# Enhancement of monacolin K production via intergeneric protoplast fusion between *Aspergillus terreus* and *Monascus anka*

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**Abstract** Intergenric protoplast fusion between *Aspergillus terreus* CA99 and *Monascus anka* M-3, the high and low producers of monacolin K respectively, was performed for enhancement of monacolin K production. The 24-hour-old mycelia of *A. terreus* CA99 and *M. anka* M-3 were treated with 0.5% lywallzyme, 0.3% snailase and 0.3% cellulase at 34℃ for 5 h and at 30℃ for 3.5 h, and their protoplasts formation reached $1.76\times10^7$/mL and $1.68\times10^7$/mL respectively. Parental protoplasts were irradiated with a 30 W UV light away from 30 cm for 3 min and then mixed. The mixture was incubated with 30% PEG 6000 for 15 min. The reviving fusants were isolated on the regeneration plates. Of the 363 fusants isolated, over 100 showed enhanced monacolin K production compared with the parental strain *M. anka* M-3. Ten of them produced monacolin K about 1.6-fold of that *M. anka* M-3 does and the monacolin K titer of two fusants (F49 and F104) increased by about 1-fold. The monacolin K yields of F49 and F104 were 460 μg/mL and 457 μg/mL respectively. In optimized fermentation medium, the monacolin K titer of F49 reached 1216 μg/mL.

**Keywords:** monacolin K, *Monascus anka*, *Aspergillus terreus*, protoplast fusion, fermentation.

Monacolin K, also known as mevinolin or lovastatin, is an inhibitor of the enzyme hydroxymethylglutaryl coenzyme A reductase (HMG-CoA reductase) that catalyzes the reduction of HMG-CoA to mevalonate during synthesis of cholesterol[1]. It has potential as a therapeutic agent for hypercholesterolemia[2]. *Aspergillus terreus* and *Monascus* are the two main microorganisms used for industrial production of monacolin K[2,3]. *Monascus* is commonly used in oriental fermented food as a natural food colorant and preservative or as a medical agent with a history over several thousands years. *Monascus* can produce many secondary metabolites such as monacolin K, red pigment and citrinin[4]. Recently, it has been drawn world-wide attention that *Monascus* can be used as a supplement in functional foods or nutraceuticals as a therapeutic agent for hypocholesterolemia[5,6].

Classical mutagenesis technique is a very effective way to improve monacolin K production in *Monascus*, but it is very laborious and time-consuming. Protoplast fusion was used widely for strain improvement in microorganisms especially in filamentous fungi. There have been many reports describing that intra- and interspecific fusions have led to phenotypic improvement in fungi[7—10]. Due to the importance of monacolin K in medicine and edibility and safety of *Monascus*, this work attempted to carry out an intergeneric protoplast fusion between *Aspergillus terreus* CA99 with high yield of monacolin K and *Monascus anka* M-3 with low yield of monacolin K to attain monacolin K high producing strain. In this paper, we report monacolin K and red pigment production of the fusants by intergeneric protoplast fusion between the two strains. To our knowledge, this is the first report to improve monacolin K production by intergeneric protoplast fusion.

## 1 Materials and methods

### 1.1 Fungal strains and conidiospore suspensions

*Aspergillus terreus* CA99[11], a high producer of monacolin K, and *Monascus anka* M-3 which produces red pigment and low level of monacolin K, were all collected in this laboratory. All strains were maintained on their corresponding spore-forming agar medium and transferred monthly. Conidiospores from 7-day-old agar slants were harvested and stored at 4℃ and utilized as stock inoculum.

### 1.2 Media

Spore-forming media for *Monascus anka* contained (per liter): 10 g of glucose, 5 g of peptone, 3

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g of beef extract, 5 g of NaCl, 18 g of agar, at pH 7.0. The spore-forming media for *Aspergillus terreus* contained (per liter): 4 g of glucose, 4 g of yeast extract, 10 g of malt extract, 18 g of agar, at pH 7.0.

Protoplasting medium (PM) for the pre-growing strains for protoplasting contained (per liter): 100 g of glucose, 10 g of peptone, 2 g of $KNO_3$, 2 g of $NH_4H_2PO_4$, 0.5 g of $MgSO_4 \cdot 7H_2O$, 0.1 g of $CaCl_2$, at pH 6.8.

Regeneration medium was the supplemented protoplasting medium with 206 g sucrose, 8 g of sodium deoxycholate and 20 g of agar.

Seed medium and fermentation medium were used for monacolin K production in *Monascus anka* and fusants. Seed medium (per liter) was composed of 10 g of glucose, 15 g of peptone, 2 g of $NaNO_3$, 1 g of $MgSO_4 \cdot 7H_2O$, at pH 6.8. Fermentation medium (per liter) contained 50 g of glucose, 10 g of yeast extract, 20 g of tomato paste, and 1 g of $CaCO_3$.

### 1.3 Protoplast fusion

Preparation of protoplast: 100 mL PM were inoculated with stock spore suspensions of *Aspergillus terreus* CA99 or *Monascus anka* M-3 and incubated overnight at 30 ℃ on a rotary shaker (180 r/min). The mycelium was harvested by mesh sieve (mesh count, 250), washed twice with sterile distilled water; resuspended in enzyme mixture (0.5% lywallzyme, 0.3% snailase and 0.3% cellulase) with the volume of 1 mL per 150 mg wet mycelium, and then incubated at 34 ℃ for 5 h (*Aspergillus terreus* CA99) or at 30 ℃ for 3.5 h (*Monascus anka* M-3) respectively with gentle shaking. The released protoplasts were photographed using a phase-contrast microscope, filtrated through four-layer sterile lens cleaning tissue to get rid of mycelium and harvested by centrifugation (2000 *g*, 5 min). The pellet of protoplast was washed twice with PBS (0.2 mol/L phosphate buffer, pH 5.8, containing 0.7 mol/L NaCl), and finally resuspended in 5 mL PBS. The number of protoplasts was counted under the microscope.

Protoplast fusion: Parental protoplasts for protoplast fusion were both inactivated by UV irradiation as follows: 5 mL of each parental protoplast suspension was adjusted to approximately the same turbidity and irradiated with a 30 W UV light 30 cm away for 3 min. After irradiation, parental protoplasts were mixed and harvested by centrifugation (2000 *g*, 5 min). The protoplast pellet was resuspended in 1.5 mL 30% PEG 6000 containing 50 mmol/L $CaCl_2$ and 50 mmol/L glycine, and incubated for 15 min at 30 ℃. Afterwards, PEG-treated protoplast suspension was diluted in PBS to an appropriate concentration, and 0.1 mL of that was plated on the regeneration medium plates and incubated for 4—6 days at 30 ℃. Colonies growing on these plates were considered as fusants.

### 1.4 Fermentation[11]

The 50 mL seed media were inoculated with stock spore suspensions and incubated for 48 h at 30 ℃ on a rotary shaker (220 r/min), and 10 mL of seed culture were then inoculated into 100 mL fermentation medium in 500-mL fermentation flasks and incubated for 9 days at 30 ℃ on a rotary shaker at 220 r/min.

### 1.5 HPLC analysis of monacolin K

For HPLC analysis, 4 mL methanol was added to 1 mL culture broth and stirred for 1 h to extract monacolin K. The extraction was centrifuged at 3500 r/min for 10 min and the supernatant was directly applied to HPLC (Waters 600E Chromatogram) for monacolin K detection[11]. The column packed with C-18 (10 μm; 4.6 i.d. mm × 150 mm) was developed with 18 mmol/L $H_3PO_4$/methanol (22.5/77.5, v/v) at 35 ℃, and the flow rate was 1.0 mL/min. Monacolin K was detected by UV absorption at 237 nm.

### 1.6 Pigment detection

For pigment determination, 1 mL of culture broth was mixed with three volumes of 95% ethanol. Extraction was performed for 1 h on a shaker at 30 ℃, and then cells were removed by centrifugation (3500 r/min, 10—15 min). The produced pigments were measured[12] using a spectrophotometer (UV-1000, Labtech Inc) at opticaldensity values of 510 nm and 410 nm.

### 1.7 Genetic stability of the fusants

To determine whether fusants were genetically stable, the fusants with improved monacolin K production were propagated for 5 generations on Spore-

forming media. For every generation, shaking flask fermentation was carried out and monacolin K titer and red pigment production of fusants were measured.

## 2 Results

### 2.1 Optimization of protoplast preparation and regeneration in *Aspergillus terreus* and *Monascus anka*

Four factors (mycelium age, enzyme treatment time, enzyme treatment temperature and lytic enzyme) were examined to determine the optimal condition for protoplast preparation and regeneration in *Aspergillus terreus* and *Monascus anka*, which is shown in Table 1. Under these optimal conditions, parental mycelia were almost completely enzymatically lysed to form protoplasts (Fig. 1). And the regeneration rates of *Aspergillus terreus* and *Monascus anka* on the regeneration medium were 18.6% and 11.9% respectively.

Table 1. The optimal condition for protoplast preparation and regeneration in *A. terreus* and *M. anka*

| Strains | Mycelia age (h) | Temperature (℃) | Enzyme treatment time (h) | Proportion of enzyme mixture | Protoplast formation[a)] | Regeneration rate (%) |
|---|---|---|---|---|---|---|
| *A. terreus* CA99 | 24 | 34 | 5 | 0.5% lywallzyme, 0.3% snailase and 0.3% cellulase | $1.76\times10^7$ | 18.6 |
| *M. anka* M-3 | 24 | 30 | 3.5 | 0.5% lywallzyme, 0.3% snailase and 0.3% cellulase | $1.68\times10^7$ | 11.9 |

a) The number in 1 mL.

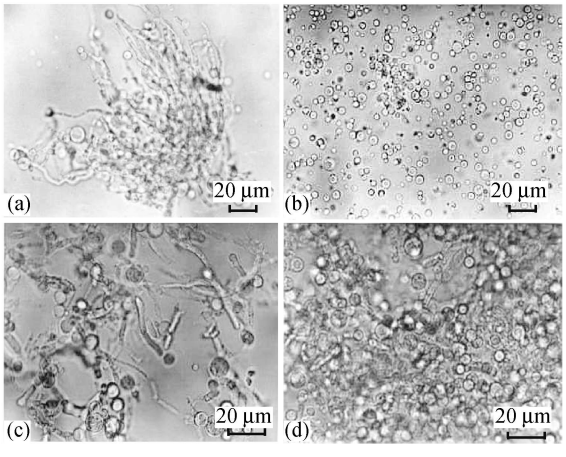


Fig. 1. Photomicrographs depicting protoplast formation. (a) The mycelia of *A. terreus* were treated with enzyme mixture for 1 h; (b) mycelia of *A. terreus* were treated with enzyme mixture for 5 h; (c) mycelia of *M. anka* were treated with enzyme mixture for 1 h; (d) mycelia of *M. anka* were treated with enzyme mixture for 3.5 h.

### 2.2 Protoplast fusion

To select the fusants with the expected genotype is a key step for protoplast fusion. Auxotrophic marker or drug resistance marker was the common way with the merit of facility to select fusants[9,10]. However, the problem is it is time-consuming and labor-intensive to obtain auxotrophic mutants or drug resistance mutants, and some of parental genotype may change during auxotrophic mutant preparation. Another way to increase the frequency of fusants recovered from protoplast fusion involves inactivating the parental protoplasts by UV light or heat treatment immediately before fusing them[13]. UV irradiation introduces lethal hits into the parental chromosomes, so the single (unfused) protoplasts lose viability on the regeneration medium. To increase the frequency of fusants recovered from protoplast fusion, we used UV radiation method to inactivate parental protoplasts immediately before fusing them. The purified parental protoplasts were irradiated with a 30 W UV light 30 cm away for 3 min to achieve more than 99.99% inactivation (Table 2). Of the 5 mL of each inactivated parental protoplast suspension were mixed, treated with 30% PEG 6000 containing 50 mmol/L $CaCl_2$, and incubated for 15 min at 30℃. Afterwards, PEG-treated protoplast suspension was diluted in PBS to an appropriate concentration, and then was plated on regeneration medium plates. Totally 363 single colonies were isolated and screened from these plates.

Table 2. UV-inactivation results of the parental protoplast

| UV irradiation time (min) | Inactivation frequency of protoplast (%) | |
|---|---|---|
| | *A. terreus* CA99 | *M. anka* M-3 |
| 1 | 99.61 | 99.84 |
| 2 | 99.96 | 99.98 |
| 3 | 99.994 | 99.997 |

### 2.3 Culture characteristic of fusants

Parental strain *A. terreus* CA99 formed a layer of light yellowish brown dusty spores in slant, and produced a little of light brown pigment; *M. anka* M-3 grew red villiform spores in slant, and produced

a lot of red pigments. We chose the single colonies that resembled parental strain *M. anka* genotypes. On the regeneration plates, these fusants had the colors from orange to red which were between the colors of their parents; unlike their parental strains, these fusants had an irregular colony form. At the early growth phase, the mycelia of fusants were orange; at the late growth phase, the mature colonies formed dustily compact surfaces which were difficult to pick up with the inoculate needle and the margin of them were white; the colonies produced abundant red pigments (Fig. 2).

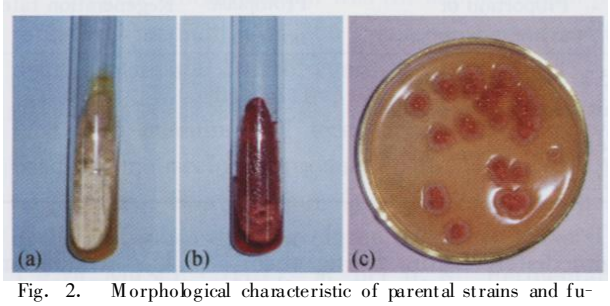


Fig. 2. Morphological characteristic of parental strains and fusants. (a) *A. terreus* CA-99; (b) *M. anka* M-3; (c) fusants. Photos were taken when colonies were grown on regeneration medium for 72 h, and floury spores were shown on the surface of the mature colonies with elongation of growth time.

Culture broth of *A. terreus* CA99 in fermentation medium looked brown, and culture broth of *M. anka* M-3 gave a red color. The culture broths of the fusants gave different extent of red color from light to dark red (Fig. 3). In fermentation medium, these fusants and their parental strains showed many differences in culture broth color, mycelia aggregation and monacolin K production. These fusants could be classified into three main kinds: one kind had dark red culture broth, and mycelia in culture broth formed uneven small grains, in which the monacolin K production was at the equal level of parental strain *M. anka* M-3, and this kind of fusants might be the fusants of *M. anka* M-3 itself; the second class had orange culture broth, and the mycelia formed uniform small grains, in which monacolin K production was higher than parental strain *M. anka* M-3; the third kind with light red culture broth, and mycelia did not form grains, in which the monacolin K production was much lower than that of parental strain *M. anka* M-3 (Fig. 3).

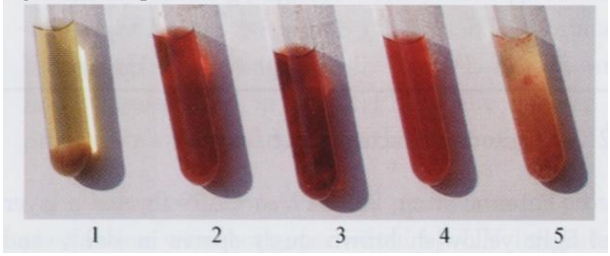


Fig. 3. Extraction of parental and fusants' s culture broth with methanol. 1, *A. terreus* CA-99; 2, *M. anka* M-3; 3 and 5, fusants; 4, monacolin K over producing fusant.

## 2.4 Monacolin K production and pigment production of fusants

Shaking flask experiment showed that among the 363 fusants isolated, 105 produced more monacolin K than parental stain *M. anka* M-3. The fusants were transferred on spore-forming medium for several rounds of sporulation to test their genetic stability. After three rounds of tranferring, 19 fusants still had a higher monacolin K production rate than *M. anka* M-3 and 10 of them showed an increased production by 60% when compared to the parental strain *M. anka* M-3, and for two strains, F49 and F104, their production of monacolin K even doubled (Table 3).

Table 3. Monacolin K and red pigment productions of fusants

| Strains | Monacolin K yield ($\mu g \cdot mL^{-1}$) | Percent | Pigment production | |
|---|---|---|---|---|
| | | | $OD_{510\,nm}$ | $OD_{410\,nm}$ |
| *M. anka* M-3 | 237 | 100 | 14.50 | 28.10 |
| *A. terreus* CA99 | 910 | 384 | 0 | 0 |
| F49 | 460 | 194 | 15.13 | 23.30 |
| F104 | 457 | 193 | 13.25 | 14.93 |
| F66 | 438 | 185 | 11.24 | 11.64 |
| F126 | 434 | 183 | 7.41 | 8.79 |
| F245 | 433 | 183 | 8.21 | 9.31 |
| F270 | 412 | 174 | 8.82 | 10.03 |
| F189 | 407 | 172 | 3.77 | 4.53 |
| F122 | 386 | 163 | 16.50 | 30.88 |
| F52 | 385 | 163 | 11.90 | 12.40 |
| F202 | 383 | 162 | 9.64 | 10.00 |

Pigment production of these fusants was also measured using a spectrophotometer at wave lengths of 510 and 410 nm (Table 3). Most fusants produced less pigment than parental strain *M. anka* M-3, while F122 produced more pigment than *M. anka* M-3. So F122 may be a prospective strain with superior productivity in both red pigment and monacolin K in industry.

In addition, to improve monacolin K production in F49, the carbon source, nitrogen source and mineral element were examined to optimize fermentation medium. The result of the orthogonal experiment showed that the optimal production medium (FMA) was compose of 10% glucose, 0.5% beef extract, 0.25% $(NH_4)_2SO_4$, 2% tomato paste, 0.1%

$CaCO_3$, 0.1% $MgSO_4$, at pH 6.5. The monacolin K yield in *M. anka* M-3 and F49 were 796 μg/mL and 1216 μg/mL in FMA respectively (Table 4).

Table 4. Monacolin K production of fusant 49 and *M. anka* M-3 in optimized fermentation medium

| Strains | Monacolin K production ($\mu g \cdot mL^{-1}$) | |
|---|---|---|
| | FM | FMA |
| *M. anka* M-3 | 237 | 796 |
| F49 | 460 | 1216 |

## 3 Discussion

To improve monacolin K production in *Monascus anka*, intergeneric protoplast fusion between *Aspergillus terreus* CA99, a high producer of monacolin K, and *Monascus anka* M-3 with low yield of monacolin K was performed. A total of 363 fusants were isolated from inactivated protoplast fusion, 19 of them produced more monacolin K than parental strain *Monascus anka*; even after four generations, they maintained the ability to produce red pigment. Though the protoplast fusion between *Monascus anka* and *Aspergillus terreus* was intergeneric, the fact that the monacolin K production of fusants increased by 1-fold indicated that intergeneric protoplasts fusion in filamentous fungi might be an effective way for genotype improvement. In this investigation, the parental protoplasts were both inactivated, so the fusants obtained from protoplast fusion included fusants formed from single strain in addition to the expected fusants. *A. terreus* CA99 formed a light yellowish brown dusty spores layer in slant, and produced a little of light brown pigments; *M. anka* M-3 formed a red villiform spore layer in slant, and produced a lot of red pigments. Taking advantage of culture characteristic difference of the parental strains, we distinguished the expected fusants from the other fusants formed from single strain by their pigment production. We selected the single colonies with the orange colors and obviated the single colonies with brown color (might be fusants from *A. terreus* itself) and dark red color (might be fusants from *M. anka* itself) for further monacolin K production.

The fungi *Monascus* species are known as the sources of various secondary metabolites including monacolin K, red pigment and other active compounds. Due to edibility and safety of *Monascus*, it has been used to produce red pigments and monacolin K. In addition to that, *Monascus* was also used to develop functional foods or nutraceuticals as a therapeutic agent for hypocholesterolemia, such as *Monascus* vinegar, *Monascus* wine and *Monascus* meat. Using the intergeneric protoplast fusion method, we have successfully obtained several genetically stable fusants with increased monacolin K production. Thus, they are very promising strains for developing functional food. However, more researches have to be done before the fusants can be used for industrial applications.

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