Supplementary effect of whey components on the monascin productivity of *Monascus* sp.

Short running title: Effect of whey on monascin productivity

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Abstract

BACKGROUND: *Monascus* sp. has been used for fermented foods for centuries. It can synthesize yellow, red and orange pigments as secondary metabolites. Here, we focused on yellow pigment monascin, responsible for anti-inflammation and antidiabetic effects, and evaluated that if whey could be a suitable substrate with or without rice powder for monascin production using *M. purpureus* AHU 9085, *M. pilosus* NBRC 4520 and *M. ruber* NBRC 32318.

RESULT: Growth and monascin production of the three *Monascus* strains were dependent on the three liquid media consisting of whey and/or rice. All strains showed the best growth in rice and whey mixed medium where *M. ruber* NBRC 32318 exhibited the highest total monascin production. Subsequent investigation on the effects of whey components indicated a mineral cocktail in whey was outstandingly stimulative for the monascin production efficiency of *M. ruber* NBRC 32318. However, this recipe exhibited lesser stimulation or even inhibition for *M. pilosus* NBRC 4520 and *M. purpureus* AHU 9085, respectively. In terms of total monascin production, rice with whey provided the highest level due to the growth promotion along with a relatively high production efficiency.

CONCLUSION: Effect of whey on the growth and monascin production was highly dependent on the *Monascus* strains. Even a mineral cocktail in whey could regulate monascin productivity in a strain specific manner. Further studies are needed to elucidate the mechanism behind the diverse response by the minerals on the monascin production of *Monascus* species.

KEYWORDS: *Monascus* sp.; monascin; whey; simulated milk ultrafiltrate

Introduction

Some species of *Monascus*, often called red mold, have been regarded as edible filamentous fungi. Rice fermented by *Monascus*, known as red yeast rice, has been commercialized in East Asia since ancient times.[1] Besides, *Monascus* is recorded to have a long history of fermenting grains especially beans named Tofunyu and Tofuyo in China and Japan, respectively.[2] In recent years, red ginseng, garlic and durian seed are being explored for the development of a novel type of fermented foods using *Monascus* sp..[3]

Monascus sp. can produce various secondary metabolites including bio-functional substances and pigments.[4, 5] Monacolin K is a colorless metabolite and has been known to lower cholesterol in the plasma due to the inhibitory effect of 3-hydroxy-3-methylglutaryl-coenzyme A reductase.[6] *Monascus* pigments have been favored as natural pigments, and widely used in the food industry as a color intensifier and food additives. Until now, more than 100 members have been identified,[7] and yellow pigments (citrinin, monascin and ankaflavin), orange pigments (rubropunctatin and monascorubrin), and red pigments (rubropunctamine and monascorubramine) are representative. Among yellow pigments, monascin and ankaflavin have been shown to have antioxidant, anti-cancer, antimicrobial, anti-inflammation, anti-obesity and antidiabetic effects[8, 9] while citrinin is thought to be hepatotoxic and nephrotoxic.[10]

Many efforts have been made to increase the secondary metabolite production of *Monascus* sp..[7] Although the review by Feng has shown that filamentous fungi can produce more abundant secondary metabolites on solid culture than liquid culture,[11] liquid culture is frequently used due to its flexible applicability to the regulation of culture conditions.[12, 13] Many parameters, including carbon and nitrogen sources, pH, temperature, and aeration, affect secondary metabolite production. Accordingly, a synthetic medium is a convenient method for establishing the ideal composition and identifying crucial factors for secondary metabolite production.

In addition to a synthetic medium, agro-industrial by-products, such as orange-peel waste, sugarcane bagasse, and whey, have been applied for the substrate[14-19] to effectively use resources while increasing secondary metabolite production. Kantifedaki *et al.*[15] have used orange peel waste as the sole source of nutrients for higher total pigment production by *M. purpureus* when comparing solid, semi-solid, and submerged fermentation. Solid fermentation showed high yellow pigment production efficiency. Velmurugan *et al.*[16] have applied corn cobs as a substrate for *M. purpureus* in solid fermentation for red and yellow pigment production and found abundant yellow pigment production, which also supports the use of solid culture. Silveira *et al.*[17] have used sugarcane bagasse supplemented with cheese whey powder as an organic nitrogen source for the production of red pigment but found that the contribution of the cheese whey powder was limited. However, some studies have claimed that cheese whey could be beneficially applied to *Monascus* pigment production.[18, 19] Thus, the value of whey for *Monascus* pigment production remains unclear.

When a solid whey culture was used, *M. purpureus* AHU 9085 produced citrinin, whereas *M. pilosus* NBRC 4520 and *M. ruber* NBRC 32318[20] produced monacolin K; however, none of these strains produced monascin (Huang and Li, unpublished results). On the other hand, the monascin-producing capability of *M. purpureus* AHU 9085 and *M. pilosus* NBRC 4520 has been reported in rice or potato dextrose liquid culture.[21, 22] Based on these results, the question of using whey for monascin production is whether it

contains unfavorable components, or a solid-state culture is unsuitable for monascin production.

In this study, we conducted specific analysis of monascin productivity to establish the value of whey as the *Monascus* substrate because a study focused on one type of total pigment productivity would be too broad and vague. Since rice powder containing submerged fermentation is likely to be suitable for high monascin production by *Monascus* M9, as shown by Chen *et al*.,[23] we compared and evaluated the effect of whey on monascin production with or without rice powder in the liquid medium using three *Monascus* strains considering the strain diversity. Furthermore, the whey was fractionated to identify the components responsible for a higher yield of monascin production.

MATERIALS AND METHODS

Strains and Media

Strains used in this study were *M. purpureus* AHU 9085 from the culture collection of Hokkaido University, *M. pilosus* NBRC 4520 and *M. ruber* NBRC 32318 obtained from Biological Resource Center (Chiba, Japan). Those were grown on potato dextrose agar (PDA; Merck KGaA, Darmstadt, Germany) at 30°C for 10 days. Spore suspensions were prepared by adding 9.0 g L^{-1} sodium chloride solution into the grown culture and counted using a hemocytometer. One hundred microliters of the spore suspension (1.3×10^5 spores mL^{-1}) were inoculated into a 10 g experimental liquid medium in a 100-mL flask.

As the basal medium, 0.5 g of commercial rice powder containing 81.9% carbohydrate, 6.0% protein, 0.7% lipids (Kouta Shouten Co., Ltd., Ibaraki, Japan) was mixed with Milli-Q water and adjusted to pH 4.0 with hydrochloric acid to obtain 10 g of the working medium. To prepare the whey medium, 0.6 g of whey powder (Meiji Co., Ltd., Tokyo, Japan) was dissolved in Milli-Q water and adjusted to pH 4.0 with hydrochloric acid to obtain 10 g of the working medium.

As the alternative to whey, a total of 10 g of the basal medium was supplemented with 0.52 g of whey permeate powder (Meiji Co., Ltd.), in which the lactose level was adjusted to the equivalent to that of whey powder. Moreover, acid whey was prepared by acidifying of raw skim milk, obtained from the experimental farm in the Field Science Center for the Northern Biosphere in Hokkaido University, to pH 4.6 with 1 mol L^{-1} hydrochloric acid at 20°C, followed by the recovery of the centrifuged supernatant. The resulting acid whey (9.5 g) was mixed with 0.5 g of rice powder and adjusted to pH 4.0 with hydrochloric acid.

The amounts of lactose, vitamins, and minerals added to the basal medium are shown in Table 1, which were decided referred to the literature.[24] As for the minerals, we used a simulated milk ultrafiltrate (SMUF) developed by Jenness and Koops[25] to mimic the mineral composition in whey. According to the instructions, SMUF should be prepared by mixing SMUF I and SMUF II. Furthermore, we divided SMUF I into group i and group ii. Group i stands for citrate-compounds-free SMUF I whereas group ii contains SMUF I without the components of group i. We also set group iii, in which the citrates in group ii are replaced by phosphates to exclude the effect of citrates. The supplemented basal media were adjusted to pH 4.0 with hydrochloric acid. Ten grams of the resulting media were transferred into a 100-mL flask.

All the media used in this study were autoclaved at 121°C for 15 min. Cultivation was performed statically at 25°C for 10 days. The results of more than six preparations of each culture condition were subjected to the statistical analysis.

Chemicals

Lactose, KH_2PO_4, K_2SO_4 and tri-potassium citrate monohydrate were purchased from Kanto Chemical Co., Inc., Tokyo, Japan. Vitamins (VB1, VB2 and sodium(+)-pantothenate), KCl, K_2CO_3 and tri-sodium citrate dihydrate were from FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan and NaH_2PO_4 was from Nacalai Tesque, Inc., Kyoto, Japan.

Measurement of lactose in whey and whey permeate powder

Lactose content was measured by the Lane-Eynon method.[26]

Recovery and measurement of wet biomass

The culture products were filtered by a strainer to recover mycelia as the intracellular fraction. The mycelia were washed by pouring 20 mL Milli-Q water. After a brief absorption of excess water with a cloth, the samples were weighed to evaluate wet biomass. The filtrate was freeze-dried and ground into powder to evaluate it as the extracellular fraction. Intracellular and extracellular fractions were stored at -20°C until use.

Extraction and quantification of monascin

Extraction of monascin was performed according to the study of Li *et al* with some modifications.[27] Intracellular and extracellular fractions were suspended in 10 mL of 80% ethanol. After heating at 60°C for 1 h, the samples were centrifuged at 15,000 × g at 25°C for 10 min to recover the supernatant followed by filtration through filter paper (No. 5C, Advantec, Tokyo, Japan). The filtrate was evaporated under a vacuum and re-dissolved in acetonitrile. The suspension was filtered by a 0.5 μm syringe filter (TOSOH Corporation, Tokyo, Japan) and subjected to high-performance liquid chromatography (HPLC) analysis. The sum of the monascin amount in intracellular and extracellular fractions was expressed as total monascin production (μg). Monascin production efficiency (μg g^{-1}) was expressed as total monascin production per wet biomass.

High performance liquid chromatography (HPLC)

Determination of monascin was performed by HPLC according to Wu[28] with some modifications. The stationary phase of HPLC was a TSK gel ODS-100Z reversed-phase column (250 mm × 4.6 mm, particle diameter: 5 μm, TOSOH Corporation, Tokyo, Japan) with temperature setting at 40°C. The mobile phase was 62.5% acetonitrile and 37.5% Milli-Q water containing 0.05% trifluoroacetic acid. Ten microliters of the samples were injected and eluted isocratically at the flow rate of 1.0 mL min^{-1}. Standard curve was made using commercial monascin (Chengdu Biopurify Phytochemicals Ltd., Chengdu, China). Detection was carried out by a UV detector at 234 nm.

Statistical analysis

The values were analyzed using the Tukey-Kramer multiple comparison test or student's t-test. The data were analyzed by JMP software (version 16.1; SAS Institute, Inc., Tokyo, Japan). Differences were considered to be statistically significant at $P < 0.05$.

RESULTS

Effects of rice and whey on growth and monascin production of three *Monascus* strains

Table 2A shows the wet biomass, total monascin production, and production efficiency of three *Monascus* strains in the three media. Despite the varied monascin amounts found in these three media, rice, as well as whey, enabled *Monascus* to produce monascin while growing.

M. purpureus AHU 9085 grew much better in the basal medium than in whey, but the basal medium containing whey produced the highest biomass. Whey caused limited monascin production and reduced the production efficiency of monascin when it was supplied in the basal medium. Thus, the basal medium was the best, and the whey had a negative effect on monascin production by *M. purpureus* AHU 9085.

In contrast to *M. purpureus* AHU 9085, *M. pilosus* NBRC 4520 grew better in whey than in the basal medium. The highest biomass was recorded using the basal medium with whey. In terms of monascin production, whey was inferior to the basal medium, as was found in *M. purpureus* AHU 9085. The total monascin production increased when whey was added to the basal medium. However, the increase was due to the increase in fungal growth as the production efficiencies of the basal medium and the basal medium with whey powder were comparable. Thus, for *M. pilosus* NBRC 4520, whey contributed to growth rather than monascin production.

Unlike the other two strains, there was no significant difference between the wet biomass of the basal medium and that of the whey for *M. ruber* NBRC 32318. The basal medium with whey yielded the highest wet biomass likewise other two strains. In terms of monascin production, there was no significant difference between the basal medium and whey. However, the total monascin production and production efficiency in the basal medium with whey increased by 11.6- and 4.1-fold, respectively, compared to the basal medium. Thus, for *M. ruber* NBRC 32318, the basal medium with whey provided remarkably superior monascin production.

Table 2B shows the distribution of monascin in the three strains. With few exceptions, monascin was commonly located in the intracellular fraction. The ratio of intracellular to extracellular monascin tended to increase as the total production increased and reached 90% under high production conditions.

Thus, the effect of whey on growth and monascin production depends on the strain. Since adding whey to the basal medium was exclusively advantageous for *M. ruber* NBRC 32318, further investigation was conducted to identify the substances in whey that stimulate monascin production by this strain.

Tracing stimulative substances in whey

Table 3A shows the wet biomass, total monascin production, and production efficiency of *M. ruber* NBRC 32318 using three different types of whey to supplement the basal medium. The addition of whey permeate resulted in significantly lower biomass. Nevertheless, the total monascin production and production efficiency were comparable in these three groups. As acid whey and whey permeate are devoid of lactic starter and proteinaceous components compared to whey powder, these components were unlikely to be involved in the stimulation effect.

Table 3B shows the wet biomass, total monascin production, and production efficiency of *M. ruber* NBRC 32318 using lactose, minerals, and vitamins in whey. The difference was exclusively found in the basal medium supplemented with SMUF, which allowed significant growth promotion and increased monascin production. The total monascin production and production efficiency were increased 4.7- and 2.2-fold, respectively. Therefore, the stimulative components were likely to be included in SMUF.

Table 3C shows the wet biomass, total monascin production, and production efficiency of *M. ruber* NBRC 32318 using SMUF I and SMUF II. The addition of either SMUF I or II did not affect the biomass compared to the basal medium. However, the addition of SMUF I resulted in remarkable monascin production and the highest production efficiency, whereas that of SMUF II was comparable with that of the basal medium.

Subsequently, Group i, Group ii, and Group iii were used to determine the stimulative candidates for monascin production (Table 3D). An insignificant difference in wet biomass was found in these groups. Group i showed more stimulative effects than Groups ii and iii, which implied that neither citrate nor sodium ion supply affects monascin productivity. Nevertheless, the stimulative extent was reduced unless whole component of SMUF I was supplied. Thus, not a single component, but the mineral cocktail in SMUF I is the key component for stimulating the monascin production of *M. ruber* NBRC 32318.

Supply of SMUF I in the basal medium to other *Monascus* species

Table 4 shows the wet biomass, total monascin production, and production efficiency of the three strains in basal medium supplied with SMUF I. Adding SMUF I into the basal medium reduced the growth of *M. purpureus* AHU 9085. The monascin production and production efficiency of this strain also dramatically decreased. In contrast, there was no significant difference in the wet biomass, irrespective of the use of SMUF I, for *M. pilosus* NBRC 4520. Adding SMUF I led to a total monascin production twice as high as that in the basal medium, attributed to elevated production efficiency. For *M. ruber* NBRC 32318, the addition of SMUF I into the basal medium induced 6.7- and 6.4-fold increases in monascin production and production efficiency, respectively. Therefore, the supplementary effect of SMUF I for monascin production is highly dependent on the strain.

DISCUSSION

To determine the effect of whey on the secondary metabolite production, for monascin in particular, we surveyed three strains belonging to three representative *Monascus* species and applied three culture media that consisted of rice powder, whey powder, or

its mixture. Although the growth preference of the three strains toward rice and whey depended on the strains, it was equally promoted in the mixed culture. Monascin was predominately found in the intracellular fraction with variable yields depending on the strain and substrate. In this study, a remarkable increase of monascin production efficiency in the basal medium with whey was exclusively recognized in *M. ruber* NBRC 32318. Application of three types of whey or representative whey components to the basal medium revealed that the minerals in SMUF I were responsible for the stimulation of monascin production efficiency. The effect of SMUF I was stimulatory to some extent for *M. pilosus* NBRC 4520 as well. However, it was rather inhibitory for *M. purpureus* AHU 9085. Therefore, whey can be advantageous for some *Monascus* strains.

Many environmental factors affect the growth and secondary metabolite biosynthesis profiles of *Monascus.* Since yellow pigment production is likely to be promoted below pH 4,[29] we seeded the tested strains into acidic cultures. Although it was already known that the three strains used in this study could grow on whey solid substrate, the catabolic capability toward whey components depends on the strain, as shown in the biomass resulting from liquid whey cultivation. Consistent with previous studies, *M. purpureus* AHU 9085 and *M. pilosus* NBRC 4520 exhibited monascin production, and the former preferred the rice liquid medium to the whey liquid medium. In contrast, *M. ruber* NBRC 32318 showed comparable monascin production in rice and whey liquid cultures, which suggested that liquid cultivation is more suitable for monascin production than solid cultivation because monascin was undetectable when this strain was seeded on the whey solid medium (Huang and Li, unpublished results). It was noteworthy that applying whey to the basal medium yielded outstanding monascin production and production efficiency by *M. ruber* NBRC 32318. Thus, the effect of whey on growth and monascin production was proven to depend on the strain.

Further studies regarding the whey component responsible for monascin production were performed on *M. ruber* NBRC 32318. As the first step, the basal medium was supplied with whey of three different origins. Compared with acid whey, commercially available whey powder is often prepared from cheese whey, which contains additional components, such as C-terminal region peptide from κ-casein and lactic starter metabolites.[30] Meanwhile, whey permeate is devoid of the majority of whey proteins, which comprise 13% of the solid component of whey.[31, 32] No significant monascin productivity difference was found between the three types of whey; therefore, whey protein, the C-terminal region peptide from κ-casein, and metabolites of lactic starter bacteria are not involved in the stimulation of monascin production. Furthermore, lactose, which accounts for around 75% of the solid component of whey, and vitamins did not assist in monascin production. In contrast, the minerals in SMUF I exhibited a stimulative effect on monascin production and were associated with the highest monascin production efficiency. Accordingly, the components in whey responsible for the promotion of *M. ruber* NBRC 32318 monascin production were not specific to dairy products but to well-known minerals.

Further studies were conducted on the mineral component in whey related to monascin production by *M. ruber* NBRC 32318. Although many studies on the effect of minerals on *Monascus* yellow pigment production have been conducted using spectrophotometric analysis,[12, 13, 27] few studies have been concerned with specific monascin production analysis. Lung *et al.*[33] have reported that the addition of Mg, Ca, Zn, or Fe to water increased monascin production when dioscorea was subjected to solid-state fermentation

with *M. purpureus*. However, for monascin production of *M. ruber* NBRC 32318, the Ca and Mg in SMUF II showed no effect, or rather canceled the stimulation effect of SMUF I to yield production levels equal to the whole SMUF. Lin *et al.*[34] have found that removing KH_2PO_4 from a rice-containing liquid medium caused a marked decline in the growth and pigmentation of *M. pilosus*, which implies potassium has a crucial role. Although the working basal medium in this study was assumed to contain 0.75, 2.3, and 5.7 μmol of calcium, magnesium, and potassium, respectively[35] as the background, SMUF provided as much as 85, 30, and 353 μmol of calcium, magnesium, and potassium, respectively. Taking this difference into account, it is possible that potassium is crucial for monascin production by *M. ruber* NBRC 32318, whereas divalent metal ions are important for *M. purpureus*. Continuous studies are needed where minerals crucial to a secondary metabolite are present in the target substance and the strain applied.

To elucidate the species- or strain-dependence on monascin production resulting from minerals, molecular analysis, as well as synthetic media-based studies, are required. The biosynthetic pathways of monascin of both *M. ruber* and *M. purpureus* have been proposed with related pigment gene clusters.[36] Adjusting the culture condition leading to up-regulation of *mppE*, the gene encoding an enoyl reductase in *M. purpureus* related to the final conversion toward monascin formation could increase monascin production.[37, 38, 39] In contrast, the orthologue of *mppE*, *mrpigH* in *M. ruber*, has been proposed as less essential for monascin production.[40] Furthermore, in addition to the proposed biosynthesis pathways, the involvement of isozymes encoded on other gene clusters to provide some intermediate products in the monascin synthesis pathway has not been ruled out. For instance, *M. ruber* encodes MrPigA, whose function is predicted as polyketide synthase that assembles a hexaketide intermediate[36] at the early stage of *Monascus* pigment production, while four polyketide biosynthetic systems were found in a *M. ruber* strain.[21] Thus, it should be investigated that if the regulation of these alternative genes in the presence of some minerals to take part in monascin production.

Finally, the genetic background of the three edible strains used in this study should be discussed. Some studies have claimed *M. ruber* and *M. pilosus* could belong to the same clade,[41, 42] based on the high homology of the nucleotide sequence of the ITS region and partial β-tubulin genes, while *M. purpureus* could be classified in another. Higa *et al.*[21] have analyzed the secondary metabolite biosynthetic gene clusters and concluded that *M. purpureus* and *M. pilosus* are chemotaxonomically different, while *M. pilosus* and *M. ruber* have similar biosynthetic and secondary metabolite gene clusters. This was supported by another study that claimed the genetic organization of the *M. purpureus* pigment cluster is significantly different from that of *M. pilosus* and *M. ruber* in one region.[43] The results were consistent with our study: *M. pilosus* and *M. ruber* exhibited different degrees of stimulatory response, while *M. purpureus* showed a negative response to the same substrate for monascin production. Similar trends were also found in mycotoxin citrinin production, as citrinin was undetectable in all culture products of *M. pilosus* NBRC 4520 and *M. ruber* NBRC 32318 in our study, while *M. purpureus* AHU 9085 produced it under most of the culture conditions tested (data not shown). Thus, *M. purpureus* often exhibits discriminated traits from *M. pilosus* and *M. ruber*, along with the opposite trend of monascin production brought by supplementation of whey constituents.

In conclusion, the applicability of whey as the substrate component for monascin strongly depends on the strain. Although monascin production efficiency was higher in

rice liquid medium supplied with SMUF I than that with whey, whey provided the highest biomass resulting in the highest total monascin production. Thus, whey is a feasible supplementary component for monascin production for some *Monascus* strains.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

REFERENCES

1 Lin Y, Wang T, Lee M, Su N, Biologically active components and nutraceuticals in the *Monascus*-fermented rice: a review. *Appl Microbiol Biotechnol* **77**: 965-973 (2008).
2 Fukami H, Higa Y, Hisano T, Asano K, Hirata T, Nishibe S, A review of red yeast rice, a traditional fermented food in Japan and east Asia: its characteristic ingredients and application in the maintenance and improvement of health in lipid metabolism and the circulatory system. *Molecules* **26**: 1619 (2021).
3 Srianta I, Ristiarini S, Nugerahani I, Sen SK, Zhang BB, Xu GR, Blanc PJ, Recent research and development of *Monascus* fermentation products. *Int Food Res J* **21**: 1-12 (2014).
4 Patakova P, *Monascus* secondary metabolites: production and biological activity. *J Ind Microbiol Biotechnol* **40**: 169-181 (2013).
5 Mostafa ME and Abbady MS, Secondary metabolites and bioactivity of the *Monascus* pigments review article. *Glob J Biotechnol Biochem* **9**: 01-13 (2014).
6 Akihisa T, Tokuda H, Yasukawa K, Ukiya M, Kiyota A, Sakamoto N, Suzuki T, Tanabe N, Nishino H, Azaphilones, furanoisophthalides, and amino acids from the extracts of *Monascus pilosus*-fermented rice (red-mold rice) and their chemopreventive effects. *J Agric Food Chem* **53**: 562-565 (2005).
7 Liu H, Zhang J, Lu G, Wang F, Shu L, Xu H, Li Z, Wang Y, Guo Q, Wu S, Jiang L, Wang C, Huang D, Liu B, Comparative metabolomics analysis reveals the metabolic regulation mechanism of yellow pigment overproduction by *Monascus* using ammonium chloride as a nitrogen source. *Appl Microbiol Biotechnol* **105**: 6369-6379 (2021).
8 Zhang X, Liu C, Tian W, Zhang H, Li P, Wang J, He W, Theoretical and experimental investigation of the antioxidative activity of monascin. *Food Funct* **11**: 5915-5923 (2020).
9 Lin C, Lin T, Pan T, Alleviation of metabolic syndrome by monascin and ankaflavin: the perspective of *Monascus* functional foods. *Food Funct* **8**: 2102-2109 (2017).
10 Xu B, Jia X, Gu L, Sung C, Review on the qualitative and quantitative analysis of the mycotoxin citrinin. *Food Control* **17**: 271-285 (2006).
11 Feng Y, Shao Y, Chen F, *Monascus* pigments. *Appl Microbiol Biotechnol* **96**: 1421-1440 (2012).
12 Adin SN, Gupta I, Panda BP, Mujeeb M, Monascin and ankaflavin—biosynthesis from *Monascus purpureus*, production methods, pharmacological properties: A review. *Biotechnol Appl Biochem* (2022) https://doi.org/10.1002/bab.2336.
13 Zhang S, Zhao W, Nkechi O, Lu P, Bai J, Lin Q, Liu J, Utilization of low-cost agricultural by-product rice husk for *Monascus* pigments production via submerged batch-fermentation. *J Sci Food Agri* **102**: 2454-2463 (2021).
14 Srianta I, Kusdiyantini E, Zubaidah E, Ristiarini S, Nugerahani I, Alvin A, Iswanto N, Zhang B, Utilization of agro-industrial by-products in *Monascus* fermentation: A review. *Bioresour Bioprocess* **8**: 129 (2021).
15 Kantifedaki A, Kachrimanidou V, Mallouchos A, Papanikolaou S, Koutinas AA, Orange

processing waste valorisation for the production of bio-based pigments using the fungal strains *Monascus purpureus* and *Penicillium purpurogenum*. *J Clean Prod* **185**: 882-890 (2018).
16 Velmurugan P, Hur H, Balachandar V, Kamala-Kannan S, Lee KJ, Lee SM, Chae JC, Shea PJ, Oh BT, *Monascus* pigment production by solid-state fermentation with corn cob substrate. *J Biosci Bioeng* **112**: 590-594 (2011).
17 Silveira ST, Daroit DJ, Sant'Anna V, Brandelli A, Stability modeling of red pigments produced by *Monascus purpureus* in submerged cultivations with sugarcane bagasse. *Food Bioproc Tech* **6**: 1007-1014 (2013).
18 Da Costa JPV, De Oliveira CFD, Vendruscolo F, Cheese whey as a potential substrate for *Monascus* pigments production. *AIMS Agric Food* **5**: 785-798 (2020).
19 Mehri D, Perendeci NA, Goksungur Y, Utilization of whey for red pigment production by *Monascus purpureus* in submerged fermentation. *Fermentation* **7**: 75 (2021).
20 Kumura H, Ohtsuyama T, Matsusaki YH, Taitoh M, Koyanagi H, Kobayashi K, Hayakawa T, Wakamatsu J, Ishizuka S, Application of red pigment producing edible fungi for development of a novel type of functional cheese. *J Food Process Preserv* **42**: e13707 (2018).
21 Higa Y, Kim Y, Altaf-Ul-Amin M, Huang M, Ono N, Kanaya S, Divergence of metabolites in three phylogenetically close *Monascus* species (*M. pilosus*, M. *ruber*, and *M. purpureus*) based on secondary metabolite biosynthetic gene clusters. *BMC Genom* **21**: 679 (2020).
22 Takahashi M, Matsumoto T, Okonogi S. (In Japanese). *Nippon Shokuhin Kagaku Kogaku Kaishi* **51**: 67-71 (2004).
23 Chen D, Xue Y, Chen M, Chen M, Li Z, Wang C, Optimization of submerged fermentation medium for citrinin-free monascin production by *Monascus*. *Prep Biochem Biotechnol* **46**: 772-779 (2016).
24 Zadow JG (ed). *Whey and lactose processing*. Elsevier Applied Science, New York (1992).
25 Jenness R and Koops J, Preparation and properties of a salt solution which simulates milk ultrafiltrate. *Neth Milk Dairy J* **16**: 153-164 (1962).
26 Lane JH and Eynon L, Methods for determination of reducing and nonreducing sugars. *J Soc Chem Ind* **42**: 32-37 (1923).
27 Li W, Li Y, Yu W, Li A, Wang Y, Study on production of yellow pigment from potato fermented by *Monascus*. *Food Biosci* **50**: 102088 (2022).
28 Wu C, Kuo Y, Lee C, Hsu Y, Pan T, Synchronous high-performance liquid chromatography with a photodiode array detector and mass spectrometry for the determination of citrinin, monascin, ankaflavin, and the lactone and acid forms of monacolin K in red mold rice. *J AOAC Int* **94**: 179-190 (2011).
29 Shi K, Song D, Chen G, Pistolozzi M, Wu Z, Quan L, Controlling composition and color characteristics of *Monascus* pigments by pH and nitrogen sources in submerged fermentation. *J Biosci Bioeng* **120**: 145-154 (2015).
30 Dziuba J and Minkiewicz P, Influence of glycosylation on micelle-stabilizing ability and biological properties of C-terminal fragments of cow's κ-casein. *Int Diary J* **6**: 1017-1044 (1996).
31 Zhuang Y, Zhou W, Nguyen MH, Hourigan JA, Determination of protein content of whey powder using electrical conductivity measurement. *Int Diary J* **7**: 647-653 (1997).
32 Marwaha SS and Kennedy JF. Whey—pollution problem and potential utilization. *Int J Food Sci Technol* **23**: 323-336 (1988).
33 Lung T, Liao L, Wang J, Wei B, Huang P, Lee C, Metals of deep ocean water increase the anti-adipogenesis effect of *Monascus*-fermented product via modulating the monascin and ankaflavin production. *Mar Drugs* **14**: 106 (2016).
34 Lin W, Ting Y, Pan T, Proteomic response to intracellular proteins of *Monascus pilosus* grown under phosphate-limited complex medium with different growth rates and pigment production. *J Agric Food Chem* **55**: 467-474 (2007).
35 Ministry of Education, Culture, Sports, Science and Technology, Japan(eds). *Standard Tables of Food Composition in Japan* 2015, 7th edn.
http://www.mext.go.jp/a_menu/syokuhinseibun/1365297.htm.
36 Chen W, Chen R, Liu Q, He Y, He K, Ding X, Kang L, Guo X, Xie N, Zhou Y, Lu Y, Cox RJ, Molnár I, Li M, Shao Y, Chen F, Orange, red, yellow: biosynthesis of azaphilone pigments in *Monascus* fungi. *Chem Sci* **8**: 4917-4925 (2017).

37 Huang T, Wang M, Shi K, Chen G, Tian X. Wu Z, Metabolism and secretion of yellow pigment under high glucose stress with *Monascus ruber*. *AMB Express* **7**: 79 (2017).
38 Chen D, Wang Y, Chen M, Fan P, Li G, Wang C, Ammonium nitrate regulated the color characteristic changes of pigments in *Monascus purpureus* M9. *AMB Express* **11**: 3 (2021).
39 Balakrishnan B, Park SH, Kwon HJ, A reductase gene *mppE* controls yellow component production in azaphilone polyketide pathway of *Monascus. Biotechnol Lett* **39**: 163-169 (2017).
40 Li L, Xu N, Chen F, Inactivation of *mrpigH* gene in *Monascus ruber* M7 results in increased *Monascus* pigments and decreased citrinin with *mrpyrG* selection narker. *J Fungi* **7**: 1094 (2021).
41 Park HG, Stamenova EK, Jong SC. Phylogenetic relationships of *Monascus* species inferred from the ITS and the partial β-tubulin gene. *Bot Bull Acad Sin* **45**: 325-330 (2004).
42 Li Z, Liu Y, Li Y, Lin F, Wu L, Screening and identification of *Monascus* strains with high-yield monacolin K and undetectable citrinin by integration of HPLC analysis and *pksCT* and *ctnA* genes amplification. *J Appl Microbiol* **129**: 1410-1418 (2020).
43 Balakrishnan B, Chen C, Pan T, Kwon H, *Mpp7* controls regioselective Knoevenagel condensation during the biosynthesis of *Monascus* azaphilone pigments. *Tetrahedron Lett* **55**: 1640-1643 (2014).

461 Table 1 Components and the amount added in the basal medium

Components				Amount per 10 g medium
Whey constituents				
lactose				0.45 g
vitamins	vitamin B1			30 μg
	vitamin B2			15 μg
	sodium (+)-pantothenate			40 μg
SMUF	SMUF I	Group i	KH_2PO_4	110 μmol
			K_2SO_4	9.8 μmol
			K_2CO_3	21 μmol
			KCl	76 μmol
		Group ii	tripotassium citrate monohydrate	35 μmol
			trisodium citrate dihydrate	58 μmol
	SMUF II	$CaCl_2 \cdot 2H_2O$		85 μmol
		$MgCl_2 \cdot 6H_2O$		30 μmol
Others				
Group iii	KH_2PO_4			105 μmol
	NaH_2PO_4			173 μmol

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Table 2A Growth and monascin production of the three *Monascus* strains.

Medium/ Strain	*M. purpureus* AHU 9085	*M. pilosus* NBRC 4520	*M. ruber* NBRC 32318
	Wet biomass (g)		
basal medium	2.5 ± 0.1^{xa}	0.9 ± 0.1^{xb}	1.1 ± 0.1^{xb}
whey	0.9 ± 0.1^{ya}	1.5 ± 0.1^{yb}	1.3 ± 0.1^{xab}
basal medium + whey	4.3 ± 0.2^{za}	3.0 ± 0.1^{zb}	3.1 ± 0.1^{yb}
	Total monascin production (μg)		
basal medium	496.0 ± 43.2^{xa}	283.3 ± 19.0^{xb}	268.3 ± 13.2^{xb}
whey	4.9 ± 1.3^{ya}	30.1 ± 7.6^{xa}	206.6 ± 22.5^{xb}
basal medium + whey	335.8 ± 55.1^{za}	1023.9 ± 338.2^{ya}	3125.3 ± 243.9^{yb}
	Monascin production efficiency (μg g^{-1})		
basal medium	198.8 ± 19.9^{xa}	312.8 ± 34.5^{xb}	252.9 ± 14.2^{xab}
whey	5.3 ± 1.3^{ya}	20.3 ± 5.2^{ya}	176.1 ± 27.6^{xb}
basal medium + whey	77.1 ± 10.9^{za}	338.5 ± 110.3^{xa}	1028.6 ± 94.2^{yb}

Table 2B Distribution of monascin in the three *Monascus* strains.

Medium/ Location	Intracellular (μg)	Extracellular (μg)	Ratio (Intracellular/Extracellular)
	M. purpureus AHU 9085		
basal medium	463.5 ± 39.0^{x}	32.5 ± 4.9^{x}	93: 07
whey	1.3 ± 0.9^{y}	3.6 ± 0.8^{y}	27: 73
basal medium + whey	315.7 ± 56.3^{z}	20.1 ± 2.1^{z}	94: 06
	M. pilosus NBRC 4520		
basal medium	214.3 ± 21.0^{x}	68.9 ± 8.0^{xy}	76: 24
whey	18.5 ± 4.5^{x}	11.6 ± 3.1^{x}	61: 39
basal medium + whey	916.0 ± 312.1^{y}	107.9 ± 31.8^{y}	89: 11
	M. ruber NBRC 32318		
basal medium	214.3 ± 13.5^{x}	54.0 ± 3.6^{x}	80: 20
whey	158.3 ± 20.0^{x}	48.2 ± 4.3^{x}	77: 23
basal medium + whey	2812.8 ± 232.9^{y}	312.5 ± 22.6^{y}	90: 10

Value is expressed as mean ± SE (n = 6, except n = 26 of the basal medium in *M. ruber* NBRC 32318 and n = 15 of the basal medium with whey in *M. ruber* NBRC 32318).
x, y and z in a column indicate a significant difference between the media for the strains (Tukey-Kramer; $P < 0.05$).
a, b and c in a row indicate a significant difference between the strains for the media (Tukey-Kramer; $P < 0.05$).

Table 3A Growth and monascin production in the basal medium with different types of whey of *M. ruber* NBRC 32318

Component added in basal medium	n	wet biomass (g)	Total monascin production (μg)	Monascin production efficiency (μg g^{-1})
whey	15	3.1 ± 0.1^{x}	3125.3 ± 243.9^{x}	1028.6 ± 94.2^{x}
acid whey	6	3.1 ± 0.1^{x}	2998.3 ± 126.7^{x}	987.4 ± 74.6^{x}
whey permeate	6	2.3 ± 0.1^{y}	2438.6 ± 199.9^{x}	1077.3 ± 83.4^{x}

Table 3B Growth and monascin production in the basal medium with different components in whey of *M. ruber* NBRC 32318

Component added in basal medium	n	wet biomass (g)	Total monascin production (μg)	Monascin production efficiency (μg g^{-1})
basal medium (control)	26	1.1 ± 0.1^{x}	268.3 ± 13.2^{x}	252.9 ± 14.2^{x}
lactose	6	1.1 ± 0.1^{x}	341.4 ± 42.2^{x}	332.0 ± 48.9^{x}
SMUF	12	2.3 ± 0.1^{y}	1256.9 ± 82.2^{y}	553.6 ± 41.3^{y}
Vitamins	6	1.0 ± 0.1^{x}	282.9 ± 18.5^{x}	301.8 ± 20.6^{x}

Table 3C Growth and monascin production in the basal medium with different components in SMUF of *M. ruber* NBRC 32318

Component added in basal medium	n	wet biomass (g)	Total monascin production (μg)	Monascin production efficiency (μg g^{-1})
basal medium (control)	26	1.1 ± 0.1^{x}	268.3 ± 13.2^{x}	252.9 ± 14.2^{x}
SMUF	12	2.3 ± 0.1^{y}	1256.9 ± 82.2^{y}	553.6 ± 41.3^{y}
SMUF I	12	1.1 ± 0.1^{x}	1786.1 ± 143.2^{z}	1612.4 ± 90.8^{z}
SMUF II	6	1.1 ± 0.1^{x}	222.1 ± 19.5^{x}	206.1 ± 20.1^{x}

Table 3D Growth and monascin production in the basal medium with different components in SMUF I *M. ruber* NBRC 32318

Component added in basal medium	n	wet biomass (g)	Total monascin production (μg)	Monascin production efficiency (μg g^{-1})
basal medium (control)	26	1.1 ± 0.1^{x}	268.3 ± 13.2^{x}	252.9 ± 14.2^{x}
SMUF I	12	1.1 ± 0.1^{x}	1786.1 ± 143.2^{y}	1612.4 ± 90.8^{y}
Group i	6	1.3 ± 0.1^{x}	971.8 ± 60.4^{z}	763.2 ± 68.2^{z}
Group ii	6	1.0 ± 0.1^{x}	292.0 ± 18.6^{x}	292.7 ± 17.2^{x}
Group iii	6	1.1 ± 0.1^{x}	133.3 ± 26.2^{x}	124.2 ± 15.9^{x}

Value is expressed as mean ± SE.
x, y and z in a column indicate a significant difference (Tukey-Kramer; $P < 0.05$).

Table 4. Growth and monascin production in the basal medium with SMUF I of the three *Monascus* strains

Medium/strain	*M. purpureus* AHU 9085	*M. pilosus* NBRC 4520	*M. ruber* NBRC 32318
	Wet biomass (g)		
basal medium	2.5 ± 0.1^{x}	0.9 ± 0.1^{x}	1.1 ± 0.1^{x}
basal medium + SMUF I	1.9 ± 0.0^{y}	1.0 ± 0.1^{x}	1.1 ± 0.1^{x}
	Total monascin production (μg)		
basal medium	496.0 ± 43.2^{x}	283.3 ± 19.0^{x}	268.3 ± 13.2^{x}
basal medium + SMUF I	19.7 ± 7.9^{y}	594.5 ± 75.0^{y}	1786.1 ± 143.2^{y}
	Monascin production efficiency (μg g^{-1})		
basal medium	198.8 ± 19.9^{x}	312.8 ± 34.5^{x}	252.9 ± 14.2^{x}
basal medium + SMUF I	10.5 ± 4.3^{y}	631.8 ± 81.8^{y}	1612.4 ± 90.8^{y}

Value is expressed as mean ± SE (n = 6, except n = 26 of the basal medium in *M. ruber* NBRC 32318 and n = 12 of the basal medium with SMUF I in *M. ruber* NBRC 32318).
x and y in a column indicate a significant difference among the media for the strains (Student's t-test; $P < 0.05$).