Biol Res 44: 377-382, 2011

Elevated yield of Monacolin K in *Monascus purpureus* by fungal elicitor and mutagenesis of UV and LiCl

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ABSTRACT

In China, *Monascus* spp., a traditional fungus used in fermentation, is used as a natural food additive. *Monascus* spp. can produce a secondary metabolite, monacolin K namely, which is proven to be a cholesterol-lowering and hypotensive agent. Hence, recently, many researchers have begun focusing on how to increase the production of monacolin K by *Monascus purpureus*. In the present study, we investigated the effect of the fungal elicitor and the mutagenesis of UV & LiCl on the amount of monacolin K produced by *Monascus purpureus*. The fugal elicitor, *Sporobolomyces huaxiensis*, was isolated from tea leaves and its filtrate was added into the culture filtrate of *Monascus purpureus* during growth to induct the production of monacolin K. The results showed that the highest amount of monacolin K produced by the liquid fermentation was 446.92 mg/mL, which was produced after the fungal elicitor was added to the culture filtrate of *Monascus purpureus* on the day 4; this amount was approximately 6 times greater than that of the control culture filtrate, whereas the highest amount of monacolin K produced by the mutated strain was 3 times greater than the control culture after the irradiation of UV light in the presence of 1.0 ‰ LiCl in the medium.

Keywords: *Monascus* spp., monacolin K, fungal elicitor, mutagenesis.

INTRODUCTION

Monascus spp., a traditional fermentation fungus, has been used as natural food and as a food additive in China for thousands of years (Zlov et al., 1996; Heber et al., 1999). In addition, red yeast rice is a proprietary scientifically natural form of *Monascus* yeast fermented on rice and is a traditional Chinese health food. The health-enhancing ability of this yeast has been widely recognized and this yeast has been used for health purposes in China for over 2000 years, dating back to the Tang dynasty in 800 AD (Maron et al., 2000). Specifically, *Monascus* yeast has been used to make rice wine, preserve the color and taste of fresh fish and meat, and maintain blood lipid levels within the normal range. According to the ancient Chinese pharmacopoeia, Compendium of Materia Medica (*Ben Cao Gan Mu*), the *Monascus* yeast may promote a healthy cardiovascular system (Li et al., 1998); this has also been confirmed by the discovery of a proprietary strain of yeast that produces metabolic agents that play a role in regulating cholesterol levels so that they are within the normal range.

In the 1970's, Professor Endo, a Japanese scientist, first discovered that certain strains of *Monascus purpureus* fermented with rice and produced a certain metabolite designated as monacolin K (commercially known as lovastatin, mevinolin, or mevacor) (Maron et al., 2000), which is a secondary metabolite of *Monascus* and *Aspergillus* spp. (molecular formula, $C_{24}H_{36}O_5$; molecular weight, 404.55) (Matilde et al., 1999; Maron et al., 2000). Monacolin K is a more active methylated form of compactin produced by *Monascus purpureus*. In addition, it is also a potent competitive inhibitor of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, which is a rate-limiting enzyme in cholesterol biosynthesis (Matilde et al., 1999; Maron et al., 2000). HMG-CoA not only inhibits cholesterol biosynthesis but also lowers blood cholesterol levels in both humans and animals. A clinical trial using a dietary additive containing a standard amount of monacolins revealed that total cholesterol, low-density lipoprotein cholesterol, and triglycerides, which play a positive role in alleviating arteriosclerosis, decreased by 18%, 23%, and 15%, respectively (Maron et al.,2000; Lin et al.,2005).

In this regard, recent researches have focused on general culture conditions and substrate evaluation for monacolin K production, and most studies have been performed by varying controllable conditions, such as temperature, C/N resource, and medium composition (Su et al., 2003; Wang et al., 2003; Chen and Hu, 2005). Because the use of the current production technology for monacolin K on an industrial scale proves expensive and hard to manipulate, a novel production process with lower costs should be investigated (Ng and Shyu, 2004).

Thus, the main objective of the present study was to develop a potential fermentation process that maximizes the production of monacolin K with a fungal elicitor, *Sporobolomyces huaxiensis*.

MATERIALS AND METHODS

Microorganisms

We used the fermented filtrate of a culture of *Monascus purpureus* containing monacolin K (65.42 mg/mL); the culture was provided by the Institute of Fungus Resources, Guizhou University (purchased from China General Microbiological Culture Collection Center; CGMCC). The culture was maintained on a yeast extract–peptone–glucose medium

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Received: September 29, 2010. In revised form: May 25, 2011. Accepted: July 05, 2011.

(Chuanjiang Chemistry Reagent Company, Chongqing, China) preserved at 4ºC.

The fungal elicitor *Sporobolomyces huaxiensis* (the anamorph of *Exobasidium vexans*), isolated from tea leaves, was maintained on potato dextrose agar medium (PDA) at 10ºC, temporarily preserved at the Institute of Fungus Resources (Guizhou University, China), and sub-cultured once every month.

Culture condition and incubation

The liquid medium containing 70 g/L glycerin, 30 g/L glucose, 30 g brewis, 8 g peptone, 8 g $NaNO_3$, and 1 g $MgSO_4$, mixed with 75 mL purified water was prepared in 250 mL Erlenmeyer flasks at an initial pH of 7.0 and used for subsequent experiments (Yu and Zhu, 1998).

To a fully sporulated (6–8 days old) agar slope culture, 10 mL of sterile distilled water was added. The spores were then scraped under aseptic conditions. The spore suspension obtained was used as the inoculum with 1.5×10^5 spores/mL.

After the preparations, the liquid substrates were autoclaved at 121°C for 20 min, cooled to room temperature, inoculated with 2 mL of the spore suspension containing 1.5×10^5 spores/mL of *M. purpureus*, and incubated at 150 rpm at 30°C for 14 days in the oscillation incubator (Suzhou Weier Scientific Inc., Suzhou, China). Unless otherwise mentioned, these conditions were maintained for all the experiments.

Sporobolomyces huaxiensis was cultured on the PDA medium at 26°C for 12 days. A suspension of 1.0×10^6 spores/mL was then inoculated in a 250 mL Erlenmeyer flask containing 75 mL potato dextrose (PD) medium, stirred at a rate of 150 rpm at 26°C for 8 days. Finally, the carbohydrate concentration was determined by the orcinol–sulfuric acid method.

All the chemicals used above were purchased from Chuanjiang Chemistry Reagent Company and the potato was purchased from the local supermarket.

Biomass estimation

The weight of the fungal culture during growth was estimated by a gravimetric analysis method by using a digital balance (HA1001, Shanghai Electronic Balance Co., Shanghai, China). After filtration of the culture broth on pre-weighed filter paper (Chuanjiang Chemistry Reagent Company), the culture was dried at 80ºC to a constant weight. Thus, the growth curve of the strain was plotted with the fermentation time as abscissa and the values of dried weight and the content of monacolin K in the samples as Y-coordinate, which was used to calculate the concentration of monacolin K in the samples.

Extraction and determination of monacolin K

Extraction and determination of monacolin K was conducted as previously described by Yu et al (Yu and Zhu, 1998). The culture filtrate in the fermented culture substrate was treated with a tissue grinder (Shanghai Hamu Scientific Inc., Shanghai, China), condensed to 50 mL, extracted with 50 mL ethyl acetate at pH 3.0 (adjusted with phosphoric acid) and then centrifuged at 150 rpm at 26ºC for 5 h with oscillation incubator. The supernatant was repeatedly washed with 5% Na_2CO_3, volatilized at 60 ºC, and dissolved in 20 mL methanol to determine the amount of monacolin K.

Monacolin K was detected by ultraviolet spectrophotometry (752A, Shanghai No.3 Analytical Instrument Factory, Shanghai, China) as previously described (Zhang and Liu, 1999). The calibration curve was plotted with values of various concentrations of reference monacolin K (Sigma-Aldrich, USA) as abscissa and OD 238 nm values as Y-coordinate. Using the curve, the monacolin K concentrations of tested samples (Sc, mg/mL) were calculated from their OD 238 nm values.

Monacolin K content (mg/mL) = Sc × 50 / 75

All the chemicals above were purchased from Chuanjiang Chemistry Reagent Company.

Treatment of M. purpureus by fungal elicitor

After liquid fermentation of *Sporobolomyces huaxiensis* for 8 days, the filtrate was sterilized, and then cultured in a liquid medium containing *M. purpureus*. One milliliter of the fungal elicitor was added to the liquid medium containing *M. purpureus* every 24 h from day 1 to day 10 (Liu et al. 2002). Thus, the optimum time for adding the elicitor was determined by the amount of monacolin K present in the tested samples. At the optimum time for adding the elicitor, 1 mL of filtrate containing the fungal elicitor according to the different concentrations of polysaccharides were mixed with the liquid medium and the optimum concentration was determined.

Mutation with ultraviolet light and LiCl

The prepared spore suspension of *M. purpureus* and the sterile magnetic needle were inserted into the sterile Petri dish, and then were placed on the magnetic blender (Shanghai Hamu Scientific Inc) approximately 30 cm away from the UV light (30 W). The exposure time was 15 s, 30 s, 45 s, 60 s, 75 s and 90 s. The spores were incubated in darkness for 3 days before being transferred into the slants. After being passed through five generations, the content of monacolin K in the mutated strains was detected after liquid fermentation and the optimum exposure time was determined.

LiCl (Chuanjiang Chemistry Reagent Company) was added into the PDA medium. The spores of the original strain were subjected to UV treatment for 45 s, and then transferred into the medium containing various concentrations of LiCl, as 0.06%, 0.08%, 0.10% and 0.12%. After the liquid fermentation of the mutated strains, the contents of monacolin K were detected.

At the end of experiment, the death rate and the positive mutation rate were calculated based on the following equations.

Death rate (%) = 100 × (1 – a) (a- total number of the mutated surviving colonies in the Petri dish / total number of the surviving colonies in the control Petri dish)

Positive mutation rate (%) = 100 × a × b / 50 (b– the number of the mutated strains with higher monacolin K content than the original strain in the 50 selected mutated strains).

Statistical analysis

Data were presented as mean ± standard deviation (SD), and $P<0.05$ was regarded as statistically significant differences. All statistical analyses were performed using SPSS package (version 11.5 for Windows, by SPSS Inc. 2002).

RESULTS

Variation in the metabolite content during the culture process

Variations in the metabolite content during the culture process are shown in Fig. 1 and Table 1. The production of monacolin K and the biomass increased slowly between day 1 and day 3.

Monacolin K is one of the secondary metabolites derived from the polyketide pathway (Shimizu et al., 2005). Between day 5 and day 13, the amount of monacolin K almost doubled during the idiophase. The monacolin K content was the maximum on day 13, after which there was a sharp decrease in the content. The growth rate of biomass gradually increased from day 4 to day 9, after which the production was stable.

Mutagenesis of ultraviolet light and LiCl

From Fig. 2, it cay be concluded that the rate of positive mutations was the most favorable when the exposure time was 45 s and the LiCl concentration was 1.0%. After the culture was irradiated with UV light for 45 s, the rate of positive mutations reached 4% when the LiCl concentration was 1.0%.

After 3 rounds of mutation by irradiating the cells with UV light in the presence of 1.0% LiCl, 5 mutated strains were obtained. The maximum yield was 219.93μg/mL for 5 generations of subsequent subcultures, as shown in Table 2. From Table 2, it cad be concluded that the biomass of the mutants decreased when the content of monacolin K increased.

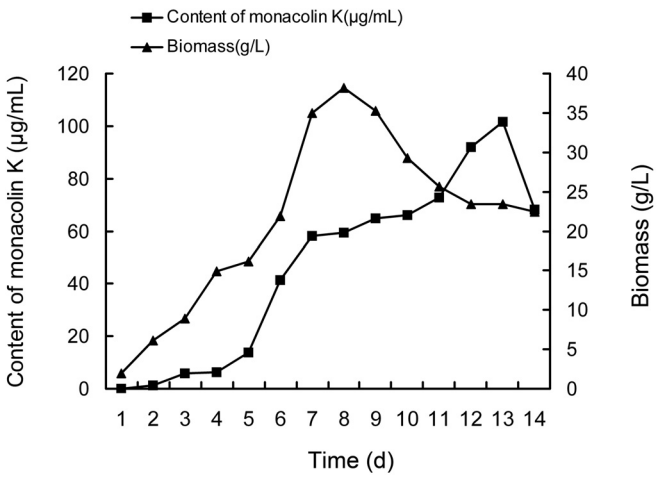

Fig. 1. Changes in monacolin K levels and biomass production with respect to time.

Effects of fungal elicitor

Estimation of optimum time for adding elicitor

Table 3 shows that monacolin K production was the maximum, i.e., 299.74 mg/mL (3 times that of the control culture, 65.42 mg/mL), when 1 mL of the fugal elicitor was added on day 4, after which monacolin K production decreased. The results indicatd that adding the fungal elicitor to the culture substrate before day 4 during the culture process produces a larger amount of monacolin K.

TABLE 1
Monacolin K and biomass production during fermentation[a]

Time (days)	Monacolin K content (SD) (μg/mL)	Biomass (SD) (g/L)	Ratio of monacolin K content to biomass ($\times 10^{-3}$)
1	0.00 (0.00)	2.01 (0.01)	0.00
2	1.23 (0.02)	6.11 (0.02)	0.20
3	6.03 (0.01)	8.94 (0.02)	0.67
4	6.13 (0.04)	14.85 (0.01)	0.41
5	13.91 (0.05)	16.21 (0.02)	0.86
6	41.42 (0.03)	21.89 (0.01)	1.89
7	58.06 (0.02)	34.91 (0.02)	1.66
8	59.23 (0.01)	38.17 (0.04)	1.55
9	64.74 (0.02)	35.20 (0.03)	1.84
10	65.96 (0.03)	29.28 (0.02)	2.25
11	72.89 (0.02)	25.70 (0.01)	2.84
12	92.17 (0.01)	23.35 (0.01)	3.95
13	101.41 (0.03)	23.44 (0.03)	4.33
14	68.27 (0.02)	22.48 (0.02)	3.04

[a] All experiments were carried out in triplicate, and data are expressed as the mean (standard deviation).

TABLE 2
Biomass and monacolin K content produced by mutated strains (ZT) of *M. purpureus* in the fifth generation of subcultures

Strains	Content of monacolin K (μg/mL)	Biomass (g/L)	Ratio of monacolin K content to biomass ($\times 10^{-3}$)
*M. purpu*reus	65.42	49.6	1.32
M. purpureus ZT32	219.93	35.6	6.18
M. purpureus ZT33	178.62	39.4	4.53
M. purpureus ZT35	201.75	37.6	5.37

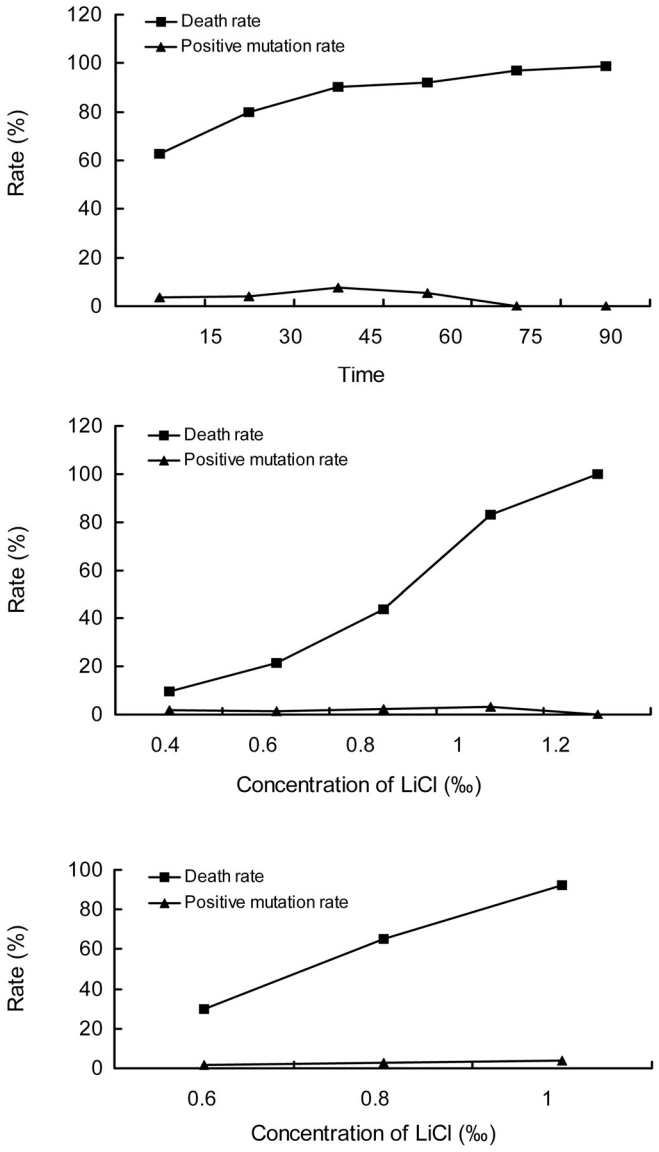

Fig. 2. Mutagenesis due to UV light and LiCl.

Effects of the fungal elicitor at different polysaccharids concentrations

On the basis of the optimum adding time, the fungal elicitor containing different concentrations of polysaccharides were added to the culture of *Monascus purpureus* on day 4. After incubation for 13 d, the samples were found to produce monacolin K.

Polysaccharides, which are considered important active substances, have been used as elicitors (Keen et al., 1972; Vanhulle et al., 2007). As shown in Table 4, on adding the fungal elicitor in the presence of different concentrations of polysaccharides, we observed changes in the monacolin K production level. The yield of monacolin K was the maximum at 446.92 mg/mL when 1 mL of the fungal elicitor was added with 105.61 mg/mL of polysaccharided; the yield was significantly higher than that produced by the control culture. These results indicated that elicitation by the fungus *Sporobolomyces huaxiensis* resulted in rapid induction of and increased monacolin K production.

TABLE 3
Effects of the elicitor on monacolin K production when added at different durations[a]

Adding time (day)	Content of monacolin K (SD) (μg/mL)
CK [b]	65.42 (0.02)
1	180.00 (0.01)
2	252.81 (0.04)
3	269.32 (0.04)
4	**299.74** (0.03)
5	195.12 (0.03)
6	159.85 (0.04)
7	102.84 (0.05)
8	117.76 (0.06)
9	64.43 (0.02)
10	58.42 (0.03)

[a] All experiments were carried out in triplicate and data were expressed as the mean (standard deviation).
[b] The results show that adding the fungal elicitor to the medium apparently enhanced monacolin K production by *Sporobolomyces huaxiensis* as analyzed by Student's *t* test ($p = 0.005$).

TABLE 4
Effects of adding the filtrate containing the fungal elicitor along with different concentrations of polysaccharides[a]

Polysaccharide content (SD) (μg/mL)	Biomass (SD) (g/L)	Monacolin K production (SD) (μg/mL) [b]
0	49.62 (0.04)	65.42 (0.02)
8.80 (0.14)	25.10 (0.06)	110.41 (0.03)
17.60 (0.06)	24.30 (0.12)	114.02 (0.04)
26.40 (0.04)	24.80 (0.11)	115.92 (0.04)
35.22 (0.03)	23.60 (0.01)	128.33 (0.05)
44.00 (0.08)	23.40 (0.02)	139.64 (0.06)
52.80 (0.04)	24.20 (0.05)	119.65 (0.02)
61.60 (0.03)	23.80 (0.04)	110.67 (0.11)
70.40 (0.06)	21.00 (0.01)	151.12 (0.08)
79.20 (0.01)	20.80 (0.12)	205.71 (0.07)
88.00 (0.05)	19.80 (0.11)	277.12 (0.06)
96.80 (0.02)	18.60 (0.13)	398.03 (0.05)
105.61 (0.02)	**16.80** (0.09)	**446.92** (0.02)
114.40 (0.03)	20.60 (0.08)	266.24 (0.03)
123.20 (0.13)	20.10 (0.01)	240.47 (0.03)
132.00 (0.14)	21.40 (0.12)	152.08 (0.04)

[a] All experiments were carried out in triplicate and data were expressed as the mean (standard deviation).
[b] The results show that adding the fungal elicitor to the medium apparently enhanced monacolin K production by *Sporobolomyces huaxiensis* as analyzed by Student's *t* test ($p = 0.005$).

DISCUSSION

Monacolin K has been proved to effectively block cholesterol synthesis. To develop more products beneficial for health, studies are now focusing on increasing monacolin K production. Studies on relevant investigations have been reported in the past; however, most of these studies have dealt with the optimization of fermentation methodologies, mutagenesis, and chemical stimulants, whereas the production levels reported were low (Lee et al., 2006; Sayyad et al., 2007). In this study, although the compound mutagenesis was applied to enhance the production of monacolin K, the content of monacolin K was still more than 3 fold that as the control culture. So, easier, simpler and more effective ways to enhance the concentration of monacolin K should be investigated.

In this study, we report for the first time a novel method that enhances monacolin K production when the fungal elicitor *Sporobolomyces huaxiensis* is used. Our results indicated that the biomass and monacolin K level during the culture of *Monascus purpureus* was the highest on day 8 and day 13, respectively (Fig. 1). Correspondingly, the optimum time for elicitation in order to obtain the maximum amount of monacolin K (6 fold as the control) was on day 4, when this secondary metabolite was initially produced in the medium.

In addition, multiple glycosylated compounds acting as signal receptors were located on the surface of eukaryotic cells (Keen et al., 1972; Miyake et al., 2005). On the basis of the results obtained in the present study, *Monascus purpureus* may initiate monacolin K metabolism on day 4. Based on the results above, it could be concluded that after adding the fungal elicitor, the polysaccharides transmitted a signal to the signal receptor of *Monascus* fungal cells, which triggered a second metabolic reaction when the signal was received by the corresponding enzyme system, thereby producing a large amount of monacolin K (Miyake et al., 2005; Zhao et al., 2006).

In conclusion, this study enphasizes that monacolin K enhancement by direcy elicitor and stimulation by the fungal elicitor may be a more effective regulatory mechanism than previously recognized (Han et al., 2002); this finding will be of significance for industrial application of monacolin K production (Chiu et al., 2006).

COMPETING INTERESTS

The authors declare that they have no competing interests.

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