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The Production of Nata Colored by *Monascus purpureus* J1 Pigments as Functional Food

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Pigments from *Monascus* sp. may color nata. The mold also produces monacolin K that inhibits HMG-CoA reductase affecting the reduction of blood cholesterol. The aim of this study was to produce the colored nata containing monacolin K. The mold was isolated from commercial *angkak.* Potato sucrose (PS) and synthetic glucose (SG) media were used to ferment nata with *Monascus purpureus* J1. Fermentation of nata in PS medium produced red nata, while that in SG medium produced orange nata. The color of nata was similar to the color of supernatant. The optimum red production was obtained after five days of incubation, while the orange production increased until the 14th day. The color concentrations of the supernatant of PS medium containing nata (35.4 μg mL^{-1}) were lower than those without nata (12.4 μg mL^{-1}). The colors of nata looked darker than the color of the supernatant. The concentration of monacolin K in the red nata and the supernatant of PS medium were 0.6 μg mL^{-1} and 4.6 μg mL^{-1} respectively, while those in the orange nata and the supernatant of SG medium were 3.2 μg mL^{-1} and 14.6 μg mL^{-1} respectively. Dry matter biomass in the PS medium was lower than that in the SG medium. Even though the color of nata looked relatively stable, analyses of the nata water extract that showed a stable condition only occurred in freezing (-20°C) and soaking in buffer solution of pH 12; boiling, water washing, and soaking in a solution of pH 3 and 7 reduced the pigment concentration. Monacolin K concentration was not stable for every treatment, especially for water washing and freezing. Eventhough it was not stable, the boiling nata contained red pigments and monacolin K of 19.7 μg mL^{-1} and 0.1 μg mL^{-1} respectively, which can be served as functional food.

Key words: colored-nata, *Monascus purpureus*, monacolin K

Functional food is currently getting more popular as a kind of food that is delicious as well as nutritious. It is expected to contain substances for both health and sickness prevention. Nata is a kind of functional food that contains bacterial cellulose from *Acetobacter xylinum*. It is jelly like, white, and chewy. The cellulose is important for water absorption in the colon. Nata is produced in coconut water (Colvin *et al*. 1977), and it cannot absorb synthetic food coloring compounds; however, it was reported that it can be colored by the red pigments of *Monascus* spp. (angkak) (Ng and Shyu 2004; Ng *et al*. 2004). There are three kinds of pigments produced from *Monascus*, namely the yellow pigments (monascin and ankaflavin), the orange pigments (monascorubin and rubropunctatin), and the red pigments (monascorubramin and rubropunctamin) (Juzlova *et al*. 1996). The pigment production occurs during the mold fermentation and is influenced by the type of substrate, pH and light (Hajjaj *et al*. 1997; Babitha *et al*. 2008).

Pigments from *M. purpureus* are stable and suitable for food coloring (Fink-Gremmels *et al*. 1991; Fabre *et al*. 1993; Juzlova *et al*. 1996). When nata is fermented with *Monascus*, its mycelia will fill the nata pores which then produce colors. The pigments of *Monascus* nata complex were more stable when washed, heated, frozen, and soaked in acid and alkali conditions than when they were colored with Cochineal Red A, a synthetic coloring agent (Ng *et al.* 2004). Different coloring nata will produce pretty gel, especially if it is used for dessert.

Another functional compound of *Monascus* is monacolin K that inhibits HMG-CoA reductase which affects the reduction of blood cholesterol. The Food and Drug Administration approved the application of monacolin K as anti hypercholesterolemic (FDA 1998). The fermentation process of *Monascus* nata gel produces functional nata gel containing cellulose, pigments and monacolin K, as well as colored liquid. The liquid might be separated as another coloring agent.

The aim of this study was to produce different colored nata containing monacolin K in the natural medium of potato sucrose (PS) and synthetic medium of glucose (SG), as well as to evaluate the stability of its pigments and monacolin K.

MATERIALS AND METHODS

***Monascus* sp. Isolates and Nata.** *Monascus* sp. was isolated from 3 commercial angkak collected from Bogor, Cibinong, and Jakarta. Nata was freshly collected from the culture without boiling from Bogor home industries.

Isolation of *Monascus* sp. from Angkak. Angkak (0.3 g) was blended in 3 ml of 0.85% NaCl solution (w/v), and the suspension was spread on PSA [20% (v/v) potato extract, 2% (w/v) sucrose, 1.5% (w/v) agar, and 0.3% (w/v) yeast extract at pH 6.25-6.30], and incubated at 30°C for seven days. The best isolate which grew best and produced pigments will be selected for one spore isolation. The isolate was suspended in 100 x dilution, centrifuged at 900 x g for 1 minute, spread on PSA and incubated at 30°C for seven days. Three colonies separately grown from one spore were subcultured on PSA slants. Each isolate was inoculated in PS medium and repeated three times. The most stable strain in repeated coloring formation and fast red pigment production was selected for further experiments. Later the species of the best strain was morphologically analysed and identified as *M. purpureus* J1.

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Production of Coloring Nata and Pigments in Liquid Media with *M. purpureus* J1. Two kinds of liquid media, PS and SG were conducted to observe the pattern of pigment production. The media were prepared for 50 mL in 250 mL flasks, 6 flasks for each medium. The PS medium was prepared like PSA but without agar. The SG medium was prepared following Ng and Shyu (2004), which were 5% (w/v) glucose, 0.05% (w/v) KCl, 0.01% (w/v) $MgSO_4$, 0.001% (w/v) $ZnSO_4$, and 0.05% (w/v) $CaCl_2$ at pH 7, modified with addition of 1.5% (w/v) NH_4Cl and 0.05% (w/v) yeast extract. Long nata ± 3.0 x 0.3 x 0.2 cm (25 g) was incorporated in 3 flasks of each medium. Thus, every treatment was repeated three times. All flasks were autoclaved at 121°C for 15 minutes, cooled and inoculated with *M. purpureus* J1 grown on PSA slants for 8 days. The flasks were incubated at 150 rpm, 30°C for 14 days. Pigments on the culture supernatant were measured at 2, 3, 5, 7, 12 and 14 days of incubation time.

The second experiment was conducted to observe the monacolin K production. The media, inoculum and nata were prepared by following the first experiment, except that the analyses were conducted on the 5th day of incubation. For the 2nd experiment, monacolin K was determined in the nata and culture supernatant, while dry biomass content was only determined in the culture supernatant.

Stability of Colored Nata and Monacolin K. The stability experiment was only conducted for the nata from the PS medium prepared in 14 flasks within 5 days of incubation time, which produced 350 g of red nata. The nata was divided into 7 treatments: washing, boiling, freezing and soaking at pH 3, 7 and 12, and the control without any treatments but stored at room temperature for 48 hours. The nata was washed by running water for 48 hours. For heating treatment, the nata was boiled for 15 minutes. For freezing treatment, the nata was frozen at -20°C for 24 hours, while for analysing the pH stability, the nata was soaked in citric acid buffer (pH 3), phosphate buffer (pH 7), and glycine buffer (pH 12) for 48 hours.

After each treatment, the nata was dried and blended in 40 ml of dH_2O for 8 minutes. The pigment and monacolin K concentrations of the extracts were determined. The relative concentration was calculated toward the concentration of the control (untreated nata).

Pigment Analysis. The pigment concentration of the supernatant or nata extract was determined using spectrophotometer after centrifuging at 15 000 x g for 10 minutes. Analyses were determined for yellow, orange, and red pigments using Quinollin Yellow (at 400 nm), Sunset Yellow (at 487 nm), and Amaranth (at 500 nm) respectively as standards. Standard curves were prepared at concentration of 2-16 μg mL^{-1}.

Analysis of Monacolin K. The analysis was conducted following that of Ng *et al.* (2004), modified in the extraction solvent as n-hexane. The colored nata (50 g) was dried by using a vacuum oven, blended and homogenized in 40 mL of dH_20. The water extract or free cell culture supernatant was then mixed with 40 mL of n-hexane and homogenized again for 8 minutes. The free cell culture supernatant was obtained after centrifuging at 15 000 x g for 10 minutes. The top phase of the mixture of water and hexane extract was collected and dried by using a vacuum oven. The dry compound was solubilized in 1 mL of acetonitrile and was ready for HPLC analysis (Agilent 1100). The running phase contained acetonitrile and ddH_2O (75:25 v/v), with flowing speed of 1 mL $minute^{-1}$, injection volume of 30 μL, and detected at UV 240 nm. The concentrations were calculated using monacolin K standard (Mevinolin from Sigma) at 0.1-0.5 mg mL^{-1} concentration.

Cell Biomass. The free nata cultured (4 mL) from the PS or the SG medium was centrifuged at 15 000 x g for 10 minutes. The pellet was dried by using a vacuum oven, and weighed. The dry weight expressed the dry biomass of mycelia and spores of the *M. purpureus* J1 present in the supernatant.

RESULTS

Isolation of *Monascus purpureus* from Angkak. Based on the growth rate and color formation, strain TP-a isolated from angkak from the Bogor market, Bogor, West Java, was observed as the best isolate. Further isolation revealed three separate colonies grown from one spore, namely the strains M1, J1, dan P1. *Monascus* purpureus J1 was selected for further experiment due to its high growth rate and production of stable pigments.

Red was observed in the supernatant of *M. purpureus* J1 when grown in the liquid of PS medium, while orange was observed in the liquid of SG medium. The expressed color in the culture supernatant came from the mixture of three pigments: yellow, orange, and red (Fig 1 and 2). The optimum concentrations of yellow, orange, and red pigments in the supernatant of PS medium with nata and without nata were observed after 5 days of incubation time (Fig 1a and b). The pigment concentrations were higher in the culture without nata addition than those with nata addition. In the supernatant of *M. purpureus* J1 grown in PS medium with nata addition, the yellow and orange pigments (13.5 and 13.4 μg mL^{-1}) were relatively similar and higher than the red pigments (12.4 μg mL^{-1}), while in that without nata addition, the orange and red pigments (31.1and 30.8 μg mL^{-1}) were higher than the yellow ones (26.4 μg mL^{-1}).

The yellow pigments appeared dominant in the culture of SG medium, while orange and red were less dominant. The optimum pigment production in the culture supernatant of SG medium was found after the 12th day (Fig 2b), while in the media added with nata the pigment concentration was still increasing on the 14th day (Fig 2a). The pigments in the SG medium with and without nata sharply increased on the 12th day. Pigment concentrations in SG medium without nata addition were higher than that with nata addition (Fig 2). On the 12th day, the orange pigments in the supernatant of SG medium without nata achieved 23.9 μg mL^{-1}, while in the nata added supernatant it was 12.4 μg mL^{-1}.

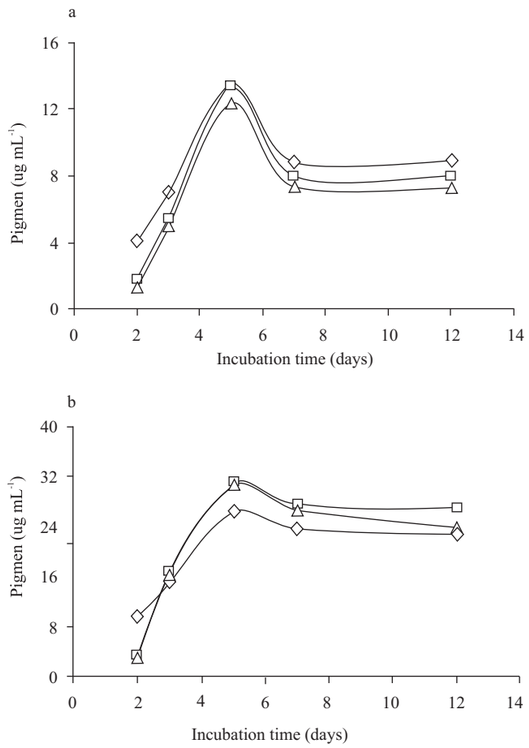

Fig 1 Color formation of *Monascus purpureus* J1 along the course of incubation time on media potato sucrose: a, with addition of nata and b, without nata. Yellow pigments (◊), orange pigments (□) and red pigments (Δ).

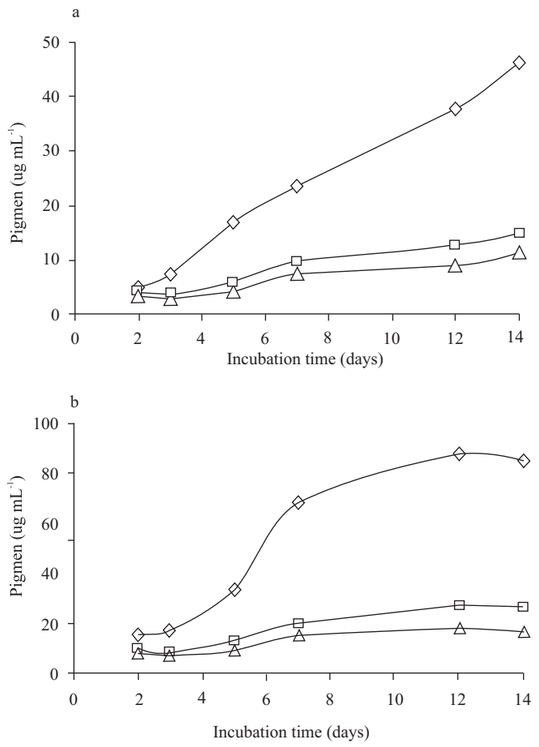

Fig 2 Color formation of *Monascus purpureus* J1 along the course of incubation time on media synthetic glucose: a, with addition of nata and b, without nata. Yellow pigments (◊), orange pigments (□) and red pigments (Δ).

Fig 3 Nata fermented with *Monascus purpureus* J1 on liquid media: a, potato sucrose and b, synthetic glucose.

The nata fermented in the PS medium appeared red (Fig 3a), while in the SG medium the nata was orange (Fig 3b). In the supernatant of SG medium, the yellow pigments were high (33.3 μg mL^{-1}), while the red pigments were low (9.4 μg mL^{-1}). In the supernatant of the PS medium, the yellow pigment content (26.4 μg mL^{-1}) was relatively similar to the red pigment content (30.8 μg mL^{-1}) (Table 1).

Monacolin K concentration in the supernatant of PS medium (4.6 μg mL^{-1}) was lower than that of the SG medium (14.6 μg mL^{-1}). The monacolin K concentration of nata in PS medium (0.6 μg mL^{-1}) was lower than that in SG medium (3.2 μg mL^{-1}), while monacolin K concentrations of the nata in both media were lower than their supernatant (Table 1). The cell biomass of *M. purpureus* J1 in the supernatant of PS medium with and without nata were 3.50 μg mL^{-1} and 5.50 μg mL^{-1} respectively (Table 1), while that of the SG medium with and without nata were 10.40 μg mL^{-1} and 9.70 μg mL^{-1} respectively.

During visual observation, the color of nata did not change in almost all treatments, such as washing, freezing and soaking at different pHs, except after boiling which showed a lighter color. However, the spectrophotometric data of the dH_2O nata extract showed different results (Table 2). All treatments decreased the pigment and monacolin K concentrations. The monacolin K concentrations were more greatly reduced than those of the pigment concentrations. Washing and freezing destroyed almost all monacolin K concentrations. The lowest value due to highest decrease of pigment concentrations was observed after soaking at pH 3, which gave 73.1, 71.4 and 67.1% reduction for yellow, orange and red pigments respectively. Other treatments reduced the pigment concentration by 37-50%. The pigments were the least decreased after freezing and soaking at pH 12; however, freezing destroyed monacolin K. The highest monacolin K concentration (the least reduction) was observed after soaking at pH 7; however, the reduction reached 83%.

Table 1 Pigment and monacolin K concentrations of nata and culture supernatant, and dry cell biomass of media fermented with *M. purpureus* J1

Treatment	Color	Pigment (μg mL^{-1})*			Monacolin K (μg mL^{-1})		Biomass (mg mL^{-1})
		Yellow	Orange	Red	Nata	Supernatant	
PS - Nata	Red	26.4	31.1	30.8	ND	ND	5.5
PS + Nata	Red	13.5	13.4	12.4	0.6	4.6	3.5
SG - Nata	Orange	33.3	13.2	9.4	ND	ND	9.7
SG + Nata	Orange	16.8	5.9	4.2	3.2	14.6	10.4

*Pigment detected from culture supernatant. ND:Not determined; PS: liquid medium of potato sucrose, SG: liquid medium of synthetic glucose.

DISCUSSION

Isolation of mold from one spore should be conducted to obtain pure isolate that produces stable pigments and monacolin K. In the preliminary experiment it was difficult to have homogeneity in the repeated samples (unpublished data). *M. purpureus* J1 appeared red when grown in PS medium, and orange in SG medium. The same colors were also visualized both in the supernatant and fermented nata. It is possible that the pigments produced by the mold in the liquid medium diffused into the nata. Another possibility is that the mold mycelia in the nata pores produced similar pigments depending on the type of carbon source (Ng *et al*. 2004). Even though the culture showed only red and orange, the spectrophotometer data showed the observed color originated from a mixture of three pigments: yellow, orange and red (Juzlova *et al*. 1996; Danuri 2008). The orange observed originated not only from the orange pigments, but also from the mixture of yellow and red pigments. Therefore, the orange supernatant and nata in the glucose medium was a result of the high yellow pigment concentrations (Fig 3, Table 1).

Table 2 Relative pigment and monacolin K concentrations of the treated nata expressed as % of control*

Treatment	Relative concentrations (%)			
	Pigment			Monacolin K
	Yellow	Orange	Red	
Control	100.0	100.0	100.0	100.0
Washing	52.6	49.5	52.4	≈ 0.0
Boiling	65.3	63.2	64.3	3.2
Freezing	99.0	93.6	95.1	≈ 0.0
Soaking in pH 3	36.9	28.6	32.9	15.1
Soaking in pH 7	59.6	59.4	60.9	17.0
Soaking in pH 12	98.9	78.7	98.1	9.5

*Control was untreated.

Different medium compositions from different carbohydrate sources produced different pigment concentrations and compositions as well as cell biomass and monacolin K concentrations. In this experiment 5% glucose produced more pigments, cell biomass and monacolin K than 2% of sucrose and potato extract (extract from 20% raw potato). Even though there was a report on induction of monacolin K production by more complex carbohydrate than that by simple sugar (glucose) which repressed the production (Miyake *et al.* 2006), a different result was observed in our experiment. Our data agreed with Danuri's (2008), who reported that better pigment production came from rice which contains higher amylase than amylopectin. The amyloserich rice is more degradable in producing glucose than amylopectin is. Addition of minerals also influenced the production of cells, pigments, and monacolin K. Higher concentration of pigments in the GS medium produced higher monacolin K due to similar synthesis pathway of these molecules. Both molecules have the same precursor, monoketide (Hajjaj *et al.* 1997).

The nata in SG medium produced a yellow color on the third day of incubation, and appeared orange on the fourth day of incubation. (Fig 2b). This result did not agree with Ng and Shyu's report (2004), which showed that the red nata was obtained in the glucose medium. Monacolin K concentration in colored nata from this experiment was much lower compared with the nata from Ng *et al*. (2004), which was 3.2 vs 157 μg mL^{-1}. These might be related to the different *Monascus* strains and different media compositions used.

Different results were reported on the stability of colored nata. According to Lin *et al.* (1992) this experiment showed that soaking at pH 12 did almost not reduce pigment concentration, while, Ng *et al.* (2004) reported an instability following this treatment. Pigment concentrations in the nata decreased when boiled, washed, and soaked at pH 7, especially when soaked in acid conditions (pH 3) in this experiment. Ng *et al.* (2004) reported that the pigments in the nata were stable when washed. Pigment concentration in the nata decreased after all treatments, but the pigments were still bound in the nata pores affecting the color of treated nata which was visually not different with the color of the control. The color of nata is visually quite stable, thus pigments from *M. purpureus* J1 is good for coloring nata products.

Monacolin K in this experiment was not stable when washed, boiled and frozen. This data did not agree with the result of Ng *et al*. (2004), which showed that monacolin K in nata was not stable when heated and frozen, but stable when washed. In our experiment, monacolin K was also not stable when soaked in different pHs, which is different from what was reported by Ng *et al*. (2004). In their study, monacolin K stability was relatively not affected by pH changes. Monacolin K instablity when heated might be due to the damaged molecule that occurred through oxidation, while the damage in freezing is caused by ice crystal formation. The decrease of monacolin K concentration when washed

and soaked at neutral pH was caused by the solubility of the pigment transfer from the nata to the buffer. Beside the solubility, soaking in acid and alkali conditions (pH 3 and 12, respectively) might damage the molecules. This experiment implied that nata could be boiled for a limited time, and ultra-heat treatment might be more protective for the monacolin K. Boiling treatment is in fact a necessity to preserve the colored nata. The boiled nata will contain cellulose, angkak pigments, and monacolin K, and therefore, could be referred to as functional food. The colored nata should better be stored in moderate temperature of 4-25^{o}C, and does not need to be washed.

Monascus purpureus J1 isolated from commercial angkak makes the nata red when fermented in potato sucrose medium and orange in synthetic glucose medium. The color does not only come from the mycelia grown in the gel, but may have resulted from the diffusion of the pigment compounds from the fermentation liquid into the nata.

The red and orange nata are functional food containing cellulose, organic pigments, and monacolin K. The functions of cellulose and monacolin K in human have been discussed, while the function of pigments is not reported. The pigments of angkak are also polyketides that might be easily oxydixed and function as an antioxidant.

Monacolin K concentration of the orange nata was higher than that of the red nata. Pigment and monacolin K production are influenced by media composition and *Monascus* strain. The high monacolin K content might be correlated with high yellow pigments and cell biomass. These results also gave more information than that reported in Ng *et al*. (2004) which only produced red nata.

Although the colored nata visually looks stable, the concentrations of the pigments are decreased when washed, boiled and soaked in acid and alkaline pHs. In addition, monacolin K is not stable to boiling and freezing, which reduced monacolin K significantly. Washing and soaking in pH 3, 7 and 12 also reduced the monacolin K concentrations. Boiling nata still makes it red which contains the three pigments, and monacolin K; therefore, it can be applied in the production of this functional food.

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