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Anka and anka pigment production

S-S Teng and W Feldheim

Institute for Human Nutrition and Food Science, University of Kiel, Duesternbrooker Weg 17-19, Kiel 24105, Germany

This study was conducted to determine the time-dependent changes of solid-state fermentation of rice with *Monascus purpureus* to produce anka and anka pigments. Growth of the fungus occurred prior to the synthesis of anka pigments. A steady increase in the yield of pigments occurred between the 5th and 15th days. After 15 days, growth of the fungus on rice substrate ceased and the yield of yellow anka pigments remained constant; however, orange anka pigments were reduced with a decreasing rate of 3.6 mg/g anka/day. *Journal of Industrial Microbiology & Biotechnology* (2001) **26,** 280–282.

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Introduction

Solid-state fermentation has been widely applied in food processing. In Asia, various fermented products of fungi have been consumed as a time-honored practice. The substrates are primarily soy bean, rice, wheat, and peanut [6]. Anka, one of these fermented products, is rice fermented with *Monascus purpureus*. On rice grains, the fungus synthesizes four anka pigments. In certain regions of Asia, anka has been used traditionally as a natural coloring agent or as one of the starter cultures for brewing red rice wine.

Time-dependent changes of fungal growth and pigment production by submerged cultivation of *Monascus* sp. were reported previously [7,13]. However, little information is available about its solid-state cultivation. *M. purpureus* was cultivated on rice substrate at 30°C for 43 days in 500-ml Erlenmeyer flasks. The yield of individual anka pigments was determined by high-performance liquid chromatography analysis [11]. Growth of the fungus on a solid substrate cannot be analyzed directly; therefore, indirect measurements were applied by measuring the time-dependent changes of anka, including yield, residual starch content, total protein content, and pH. Thus, the results provide a better understanding of pigment production and growth of *M. purpureus* on rice.

Materials and methods

Organism and cultivation

M. purpureus DSM 1379 was purchased from Deutsche Sammlung von Mikroorganismen und Zellkulturen (Braunschweig, Germany). The fungus was grown on agar slants, which contained (w/v) 2% yeast extract (Difco), 5% glucose, 0.1% KH_2PO_4, 0.5% $MgSO_4 \cdot 7H_2O$, 0.1% $CaCl_2$, and 1.5% agar (Difco Laboratories, Detroit, USA) in distilled water. Following 5 days of cultivation at 30°C, slants were stored at 4°C for less than 3 days. A spore suspension was prepared with 5 ml of sterile distilled water and then measured using a hemacytometer. The spore suspension was then adjusted to 2×10^7 spores/ml and used as inoculum.

The preparation of rice substrate was reported previously [12]. Rice substrate was inoculated with 2 ml of the spore suspension per 20 g. Fermentation was performed in 500-ml Erlenmeyer flasks plugged with cotton. The inoculated substrate was evenly divided into six groups and cultivated for 5, 10, 15, 20, 25, and 30 days, respectively. Each day, the inoculated substrate was manually shaken until all the rice grains were separated from each other. After the fermentation had been completed, the fermented material was freeze-dried at −60°C for 24 h. The resulting red dried rice was anka, which was stored at −26°C until analysis was conducted. Before analysis, anka was thawed at room temperature in a desiccator and ground into powder using a laboratory mill with water cooling (Eydam, Kiel, Germany). The processes to determine yield, residual starch content, total protein content, and pH of the anka obtained are described in the following sections. In addition, anka cultivated for 8, 12, 14, 17, and 43 days was analyzed to study pigment production. The rice substrate inoculated, but not cultivated, was freeze-dried after inoculation and used as reference substrate. Extraction and analysis of anka pigments were discussed previously [11].

Yield of anka

The average weight of the uncooked dry rice used in each flask was recorded. The fermented materials were freeze-dried and the resulting anka was weighed. The yield of anka was expressed in % (w/w). It was calculated using the following formula: yield of anka = weight of anka obtained/weight of uncooked dry rice used × 100%.

Residual starch content of anka

Anka powder (150 mg) was hydrolyzed with 100 μl thermostable α-amylase (EC 3.2.1.1, Termamyl 120 l; Novo, Denmark) at 100°C for 60 min and then with 50 μl amyloglucosidase (EC 3.2.1.3; Boehringer Mannheim, Germany) at 60°C overnight. The amount of glucose was determined with the help of glucose oxidase reagent (Merck, Darmstadt, Germany) [1]. The residual starch content was calculated as the amount of glucose × 0.9 and expressed in % (g starch content/100 g anka).

Correspondence: S-S Teng, Department of Tourism, Aletheia University, 32 Zahnlistr, Tamsui, Taipei, Taiwan
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Total protein content of anka

Anka powder (0.2 g) was suspended in 25 ml distilled water using a homogenizer (type TP 18/10; Janke and Kunkel, Kiel, Germany). Two-milliliter anka suspension was then digested with 2 ml NaOH (1 M) at 100°C for 5 min to break the cellular wall material and to solubilize the protein [8]. After cooling, protein was determined by the method of Bradford [2] using bovine serum albumin as reference protein. The results were expressed in % (g protein/100 g anka).

pH of anka

With the help of a homogenizer (type TP 18/10; Janke and Kunkel), 0.2 g anka powder was suspended in 25 ml distilled water. The pH of the anka suspension was determined using a pH meter.

Statistics

All the tests were conducted in seven replications. The *t*-test was used to analyze the significance of the time-dependent changes of anka and yields of pigment production.

Results

Yield of anka

Figure 1 gives the time-dependent changes of anka. The yield of anka pigments in 43 days is shown in Figure 2.

In the first 15 days of cultivation, the yield of anka decreased linearly by approximately 4.5% per day. From the 15th to 30th days, the yield of anka decreased from 30.2% to 24.6%.

Residual starch content of anka

If inoculated substrate is cultivated for 20 days, the residual starch content of anka becomes 4.8%. It was not changed significantly by cultivation longer than 20 days. During the first 15 days of cultivation, the residual starch content of anka was reduced from 76.3% to 7.6%, i.e., 90% of the starch in rice substrate was metabolized by fungus.

Total protein content of anka

The total protein content of anka was reduced from 10.5% to 6.0% during the first 5 days of cultivation. Later, it increased, first rapidly then slowly, until the 20th day, after which it decreased from 16.9% to 15.6% and remained constant.

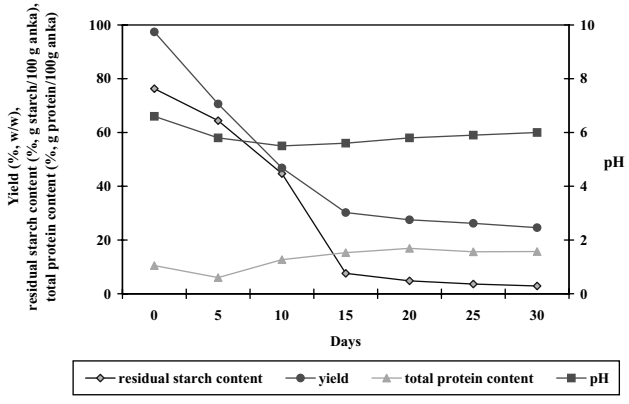

Figure 1 Time-dependent changes of anka.

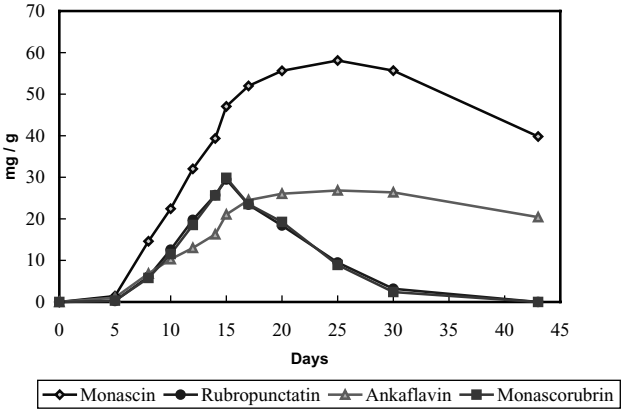

Figure 2 Yield of anka pigments in 43 days.

pH of anka

The pH of anka remained stable in the course of cultivation. The initial pH of the rice substrate was 6.6. It decreased in the early phase of cultivation, then slowly increased and remained stable. The lowest pH value (pH 5.5) was observed in anka that was cultivated for 10 days. The pH values of anka cultivated for 25 and 30 days were 5.9 and 6.0, respectively, which were not significantly different from each other.

Yield of yellow anka pigments

The yield of yellow monascin was approximately twice as much as the yellow ankaflavin in all the fermented materials cultivated for various cultivation periods (Figure 2). During the first 5 days of cultivation, a small amount of yellow pigments was synthesized. Between the 5th and 17th days, a steady increase in the yield of these two pigments was observed to have a daily increase of 4.2 and 2.0 mg pigments per gram of anka, respectively. There was no statistically significant difference in the yield of yellow pigments of anka that was cultivated for 20, 25, or 30 days. The average yields of two yellow pigments were 56.4 and 26.4 mg/g anka between 20 and 30 days. If the inoculated substrate is cultivated for 43 days, the yield of the two yellow anka pigments decreases to 39.8 and 20.4 per gram anka, respectively.

Yield of orange anka pigments

During the first 5 days of cultivation, orange pigments, similar to two yellow anka pigments, were produced in small amounts (Figure 2). A steady increase in the yield of orange pigments occurred between the 5th and 15th days, with the increasing rate of 2.9 mg of each orange pigment per gram anka per day. The concentration of these two orange pigments decreased steadily in anka that was cultivated longer than 15 days, i.e., an average of 1.8 mg individual pigment per gram anka per day. During the 43 days of cultivation, the yields of orange rubropunctatin were not significantly different from those of orange monascorubrin. After 43 days, no orange anka pigments could be detected.

Discussion

Over the first 5 days of cultivation, residual starch content, total protein content, and pH value of anka decreased considerably. The

reduction in these measurements demonstrated the intensive metabolism and growth of fungus. However, during this time, only a small amount of anka pigments was detected. This is consistent with the observation of Hiroi *et al.* [4] that growth of fungus occurred prior to synthesis of pigments.

Between the 5th and 15th days of cultivation, the residual starch content of anka decreased steadily, as did the yield of anka. Meanwhile, the minimum pH value was observed. Since starch is the main component of rice, the changes of residual starch content and of pH confirmed the metabolism of the fungus. The total protein content of anka consisted of two things: the residual rice protein and the biomass of fungus. The residual rice protein represented the utilization of rice substrate by fungus and the biomass of the fungus indicated growth of the fungus. The increase in total protein content of anka during this period of cultivation resulted from the increase of fungus biomass and the decrease of rice protein. Production of all four anka pigments increased steadily. These observations attested to the vigorous metabolism of the fungus and the utilization of rice substrate for both fungal growth and pigment formation.

Generally, when the inoculated substrate was cultivated longer than 15 days, nutrients in the rice substrate were almost exhausted. Therefore, growth of the fungus and formation of pigments were limited. The composition and pH of anka and the concentration of yellow anka pigments stayed at a relatively stable level. However, the yield of two orange anka pigments was reduced severely. Lin [7] and Chen and Johns [3] used submerged culture to produce anka pigments. They also observed a marked reduction in total yield of pigment after long cultivation. The reduction of two orange anka pigments might result from instability of these pigments at increased pH due to prolonged cultivation [10].

In flask fermentation to produce anka at 30°C, vigorous metabolism and growth of the fungus began early in cultivation and ceased after 15 days. A steady increase in pigments occurred between the 5th and 15th days. Maximum yields of pigments and of anka were attained after 15 days. In regard to anka pigment production, the inoculated substrate should be incubated for not less than 10 days and not longer than 15 days.

In 1932, Salomon and Karrer [9] isolated monascin from anka. Monascin was the dominant among the four anka pigments. After a series of purification treatments of the pigments, monascin was the only pigment that remained. In addition, it was also possible that the anka that they used as substrate for pigment extraction was not optimally cultivated. Due to prolonged cultivation, barely any orange pigment was found. As a result, only yellow anka pigments were extracted and only monascin was purified.

In 1962, Kumasaki *et al.* [5] speculated that there was an interconversion between orange and yellow anka pigments. The results of this study showed otherwise: during 43 days of cultivation, the yield of orange rubropunctatin always remained the same as that of orange monascorubrin. In contrast, the amount of yellow monascin produced was about twice as much as the yellow ankaflavin. The difference in concentration of two yellow and two orange anka pigments revealed that an interconversion of orange from yellow pigments or the reverse was impossible.

Acknowledgements

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References

1 Bach Knudsen KE, P Aman and BO Eggum. 1987. Nutritive value of Danish-grown barley varieties: I. Carbohydrates and other major constituents. *J Cereal Sci* 6: 173–186.
2 Bradford MM. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein–dye binding. *Anal Biochem* 72: 248–254.
3 Chen M-H and MR Johns. 1994. Effect of carbon source on ethanol and pigment production by *Monascus purpureus*. *Enzyme Microb Technol* 16: 584–590.
4 Hiroi T, T Takahasi, T Sima, T Suzuki, M Tsukioka and N Ogasawara. 1981. Production of red koji in solid culture. *Nippon Nogeikagaku Kaishi* 55: 1–6.
5 Kumasaki S, K Nakanishi, E Nishikawa and M Ohashi. 1962. Structure of monascorubrin. *Tetrahedron* 18: 1171–1184.
6 Leistner L. 1986. Schimmelpilz-gereifte Lebensmittel. *Fleischwirtschaft* 66: 168–173.
7 Lin C-F. 1973. Isolation and cultural conditions of *Monascus* sp. for the production of pigment in a submerged culture. *J Ferment Technol* 51: 407–414.
8 Raimbault M and D Alazard. 1980. Culture method to study fungal growth in solid fermentation. *Eur J Appl Microbiol Biotechnol* 9: 199–207.
9 Salomon H and Karrer P. 1932. Pflanzenfarbstoffe: XXXVIII. Ein Farbstoff aus "rotem" Reis, Monascin. *Hev Chim Acta* 15: 18–22.
10 Teng S-S. 1995. Ankafarbstoffe — Production, Analytik und Faerbung der Lebensmittel. Doctor dissertation, Kiel University, Germany.
11 Teng S-S and W Feldheim. 1998. Analysis of anka pigments by liquid chromatography with diode-array detection and tandem mass spectrometry. *Chromatographia* 47: 529–536.
12 Teng S-S and W Feldhiem. 2000. The fermentation of rice for anka pigments production. *J Ind Microbiol Biotechnol* 25: 141–146.
13 Yoshimura M, S Yamanaka, K Mitsugi and Y Hirose. 1975. Production of monascus pigment in a submerged culture. *Agric Biol Chem* 39: 1789–1795.