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Development and production of cholesterol-lowering *Monascus*-nata complex

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Summary

This study develops a new foodstuff, the *Monascus*-nata complex, which combines the functions of cholesterol-lowering monacolin k and bacterial dietary-fibre. Two *Monascus* strains, *M. ruber* and *M. pilosus* were fermented within cubical bacterial cellulose, nata de coco, obtained from *Acetobacter* fermented coconut juice, in a conditioned medium. The production levels and stability of monacolin k in the cultured *Monascus*-nata complex were determined to develop optimal fermentation conditions. The results indicated that a medium that comprised 5% glucose and 1.5% ammonium phosphate at pH 6.0–7.0 produced the most monacolin k (157 mg/l) for *Monascus pilosus* NCHU M-35. However, monosodium glutamate (MSG) and 0.001% $ZnSO_4$ inhibited the intracellular accumulation of monacolin k. Monacolin k within the *Monascus*-nata complex was relatively resistant to washing and changes of pH, but thermal processing and freezing storage markedly reduced the amount present. This novel *Monascus*-nata complex is potentially a healthy foodstuff.

Introduction

Monascus, a fungus, produces pigments that are traditionally grown by solid culture on bread, steamed rice or cereal. It was widely used as a food additive as a colouring and flavouring agent in foods and beverages in ancient China (Hopwood & Sherman 1990; Chen & Johns 1993). The pigments are released into the fermentation medium through its mycelia (Su 1978) and the stability and production of the pigments have been extensively studied (Wong *et al.* 1981; Lin & Demain 1991).

In the 1970s, monacolin k (mevinolin), an important metabolite of *Monascus* sp., was identified (Endo 1979) and shown to be able to inhibit the synthesis of cholesterol (Endo 1979; Albert *et al.* 1980; Endo *et al.* 1985, 1986). It also provides benefits to sufferers of cardiovascular disorders (Lin 1986). The critical reaction in the pathway of cholesterol synthesis is the formation of mevalonic acid from 3-hydroxy-3-methylglutaryl CoA (HMG-CoA) by HMG-CoA reductase. Monacolin k is structurally similar to HMG-CoA and plays a role as a competitive inhibitor, which competes with HMG-CoA and reduces the synthesis of cholesterol (Fears 1983). Since the discovery of this therapeutic function, the application of healthful *Monascus* sp. food has been broadly promoted in Asian countries (Juzlova *et al.* 1996).

Nata, which is prepared from *Acetobacter xylinum*, is a popular snack in the Philippines and other countries. It is also widely used in food processing because of its distinctly soft texture and high fibre content (Okiyama *et al.* 1992). This dietary fibre has been found to be effective in normalizing blood glucose, preventing constipation and preventing colonic cancer (Marlett *et al.* 2002). Our previous study involved the fermentation of *Monascus* on nata to produce the *Monascus*-nata complex (Sheu *et al.* 2000). After its formation, the *Monascus* mycelia grow through the cellulose network of the nata. This *Monascus*-nata complex exhibits superior colour stability and potential as a vegetarian foodstuff (Sheu *et al.* 2000).

This study seeks to increase the availability of monacolin k in the *Monascus*-nata complex. The optimal culturing conditions for monacolin k production were investigated, and the stability of monacolin k in the *Monascus*-nata complex, which is resistant to various processing treatments, was also studied.

Materials and methods

Raw materials and microorganism

Nata slices with dimensions of $30 \times 25 \times 1$ cm (stored in acetic acid) were obtained from the Cana Food Company (Tainan, Taiwan). It was washed under running water for 24 h to remove residual acetic acid, and then cut into $2 \times 2 \times 1$ cm pieces before fermentation.

Monascus ruber CCRC 31532 was obtained from the CCRC (Culture Collection and Research Center, Hshin Chu, Taiwan) and *Monascus pilosus* NCHU M-35 was obtained from National Chung Hsing University. The fungi were cultured on a potato dextrose agar (PDA) plates (Difco, USA) at 26 °C for 3 days, and then used for inoculation.

Fermentation

The medium was prepared using sterilized double-distilled water with glucose as the carbon source (5%) and peptone as the nitrogen source (1.5%). Later, sucrose, maltose or lactose was substituted for the glucose, and ammonium chloride, ammonium phosphate or monosodium glutamate (MSG) was substituted for the peptone. The medium included microelements as follows: 0.05% $CaCl_2$, 0.05% KCl, 0.001% $ZnSO_4$ and 0.01% $MgSO_4$. The pH was adjusted using 1 M HCl and NaOH. Ten pieces of nata were put into 150 ml of the medium in a 500 ml flask. After it had been sterilized at 121 °C for 15 min and cooled to room temperature, each flask was inoculated. Fermentation was carried out in a 150 rev/min rotary shaker at 26 °C for 6 days.

Extraction and HPLC analysis

Six pieces of fermented *Monascus*-nata complex were collected and homogenized (POLYTRON®, Switzerland) with 100 ml deionized water. Later, 100 ml of CH_2Cl_2 was added for extraction. After centrifugation at $3000 \times g$ for 8 min, the organic phase was collected for concentration (Büchi Rotavapor R-124 and Büchi 168 Vacuum/Distillation Controller, Switzerland) until it was dried. One millilitre of acetonitrile was added to dissolve monacolin k for HPLC analysis. HPLC (Intelligent HPLC System LC-800 series, JASCO, Japan), equipped with a system controller (model 801-SC); pump (PU-980); column (LichroCart RP18, 125×4 mm, 5 μm, Merck, Germany); a u.v. detector (870-UV, Jasco, Japan); column oven (TU-100); recorder (SISC ver. 2.1) and an autosampler (851-AS, JASCO, Japan) was used for the analysis (mobile phase acetonitrile: 0.1% phosphoric acid, 75:25 (v/v); a flow rate of 0.7 ml/min; an injection volume of 20 μl, and detection wavelength of 237 nm). A standard curve was obtained by using dilutions 10, 5, 2.5 and 1 ppm (mg/l) of monacolin k (Sigma, USA) in 100 ml acetonitrile.

Monacolin k stability test

The resistance of monacolin k in fermented *Monascus*-nata was tested by washing, heating, freezing and incubating at various pH values. Samples were washed under running tap water at a constant flow rate of 10 l/h for 60 h to determine washing resistance. In the heat resistance test, samples were cooked in boiled water for 15 min and autoclaved at 121 °C for 15 min. In the freezing test, the samples were frozen at −20 °C for 24 h. Samples were soaked in solutions with pH values 3.0–7.0 for 48 h. The monacolin k content in the complexes was determined by HPLC over a particular period.

Results and discussion

Production of Monascus-*nata complex*

Diced nata was fermented in *Monascus*-inoculated broth at 26 °C for 6 days. After fermentation, *Monascus* mycelia had penetrated the network of capillaries in nata, yielding the final *Monascus*-nata complex. The complex was uniformly and brightly coloured (See Figure 4).

Optimization of culture conditions

The effects of carbon sources, nitrogen sources, microelements and pH on the production of monacolin k by *M. pilosus* NCHU M-35 and *M. ruber* CCRC 31532 were investigated. When glucose, sucrose, maltose and lactose were utilized as alternative sole carbon sources (5%), the highest yield of monacolin k was produced with glucose as the carbon source (157 mg/l for *M. pilosus* NCHU M-35 and 148 mg/l for *M. ruber* CCRC 31532) (Figure 1a). Furthermore, ammonium chloride, ammonium phosphate and peptone at 1.5% were similarly effective (Figure 1b) nitrogen sources, while MSG was poorly utilized; these results were consistent with those of Chen & Johns (1993). The lower utility of MSG in production of monacolin k by *M. pilosus* NCHU M-35 and *M. ruber* CCRC 31532 (77 and 93 mg/l, respectively) may be associated with its promotion of growth and the formation of pigments (Lin *et al.* 1992).

With respect to the microelements, KCl exhibited the highest production of monacolin k (160 mg/l for *M. pilosus* NCHU M-35 and 163 mg/l for *M. ruber* CCRC 31532) but $ZnSO_4$ yielded the lowest production of monacolin k (127 mg/l for *M. pilosus* NCHU M-35 and 103 mg/l for *M. ruber* CCRC 31532) (Figure 1c). These results were similar to those of Bau & Wong (1979), who found that zinc ions diminished the growth, pigmentation and antibacterial activity of *Monascus*. The addition of microelements did not markedly promote the production of monacolin k. Furthermore, the optimal pH for production of monacolin k ranged from 6.0 to 7.0 (Figure 1d, monacolin k production >150 mg/l), whereas a pH of lower than 5.0 yielded relatively low levels of monacolin k. Therefore, the results indicate that *M. pilosus* NCHU M-35 yielded more monacolin k than did *M. ruber* CCRC 31532.

Most studies (Su 1978; Wong *et al.* 1981; Lin & Demain 1991; Lin *et al.* 1992; Chen & Johns, 1993, 1994) have focused on the production of pigments rather than monacolin k, so this study, in which a large amount of monacolin k is obtained, may be important for developing a functional *Monascus*-nata complex.

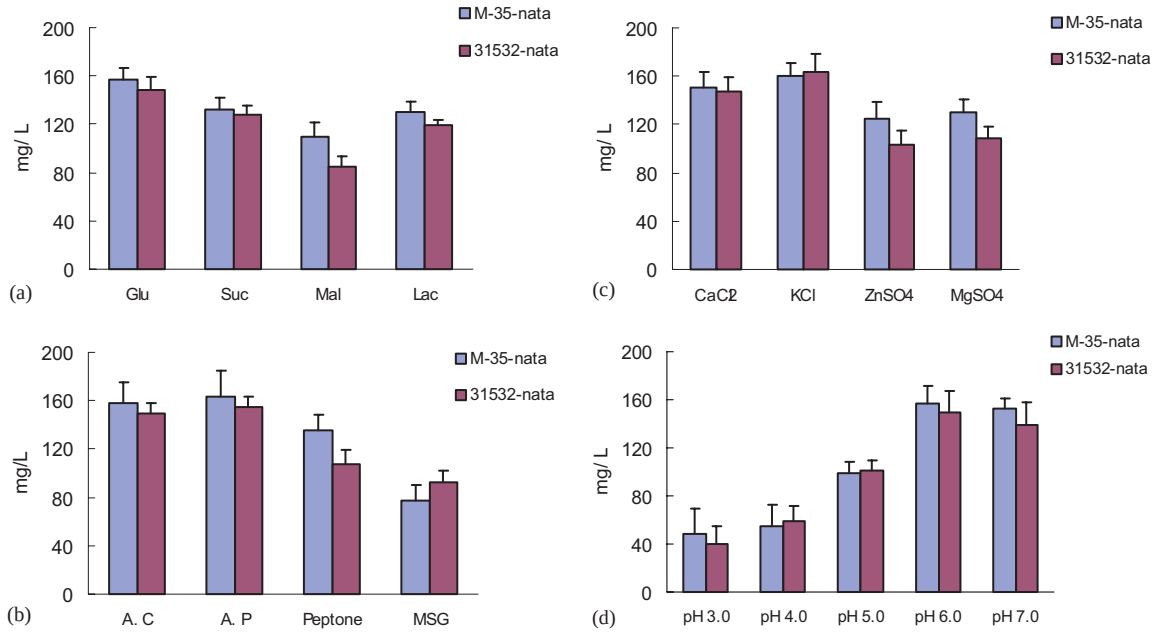

Figure 1. Monascus pilosus NCHU M-35 was fermented on nata and the monacolin k was produced using various (a) carbon sources (Glu: glucose; Suc: sucrose; Mal: maltose and Lac: lactose); (b) nitrogen sources (A. C: ammonium chloride; A. P: ammonium phosphate); (c) microelements and (d) pH treatments of medium broth. Vertical bars represent SD ($n = 3$).

Stability of monacolin k

The resistance of monacolin k toward processing treatments of the *Monascus*-nata complex was further investigated. After 60 h of washing with water, the monacolin k content fell from 160 to 119 mg/l, and from 147 to 109 mg/l for M-35-nata and 31532-nata complexes, respectively. These results indicate that the *Monascus*-nata complex exhibited washing resistance (Figure 2a). However, monacolin k was relatively thermally unstable. Treating the M-35-nata complex with boiling water for 15 min reduced monacolin k to half of its original content (80 mg/l) while autoclave treatment removed almost all monacolin k (Figure 2b). Freezing storage reduced the content of monacolin k to 97 mg/l (Figure 3a). After soaking for 48 h in solutions with pH from 3.0 to 7.0, most of the original monacolin k content in the complex was retained, although at pH 3.0, a slight reduction in content was observed (Figure 3b).

The colour of the *Monascus*-nata complex was also relatively resistant to washing (Figure 4a). When washed, the complex retained the red pigment, with a slightly increased L value and slightly decreased in its a value using a Hunter Lab colour spectrophotometer (data not shown). The results indicate that *Monascus*-nata lost relatively little colour. When thermally treated

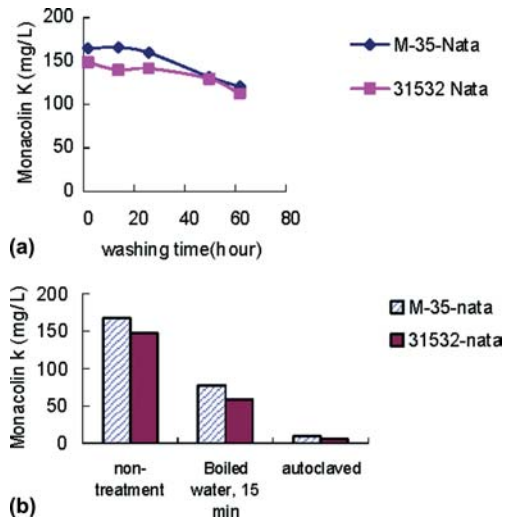

Figure 2. Monacolin k content in *Monascus*-nata complex after (a) 60 h of washing in water; (b) thermal treatment.

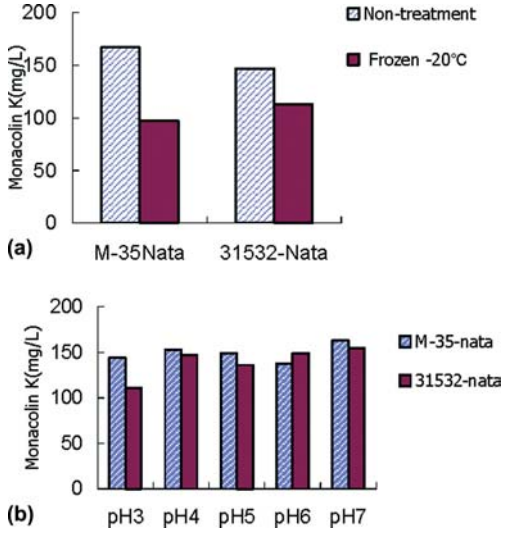

Figure 3. (a) −20 °C treatment for 48 h; (b) treatment by soaking in solutions of various pH values for 48 h.

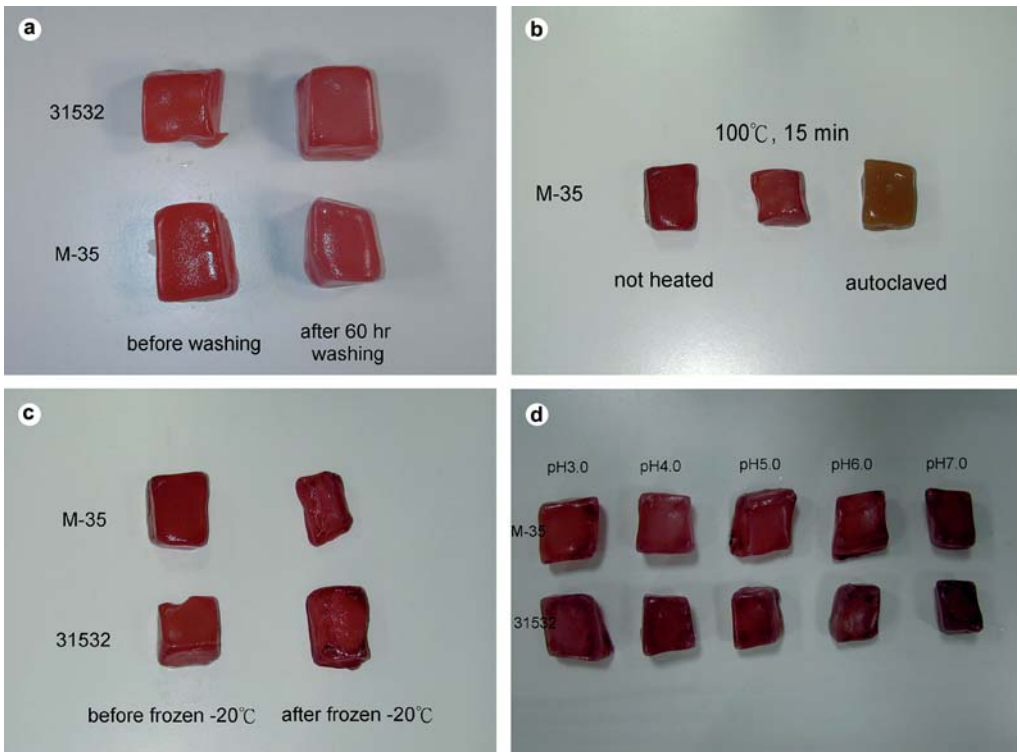

Figure 4. Effect of treatment of M-35-nata complex by (a) washing for 60 h; (b) heat treatment; (c) freezing and (d) solutions with various pH values for 48 h.

using an autoclave, much of the red pigment in the complex was lost (Figure 4b). Freezing and treatments at various pH values caused shrinkage and yielded slightly dark red pigmentation (Figure 4c and d).

The stability of monocolin k in the *Monascus*-nata complex fermented by *M. pilosus* NCHU M-35 and *M. ruber* CCRC 31532 generally appeared to follow similar trends. These results show that fresh *Monascus*-nata complex possesses great potential for use in food processing, because it has more stable monacolin k and colour (Figure 4), which are nevertheless reduced somewhat during thermal processing.

Conclusion

Monascus-nata complex, which combines the functional characteristics of monacolin k and dietary fibre, is a potential novel functional foodstuff. However, it is relatively thermally unstable, further studies are required to prevent the impact of temperature on monacolin k in *Monascus*-nata. Its desirable appearance and functional properties may make *Monascus*-nata important and popular in the future, and worthy of popularization.

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