was added to 400 μl of the reaction solution, boiled at 100°C for 10 min, and then cooled. Four hundred microliter of Nelson's Reagent (yellow) was added to the mixture and vortexed. Finally, absorbance at 520 nm was measured using a spectrophotometer (UV-1201, Shimadzu). One unit of glucoamylase activity was defined as the amount of enzyme required to release 1 μmol of glucose per min from starch.

It appeared that wild type *S. cerevisiae* had low activity (0.6 units/ml) due to glucose and other molecules from the medium present in the supernatant. On the other hand, the recombinant *S. cerevisiae*, showed approx. 6 times higher than the activity of wild type *S. cerevisiae* (3.75 units/ml).

Monascus red pigment production on sucrose medium

For the production of red pigment, *Monascus* was cultured in a liquid medium containing sucrose as a carbon source with the addition of culture broth of *S. cerevisiae*. Before this liquid coculture, seed culture and main culture were performed. For seed culture, 75 ml of Mizutani medium (5% glucose, 2% peptone, 0.8% KH_2PO_4, 0.05% $MgSO_4 \cdot 7H_2O$, 0.2% CH_3COOK, and 0.1% NaCl; pH 6.6) (10) in 500 ml Sakaguchi flasks was inoculated from agar slants of *Monascus* sp. J101. Cultivation proceeded for 48 h on a reciprocal shaker (32-WBS-40, International science) at 30°C and 120 rpm.

For main culture growth, liquid cultures were performed in Hiroi medium (liquid sucrose medium). Seventy five mililiter of liquid sucrose medium in 500 ml baffled flasks was inoculated with 3.75 ml of seed broth and cultivated for 96 h on a rotary shaker (HM-90R, Hanil) at 30°C and 180 rpm.

Finally for liquid coculture, 10 ml of wild type or recombinant *S. cerevisiae* culture broth was added to 500 ml baffled flasks containing 75 ml of Hiroi medium inoc-

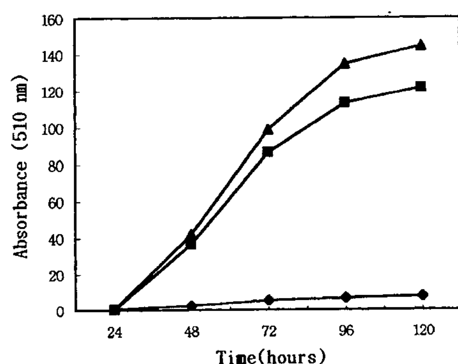

Fig. 2. Time course of red pigment production on sucrose medium. Each culture broth from a sucrose medium was treated with 95% ethanol for 1 h to extract red pigments. The red pigment concentration was measured by a spectrophotometer at a 24 h interval using cultures consisting of (◆) *Monascus* only, (■) *Monascus* with wild type *S. cerevisiae*, (▲) *Monascus* with recombinant *S. cerevisiae*.

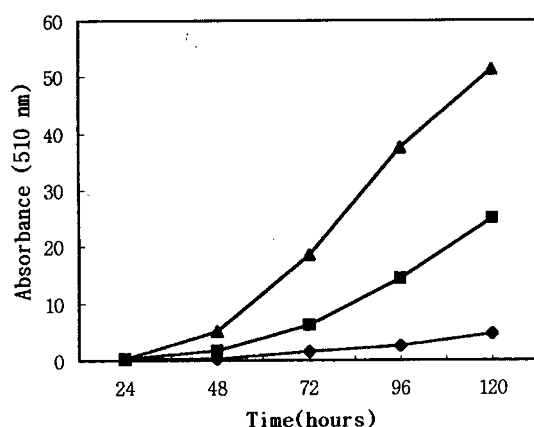

Fig. 3. Time course of red pigment production on uracil-free medium. Culture broth from a uracil-free medium was treated with 95% ethanol for 1 h to extract the red pigment. The concentration of the pigment was measured using a spectrophotometer at an interval of 24 h of cultures grown with (◆) *Monascus* only, (■) *Monascus* with wild type *S. cerevisiae*, (▲) *Monascus* with recombinant *S. cerevisiae*.

ulated with *Monascus* at 24 h of cultivation. Cultivation was then continued for another 96 h. For pigment extraction from liquid culture at intervals of 24 h. 10 ml of culture broth and 20 ml of 95% ethanol were placed into a 250 ml Erlenmeyer flask at a final ethanol concentration of approx. 63% and extracted for 1 h on a shaker (HM-90R, Hanil) at 30°C and 180 rpm (15). The resulting solutions were centrifuged to remove cells and pigment solutions were obtained. Absorbance at 510 nm was measured using a spectrophotometer (UV-1201, Shimadzu) to determine red pigment concentration (7), with fresh medium used as the negative control. Pigment concentration was presented as OD_{510} values multiplied by each dilution factor.

Pigment production seemed remarkably enhanced in both cocultures with wild type *S. cerevisiae* and recombinant *S. cerevisiae*, compared to the control with *Monascus*. As shown in Fig. 2, in a liquid sucrose medium with added culture broth red pigment production had enhanced approx 19% for recombinant *S. cerevisiae* than for wild type *S. cerevisiae*. Furthermore, pigment coproduction of recombinant *S. cerevisiae* had enhanced approx 19 times compared to the *Monascus* control.

The main problem with using coculture in a liquid culture is that pigment production can decrease because *S. cerevisiae* cells can outgrow *Monascus* cells when the inoculation time of *S. cerevisiae* is too early, or the inoculum size is too large. If the inoculum size and times of *S. cerevisiae* culture broths can be optimized in fed-batch cultures for red pigment production, pigment yields can be improved.

***Monascus* red pigment production on uracil-free medium**

In order to overcome the problems involved in plasmid retention and glucoamylase secretion of recombinant *S. cerevisiae* (3), red pigment production was performed in uracil-free cultures. Seed culture, main culture and liquid coculture were performed by the same procedure as cultures in a sucrose medium. As shown in Fig. 3, in a liquid uracil-free medium red pigment production had enhanced approx 35% for recombinant *S. cerevisiae*, compared with the wild type *S. cerevisiae*. However, when culture broths were added, total red pigment productivity in a uracil-free medium was significantly lower than in the sucrose medium as shown in Fig. 2 and 3. Total red pigment production in a uracil-free medium was lower than in a sucrose medium due to the fact that the uracil-free medium is not optimized for the growth of *Monascus*. To resolve this problem a vector with a selectable marker, expressed without the inhibition of *Monascus* growth, should be used.

We are currently investigating processes by which glucoamylase or yeast cell induces *Monascus* red pigment production.

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