International Journal of Science and Research (IJSR)
ISSN (Online): 2319-7064
Index Copernicus Value (2015): 78.96 | Impact Factor (2015): 6.391

Identification of Various Colored *Monascus* sp and its Biopigments and Citrinin Production in RMR

Susana Ristiarini[1], M. N. Cahyanto[2], Jaka Widada[3], Endang S. Rahayu[2*]

[1,2]Faculty of Agricultural Technology, Widya Mandala Surabaya Catholic University, Jl. Dinoyo 42, Surabaya 62825, Indonesia

[2]Department of Food and Agricultural Product Technology, Faculty of Agricultural Technology, Universitas Gadjah Mada, Jl. Flora No.1 Bulaksumur, Yogyakarta 55281, Indonesia

[3]Faculty of Agriculture, Universitas Gadjah Mada, Jl. Flora, Bulaksumur, Yogyakarta 55281, Indonesia

[2]Department of Food and Agricultural Product Technology, Faculty of Agricultural Technology, Universitas Gadjah Mada, Jl. Flora No.1 Bulaksumur, Yogyakarta 55281, Indonesia

Abstract: ***The isolation of the orange-colored Monascus spp (YK21A, YK27A, and PN29A) and white-colored Monascus spp (YK21B, YK27B, and PN29B) has been done from commercial red mold rice (RMR). The purpose of this study was to identify the species of Monascus spp and study the biosynthesis of pigments and citrinin in RMR. The species identification is done molecularly using amplification of the partial β-tubulin gene. Solid fermentation with rice substrate for 14 days was used to determine the biosynthesis of pigments and citrinin. Based on the results of molecular identification, it was known that both orange- and white-colored Monascus were Monascus purpureus. The total pigments of Monascus obtained from fermentation were 267.08-367.00 AU/g and 56.54 - 86.83 AU/g, with ˚hue values ranging from 25.4-32.0 and 34.4-49.2 respectively to orange and white ones. While the resulting citrinin was 11.44-113.68 ppm and 0.38-0.86 ppm respectively for the orange and white ones. Strong correlation between total pigment and citrinin (R^2 = 0.8046) and between hue and citrinin (R^2 = -0.8853) were indicated.***

Keywords: *Monascus purpureus*, pigments, ˚hue, citrinin**.**

1. Introduction

Monascus spp. are mostly used to make fermented products, such as *angkak* or red mold rice (RMR), rice wine, and kaoliang brandy in Asia countries. RMR is a fermentation product of *Monascus* with cooked rice as a substrate. During fermentation, *Monascus* sp. exhibits main secondary metabolites used for food colorant. RMR is not only used for a food supplement, but is also used to recover digestive system, promote blood circulation, and lower blood cholesterol levels [1-2].

Monascus is classified into *Ascomycetes* group and family *Monascaceae*. Generally, *Monascus* species used in producing RMR are *M. purpureus, M. pilosus*, *M. ruber*, *M. anka* or *M. kaoliang* [3-7] which also exhibit pigment. *Monascus* sp. could grow quickly on red yeast rice extract agar (RYREA) and malt extract agar (MEA) media, but grow slowly on potato dextrose agar (PDA) media. At the beginning phase of growth, *Monascus* sp. colony appears white, then turns into pale pink, red purple, or grayish black at mature phase depending on the species [8].

Pigment of *Monascus* is synthesized through polyketide pathway yielding hexaketide chromophore and through lipid biosynthesis pathway yielding β-ketoacid. The esterification of two compounds produces monascorubrin, an orange pigment [9]. Six classes of pigments synthesized by *Monascus* are monascorubrin and rubropunctatin (orange), ankaflavin and monascin (yellow), rubropunctamin and monascorubramin (red) [10-11].

Monascus spp. is also able to produce polyketide myctoxin, citrinin, that is nephrohepatotoxic, so widely that it can be a potential threat to the acceptance of RMR. The synthesis of citrinin is highly affected by microorganism type, culture medium, and fermentation condition [9, 12]. *M. purpureus* and *M. ruber* potentially produce similar citrinin, but differ with *M. pilosus*.

The isolation of *Monascus* sp. from commercial RMR in Indonesia on PDA media produces different *Monascus* colonies varying in color. Most of them are orange, and less of them are white. On this present study, the determination of species from two colored *Monascus* colonies and the identification of synthesis of pigment and citrinin in RMR production were done.

2. Material and Methods

2.1 Cultivation of the *Monascus* spp.

Six isolates from commercial angkak used in this study, namely *Monascus* sp. YK21A, *Monascus* sp. YK21B, *Monascus* sp. YK27A, *Monascus* sp. YK27B, *Monascus* sp. PN29C and *Monascus* sp. PN29B were cultivated on agar slant of Potato Dextrose Agar (PDA) medium, incubated at room temperature (28-30˚C) for 7 days, stored at 4˚C and rejuvenated every four weeks. As a reference culture for species identification, *Monascus purpureus* FNCC60008 obtained from Food and Nutrition Culture Collection, Universitas Gadjah Mada, Yogyakarta, Indonesia was used for validation.

2.2 Identification of *Monascus* spp.:

2.2.1 Isolation of genomic DNA:
The extraction of DNA genome for each isolates followed protocol of Cenis [13] with a slight modification as follows. The mycelia from 7-days *Monascus* in potato dextrose broth (PDB) were centrifuged at 13,000 rpm for 5 minutes until the pellet formation was done. The pellet was washed by 500 µL of buffer TE twice and then extraction buffer was decanted and the mycelia were grounded manually by using conical grinder. After that, Na-acetate was added and kept stored at freezer for 10 to 15 minutes at -20˚C. The mixture was centrifuged for 5 minutes and the supernatant contained DNA was transferred to a fresh tube and isopropanol (similar volume) was soaked into it, then kept at room temperature for 15 minutes. DNA pellet was centrifuged briefly and then washed by 70% ethanol. The pellet was dried by using vacuum drier and resuspended in 50 µL TE buffer.

2.2.2 DNA Amplification
DNA amplification was done according to[14] by polymerase chain reaction (PCR). Partial beta tubulin forward primer (5'-CAACTGGGCTAAGGGTCATT-3') and reverse primer (5'-GTGAACTCC ATCTCGTCCATA-3') were used to amplify DNA. The PCR was performed in a 25 µL reaction mixture containing 2 µL DNA (about 50 ng genomic of *Monascus* DNA as a template), 0.2 mM primer, 2 units Taq DNA polymerase, 800 mM dNTP, and free-RNAse aquabidest was added up to 25 µL. The amplification reaction was performed using Bio-RAD thermal cycler. The reaction was performed including pre-denaturation at 95°C for 2 minutes, followed by 30 successive cycles: denaturation at 95°C for 1 minute, annealing at 52°C for 30 seconds, extension at 72°C for 2 minutes, and final extension at 72°C for 10 minutes. An aliquot of the PCR amplification product was electrophoresed in a 1% agarose gel, stained and observed under ultraviolet light to compare with DNA ladder. The molecule size was approximately 10 kb. The sequencing was carried out by using two aforementioned primers.

2.2.3 Sequencing
DNA sequencing was performed at Laboratory of Sequencing Service, 1st Base-Malaysia. The sequencing was carried out by using primer partial β-tubulin. The sequencing results were checked and edited for making consensus sequences by using Bio-edit program, then were aligned with Clustal W to investigate the presence of sequence variations from samples obtained by using MEGA 6.0 program. DNA sequence was identified by using BLAST program on NCBI.

2.3 RMR preparation:

A long grain white rice IR64 was purchased from local market. Afterwards, it was washed under tap water and was wind-dried at 40°C until moisture content of 13.5% (according to initial state of moisture content). Fifty-grams of rice was transferred to Erlenmeyer flask and the surrounding relative humidity was set about 50%. The sterilization of rice was done using oven at 121°C for 15 minutes. After cooling, the substrates were inoculated with 10% of spore suspensions (1×10^5 spores per gram substrate) and then was re-incubated for 12 days at 30°C. After 12 days, the RMR was dehydrated in oven at 45°C for 12 to 14 hours. The dried RMR was weighed to determine dry mass percentage and next, it was grounded into fine powder for moisture content measurement [15]. The abovementioned procedure was performed in duplicate for 2 batches fermentation.

2.4 Color measurement

The color measurement of sample using Chroma Meter CR-400 followed the manual instructions of the manufacturer (Minolta Co. Ltd., Osaka, Japan). The Chroma Meter was calibrated with white color standard plate CR-400 prior to use. The color space reading included L* (lightness): a range of 0 (black) to 100 (white), a* (redness) indicating the direction of +/- (red/green) and b* (yellowness) indicating the direction of +/- (yellow/ blue). The hue angle (˚hue) was determined as ($\tan^{-1}$ b*/a*) and the chroma as $\sqrt{(a^2 + b^2)}$ showing the color and saturation of color, respectively [16, 17]. Each sample was measured thrice for each batch replication.

2.5 Pigments extraction:

RMR was extracted by diluting 75% ethanol at a ratio 1:5 (w/v). The mixture was shook at 200 rpm by using rotary shaker for 1 hour and then was sieved through Whatman No. 1 filter paper. The extract was centrifuged at 10,000 rpm for 5 minutes and measured using spectrophotometer (Shimadzu UV - 1280) at λ 400 nm for yellow pigment, 470 nm for orange pigment, and 500 nm for red pigment. More dilution was needed when the absorbance value exceeded a value of 1.00. The absorbance unit was expressed as OD value multiplied by dilution factor and volume extract per gram sample [18].

2.6 Citrinin measurement:

Citrinin quantification was done using ELISA method assisted by Ridascreen®Fast Citrinin (r-biopharm) procedure. Absorbance at λ 450 nm value was recorded in a micro plate reader (optic ivymen system 2100-C). The limit detection was 15 ppb. The absorbance values were converted to levels of citrinin by using the software of Ridasoft Win.NET - FAST citrinin. The tests were performed twice and the data were presented as a mean ± SD.

3. Results and Discussion

3.1 Identification of *Monascus* spp.

DNA genome from 6 isolates was successfully amplified and sequenced with a pair of partial beta tubulin primer. The sequence length obtained was 919 pb. Alignment from six isolates with Clustal W showed an absent variation of DNA sequence and they were identical with reference isolate used. The DNA sequencing results with beta tubulin primer confirmed that six isolates were *Monascus purpureus*. Similarity percentage as high as 99.9% indicated for *M. purpureus* CICC40269 (AC KP259286.1), *M. purpureus*

BCBC31747 (AC JX221439.1), *M. purpureus* BCBC31746 (AC JX221438.1), and *M. purpureus* BCBC31542 (AC JX221432.1). In short, *M. purpureus* was not always assigned with orange in color because the presence of 3 white-colored isolates (YK21B, YK27B dan PN29B) were also identified as *Monascus purpureus*. For another instance, the white-colored colony from *Monascus* species is *M. mucoroides* [3]. The distinctive feature among three isolates with *M. mucoroides* was ascospore size. The ascospore size of *M. mucoroides* (8 µm) was greater than that of *M. purpureus*.

3.2 The effect of colony appearance on RMR

From the results obtained, fermentation condition used on this present study did not significantly affected the yield of RMR (Table 1). It showed that all isolates were able to convert rice as a substrate to fermented products. Macroscopic balance during fermentation was briefly explained by [19] where the reaction between rice and O_2 are account for biomass, CO_2, H_2O, ethanol, and acetic acid. The PN29B isolate had the highest dry mass percentage which meant that it had lowest activity in substrate degradation. Those attributes were contrast to PN29C. Dry mass is absolutely affected by fermentation condition and *Monascus* strain. This present study obviously confirmed that dry mass was affected by *Monascus* strains.

Table 1: Dry mass and moisture content of RMR

Isolates	Dry mass (%)	Moisture (%)
YK21A	46.10 ± 2.63	8.69 ± 0.76
YK27A	48.98 ± 10.62	9.73 ± 0.09
PN29C	36.69 ± 2.89	9.78 ± 0.76
YK21B	38.05 ± 3.83	9.30 ± 1.49
YK27B	44.11 ± 2.84	8.51 ± 0.20
PN29B	55.18 ± 5.88	8.85 ± 0.90

3.3. Color value of RMR

The color difference observed in isolated colony showed the difference of pigment synthesis during fermentation (Fig. 1). A noticed difference could be observed among orange- and white-colored isolates of RMR. The difference was especially seen on lightness and hue values. The high value of lightness and hue showed pale red on RMR. In contrast, the low value of lightness and hue showed intense red on RMR. Pigment biosynthesis in producing colors on RMR was also similar (Fig. 2).

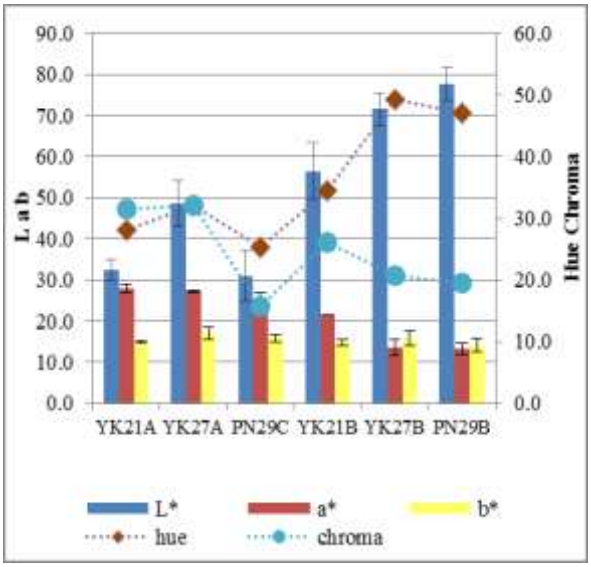

Figure 1: Color value L, a, b, hue, and chroma of RMR. Data were expressed as mean (n=3) with error bars as standard deviation.

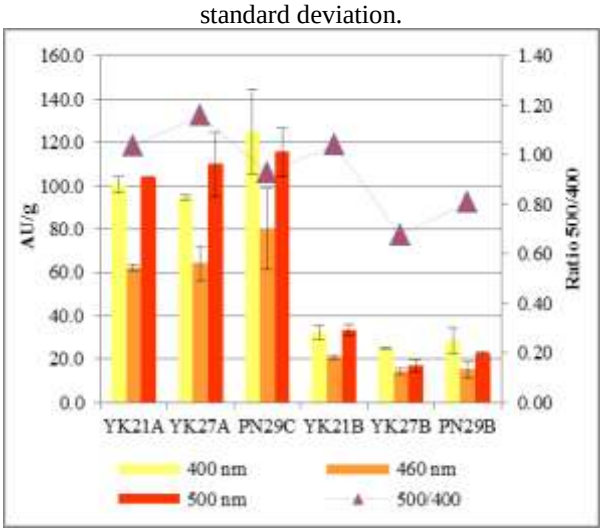

Figure 2: Color value of 75% ethanol extract of RMR. Data were expressed as mean (n=3) with error bars as standard deviation..

The ratio 500nm/400nm of YK21B isolate was more than 1 which meant that the extract was red, but the hue value of YK21B was higher than that of three orange-colored isolates of *Monascus*. Even though the value of ratio 500/400 was more than 1 but the total pigment was low, it could be summed up that the resulting color of RMR from YK21B isolate was pale red.

3.4 Citrinin synthesis

Citrinin content from colored *Monascus* colonies was shown in Table 2.

Table 2: Total Pigment and citrinin of RMR

Isolates	Total pigment (AU/g)	Citrinin (ppm)
YK21A	267.08 ± 5.05	27.14 ± 5.73
YK27A	269.32 ± 21.28	11.44 ± 10.17
PN29C	367.00 ± 22.00	113.68 ± 21.04
YK21B	86.83 ± 0.42	0.38 ± 0.17
YK27B	56.54 ± 3.98	0.86 ± 0.50
PN29B	62.31 ± 14.73	0.44 ± 0.11

The biosynthesis of pigment and citrinin obtained from six isolates showed a strong correlation. The correlation between total pigment synthesis and citrinin (R^2 = 0.8046) demonstrated that the capability of red-colored *Monascus* isolates in producing secondary metabolites were higher than that of white-colored ones because the red ones produced higher level of total pigment and citrinin than the white ones. Corresponding to the result, a strong association between hue and citrinin (R^2 = - 0.8853) was indicated. The lower the hue value (the redder the colony), the higher the citrinin content.

4. Conclusion

The white-colored *Monascus purpureus* was not capable to yield color and high pigment product. However, the result of present study suggested that white-colored *Monascus* isolate can be potentially used for further research in terms of production of other beneficial compounds.

5. Acknowledgment

The research was financially supported by UKWMS as a part of post graduate fund and doctoral research grant.

References

[1] Ma, J., Li, Y., Ye, Q., Li, J., Hua, Y., Ju, D., Zhang, D., Cooper, R. and Chang, M. "Constituents of red yeast rice, a traditional Chinese food and medicine". J. Agric. Food. Chem. 48 (11): 5220-5225, 2000.

[2] Venkateswaran, V. and Vijayalakshmi, G. "Finger millet (*Eleusine coracana*)–an economically viable source for antihypercholesterolemic metabolites production by *Monascus purpureus*". J. Food Sci. Technol. 47 (4): 426-431, 2010.

[3] Hawksworth, D. L., and Pitt, J. I. "A New Taxonomy for *Monascus* species based on cultural and microscopical characters". Aust. J. Bot. 31: 51-61, 1983.

[4] Suharna, N, Y. Kikuchi and T. Fukatsu. "Molecular phylogenetic analysis of *Monascus* fungi based on internal transcribed spacer region". Biotropia 24: 62-68, 2005.

[5] Lin, Y.L., Wang, T.H., Lee, M.H., and Su, N.W. "Biologically active components and nutraceuticals in the *Monascus*-fermented rice: a review". Appl. Microbiol. Biotechnol 77: 965-973, 2007.

[6] Samsudin, N.I.P. dan Abdullah, N. "Prevalence of viable *Monascus* van Tieghem species in fermented red rice (Hong Qu Mi) at consumer level in Selangor, Malaysia". Journal of Biochemistry, Microbiology and Biotechnology 2 (2): 57-60, 2014.

[7] Joaquin, J. L. P., Mangahas, J. A. V., Undan, J. Q., Undan, J. R., and Reyes, R. G.. "Molecular delineation of Monascus from central Luzon, Philippines based on Internal Transcribed Spacer (ITS) region". International Journal of Biology, Pharmacy and Allied Sciences 5 (10): 2581-2590, 2016

[8] Doughari, J. H. "The occurrence, properties and significance of citrinin mycotoxin". Plant Pathology & Microbiology 6: 321 doi: 10.4172/2157-7471.1000321, 2015.

[9] Hajjaj, H., Klaebe, A., Goma, G., Blanc, P.J., Barbier, E., and Francois, J. "Medium-chain fatty acids affect citrinin production in the filamentous fungus *Monascus ruber"*. Applied and Environmental Microbiology 66 (3): 1120-1125., 2000.

[10] Juzlova, P., Rezanka, T., Martinkova, L. and V. Kren. "Long-chain fatty acids from *Monascus purpureus*. Phytochemistry" 43 (1): 151-153, 1996.

[11] Wongjewboot, I., and Kongruang, S. "pH stability of ultrasonic Thai isolated *Monascus purpureus* pigments". International Journal of Bioscience, Biochemistry and Bioinformatics 1 (1): 79-83, 2011.

[12] Devi, P., D'Souza, L., Kamat, T., Rodrigues, C. and Naik, C.G. "Batch culture fermentation of *Penicillium chrysogenum* and a report on the isolation, purification, identification and antibiotic activity of citrinin". Indian Journal of Marine Sciences 38 (1): 38-44, 2009.

[13] Cenis, J.L. "Rapid extraction of fungal DNA for PCR amplification". Nucleic Acids Research, 20 (9): 2380. 1992.

[14] Park, H.G., Stamenova, E.K. and Jong, S.C. 2004. "Phylogenetic relationships of *Monascus* species inferred from the ITS and the partial β-tubulin gene". Bot. Bull. Acad. Sin 45: 325-330.

[15] AOAC Official Methods of Analysis. Association of Official Analytical Chemists (AOAC). Published by the Association of Official Analytical Chemist. Washington DC, USA. 1995.

[16] Fabre, C.E., Santerre, A.L., Loret, M.O., Baberian, R., Pareilleux, A., Goma, G., and Blanc, P.J. "Production and food application of the red pigments of *Monascus ruber*". Journal of Food Science 58 (5): 1099-1110, 1993.

[17] Teixeira, C.C.C., Teixeira, G.A. and Freitas, L.A.P. "Improving the production and CIELAB* color parameters of *Monascus ruber* pigments using a fractional factorial design". Asian Journal of Biomedical and Pharmaceutical Sciences 2 (15): 62-68, 2013.

[18] Dikshit, R., and Tallapragada, P. "*Monascus purpureus*: A potential source for natural pigment production". J. Microbiol. Biotech. Res. 1 (4): 164-174, 2011.

[19] Rosenblitt, A., Agosin, E., Delgado, J. and Ricardo, P-C. "Solid substrate fermentation of *Monascus purpureus*: Growth, carbon balance, and consistency analysis". Biotechnol. Prog. 16: 152-162, 2000.

Author Profile

Susana Ristiarini, Post graduate student at Food Science, Universitas Gadjah Mada, Yogyakarta, Indonesia. Lecturer at Widya Mandala Surabaya Catholic University, Indonesia.

Dr. M.N. Cahyanto, a lecturer of The Department of Food and Agricultural Product Technology, Faculty of Agricultural Technology, Universitas Gadjah Mada, Yogyakarta, Indonesia. He obtained his doctoral degree in Osaka University, Japan. His expertise in research yield about Fermentation and Bioprocess Engineering.

Jaka Widada, Ph.D., a lecturer at Department of agricultural microbiology, Faculty of Agriculture, Universitas Gadjah Mada, Yogyakarta. He is an expert in environmental biotechnology He obtained his doctoral degree in The University of Tokyo, Japan.

Prof. Dr. Endang S. Rahayu, M.Sc. a lecturer of The Department of Food and Agricultural Product Technology, Faculty of Agricultural Technology, Universitas Gadjah Mada, Yogyakarta, Indonesia. She obtained her doctoral degree in The University of Tokyo, Japan. She is an expert in the field of food microbiology