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Studies on the effect of methionine level on cheese colour as a solid substrate of *Monascus purpureus* JK2 fermentation

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Abstract

Monascus purpureus, a species of red filamentous mould, has been widely used in fermented food as a potential source of natural pigments. The pigments can be produced by solid-state fermentation using a solid culture substrate; an example includes cheese that provides a substantial amount of nutrients required for its growth and red pigment synthesis. *Monascus purpureus* needs appropriate organic nitrogen in the form of amino acids at a particular concentration. This study aimed to determine the effects of methionine level on cheese colour as the solid substrate in *M. purpureus* fermentation. The mould strain *M. purpureus* JK2 was cultivated from cheese without the addition of methionine and added with 0.1, 0.3, 0.5, and 1% methionine, then incubated at room temperature (25–30^{o}C) for 14 days. The growth of *M. purpureus* JK2 in cheese as a solid substrate was visually observed. Colour changes were distinguished quantitatively based on the CIELAB colour characterization system. The results showed that *M. purpureus* JK2 could grow and synthesize red pigments well in cheese as a substrate without the addition of methionine, which otherwise slowly decreased (0.1, 0.3 and 5% methionine) and inhibited the growth (1% methionine). The cheese substrate without the addition of methionine had a similar hue angle to 0.1 and 0.3% methionine and was relatively redder than with 0.5% methionine. However, the addition of 1% methionine did not induce pigmentation. Furthermore, the *M. purpureus* JK2-fermented cheese as a substrate can be used in cheese making development as culture products.

1. Introduction

Monascus are species of moulds frequently found discovered in fermented food, foodstuffs rich in starch, mouldy high-moisture food, mouldy silages, and soil (Wang and Lin, 2007), and naturally exist in dairy products (Manan *et al*., 2017). Their mycelial growth and concomitant pigment synthesis depend on the availability of nutrients in the form of carbon and nitrogen sources. Glucose is considered the most common and best carbon source for pigment growth and formation, but *M. purpureus-purplish*-red moulds-can use some other carbon sources, such as fructose, galactose, maltose, sucrose, mannitol, glycerol, raffinose hydrate, xylose, ribose, starch, lactose, and hydrolyzed lactose (Omamor *et al*., 2008; Chatterjee *et al*., 2009; Yang *et al*., 2015; da Costa and Vendruscolo, 2017).

Nitrogen plays a crucial role in cellular synthesis through which pigments are produced. Some studies report several favourable nitrogen sources for pigment formation with different effects on the resultant pigments, such as potassium nitrate (KNO_3), ammonium nitrate (NH_4NO_3), ammonium sulfate $(NH_4)_2SO_4$, monosodium glutamate (MSG), peptone, ammonium chloride (NH_4Cl), and sodium nitrate ($NaNO_3$). Many amino acids have also been added in fermentation to enhance pigment production, including DL-alanine, DL-2-amino-n butyric acid, L-arginine-mono-hydrochloride, DL-aspartic acid, L-cysteine monohydrochloride, L-cysteine, 3-(3,4-dihydroxy phenyl) DL-alanine-L-glutamic acid, glycine, L-histidine monohydrochloride, L -hydroxyl proline, L-leucine, L-isoleucine, DL-norleucine, L-lysine monohydrochloride, DL-ornithine monohydrochloride, L-proline, DL-β-phenylalanine, D-

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serine, L-tyrosine, DL-valine-DL-threonine, DL-tryptophan, and L-methionine. Methionine can provide S -adenosyl methionine (SAM), which is a precursor of secondary metabolites: pigments, monacolin K, and citrinin (Chatterjee *et al*., 2009; Jirasatid *et al*., 2013; Tallapragada *et al*., 2017).

Monascus purpureus produces six pigments that can be grouped into three pairs: orange (rubropunctatin and monascorubrin), red (rubropunctamine and monascorubramine), and yellow (ankaflavin and monascin) (Wang and Lin, 2007). The red pigment (monascorubramine) is highly favoured in fortified food production both as a natural food supplement, in the form of ground red rice, and a colourant, in the form of extract (Chatterjee *et al*., 2009). Most studies focus on red pigment production from the mould through solid-state fermentation with rice as substrate (Lotong and Suwanarit, 1990; Yang *et al*., 2015; Saithong *et al*., 2019). Besides rice, there are other substrates studied for *M. purpureus* fermentation, namely Dioscorea tubers (Lee *et al*., 2007), adlay (Chinese pearl barley, *Coix lachrymajobi* L) (Pattanagul *et al*., 2008), jackfruit seeds (Subhasree *et al*., 2011), and sorghum (Srianta and Harjono, 2015). Also, the cultivation of *M. purpureus* can produce red pigments on soy protein and whey cheese (culture media) through submerged fermentation (Lopes *et al*., 2013).

Substrates provide nutrients for microbial growth in culture and serve as the place to contain the cell. Solid-state fermentation creates a highly adequate habitat for fungi, which, due to their hyphal growth, can propagate on the surface and penetrate a substrate, thus raising pigment productivity. However, not all carbon and nitrogen sources provided by a substrate support the growth of *M. purpureus* and metabolite synthesis, including red pigment production. Regardless of type and concentration, organic nitrogen in the form of amino acids is known to severely affect fermentation parameters (Tallapragada *et al.,* 2017; Agboyibor *et al*., 2018).

Currently, there is no study report on the utilization of methionine-added cheese as a solid substrate for *M. purpureus* growth. This study aimed to determine the effect of methionine level on the colour of cheese as a substrate throughout the fermentation process. The research product is expected to create a more straightforward culture product preparation method for developing *Monascus*-ripened cheese (red mould cheese).

2. Materials and methods

2.1 Microorganisms

The microorganism used in this work was *Monascus purpureus* JK2, obtained from Widya Mandala University, Surabaya, Indonesia, that was isolated from red yeast rice at a pharmacy shop in Jakarta. This strain was cultivated on potato dextrose agar (PDA; Merck) slants (previously sterilized at 121^{o}C for 15 mins) at room temperature (25–30^{o}C) for 14 days. Spore suspensions were obtained by adding sterilized water into the grown culture on the PDA slant, then enumerated using a hemocytometer and expressed as 10^7 spores per mL.

2.2 Preparation of cheese as a solid substrate

The cheese-making process followed the procedures described in Agustinah and Winarno (2010) with modification. The cheese was manufactured with UHT milk (low-fat, high-calcium, plain; Ultra Jaya), skim milk powder (Indo Prima, Semarang, Indonesia), acetic acid solution, commercial rennet tablets (+Qso), and refined salt (Refina). Skim milk powder was first sterilized at 105^{o}C for 10 mins, then 15% skim milk powder was mixed with UHT milk and stirred until dissolved. The milk mixture was heated in the water bath at 43^{o}C for 30 mins, then added with acetic acid (pH of about 6.17), stirred gently, added with 0.03% rennet, stirred slowly, and kept heated at 43-45^{o}C for 2-3 hrs until it turned into curd. Afterwards, the curd was cut to separate it and the whey, heated at 50^{o}C for 2 hrs to increase the released whey and then stored overnight at 4 –6^{o}C to drain the whey altogether without a press. The curd mat was weighed, added with 1.5% salt, and stirred until homogeneous. The resultant fresh cheese was placed in Petri dishes (20 g), and each was heated with a cabinet dryer at 40^{o}C for at least 2 hours until the weight was reduced to around 10–11 g (water content of about 43%). Afterwards, it was sterilized at 105^{o}C for 10 mins before being cooled to room temperature. The sterilized cheese was dry-milled for around 30 s, and then the resultant cheese granule was put into sterilized conical flasks. The sterilized cheese granules were added 0.1%, 0.3%, 0.5% and 1% methionine at the beginning of the fermentation and inoculated with *M. purpureus* (10^7 spores per mL). *Monascus purpureus* was cultivated in duplicate on cheese as a substrate at room temperature (25–30^{o}C) for 14 days.

2.3 Colour measurement

The cheese pigmentation was characterized quantitatively using the CR 400 Chroma Meter (Konica Minolta Co. Ltd., Osaka, Japan) using three measurement parameters of L*, a* and b*; the values of

which were used to calculate chroma (C*) and hue (angle, h). L* indicates lightness—expressed in 0 (black) to 100 (white), a* denotes the red/green coordinate in the CIELAB colour space (+a* = red direction, -a* = green direction), and b* marks the yellow/blue coordinate (+b* = yellow direction, -b* = blue direction). C* indicates the saturation or purity of the colour, which was determined as $\sqrt{(a^*)^2+(b^*)^2}$. This value is 0 at the centre and increases with distance from the centre. Meanwhile, h differentiates into 0^o for +a (red), 90^o for +b (yellow), 180^o for -a (green), and 270^o for -b (blue) and was determined as tan-1 (b*/a*) (Jung *et al*., 2003; Ristiarini *et al*., 2017).

2.4 Statistical analysis

The analysis of variance (ANOVA) feature in the statistical software SPSS (Statistical Product and Service Solution) was used to examine the significance of the methionine level's effects on the colour of a solid substrate in fermentation. Duncan's multiple range test was performed to compare the difference between means at a probability level of 0.05. All statistical analyses were conducted in SPPS v.22.

3. Results and discussion

3.1 Fermentation condition

The fermentation state affects the growth and secondary metabolite synthesis of *M. purpureus.* It includes temperature, water activity (a_w), water content (especially for solid-state fermentation), and pH. In this study, cheese was conditioned in such a way that the mould could grow and produce secondary metabolites, as indicated by the red colour. High pigment production in solid-state fermentation results from the release of pigments into a substrate (Agboyibor *et al*., 2018). The fermentation condition of *M. purpureus* using cheese as substrate was presented in Table 1.

Table 1. The fermentation condition of *Monascus purpureus* JK2 using cheese as substrate

Initial conditions	Cheese granule (raw material)	Methionine-added cheese (as substrate)
Water activity (a_w)	0.90±0.002	0.93±0.009
Water content (%)	35.72±0.250	42.59±1.070
pH	5.17±0.012	5.50±0.007
Cultivation temperature (^{o}C)	-	25–30
Cultivation time (days)	-	14 days

The water activity (a_w) is the amount of free water responsible for the growth and metabolism of microbes, e.g., fungi, and biochemical reactions occurring in a food product. The initial a_w of the cheese was at 0.93±0.009, which supported the growth and formation of *M. purpureus spores*. Beuchat (1983) reported that higher a_w is commonly required for spore formation than spore germination. Panagou *et al*. (2003) also conditioned a_w at the range of 0.937–0.970 to cultivate *Monascus ruber* on a solid culture medium.

The initial moisture content of a substrate plays a role in regulating pigment synthesis during fermentation (Lotong and Suwanarit, 1990). The cheese's water content was 42.59±1.070%, sufficient for *M. purpureus* to produce red pigments. In a solid-state fermentation of *M. purpureus*, Jirasatid *et al*. (2013) adjusted the moisture content of rice to 40-42%, allowing this mould to grow and synthesize secondary metabolites. On the contrary, Ganrong *et al*. (2005) suggested that, as a solid medium, rice best has a moisture content hovering around 65%. Loose granules of red rice favour mass transfer (intake of oxygen and release of carbon dioxide). However, high initial moisture content can inhibit pigment synthesis because of increasing glucoamylase production, consequently, releasing glucose in an amount that inhibits pigmentation (Lotong and Suwanarit, 1990).

The cheese's pH was 5.5 or very suitable for the mould's growth and red pigment synthesis. As confirmed by Dikshit and Tallapragada (2011), pH regulates the physiology, conidial development, and pigment synthesis of fungi. *Monascus purpureus* can survive a wide range of pH (4.5–8.5). Maximum red pigmentation is observed at room temperature and pH of 5.5.

The fermentation temperature is a central and even sensitive factor of microbial growth and secondary metabolite production. Increasing temperature can promote *M. purpureus* growth; however, the optimum temperature for maximum secondary metabolites stimulation is usually lower than that required for growth. The mould species proliferates at 30^oC, and its metabolites are synthesized at 26^oC (Ganrong *et al*., 2005; Jirasatid *et al*., 2013). For this reason, setting the cultivation temperature of *M. purpureus* on cheese at 25–30^oC is deemed favourable for growing and forming red pigments as a secondary metabolite. Dikshit and Tallapragada (2011) reported that *M. purpureus* produces the highest pigment yields and biomass at 28–30°C, and this temperature range is found to be optimum for growth and pigment production. *M. purpureus* does not grow at 16°C and 50°C. Although the growth and red pigment production vary with temperature, optimum production is reported at 30^oC (Chatterjee *et al*., 2009).

In the current study, *M. purpureus* was cultivated on cheese as a solid substrate for 14 days at room

temperature; this period gave a sufficient amount of time for the mould to grow and synthesize red pigments. Jirasatid *et al*. (2013) suggested that prolonged cultivation time leads to increased cell growth and secondary metabolites production. The cultivation time required to optimally synthesize metabolites, including pigments, monacolin K, and citrinin, is 14–15 days.

3.2 Growth of Monascus purpureus in methionine-added cheese as a solid substrate

The growth of *M. purpureus* JK2 in cheese as a solid substrate was visually observed every day for 14 days. From the beginning until the third day of incubation, the mould did not show any colour changes; the signs of pigmentation appeared on Day 4. The cheese substrates without and with the addition of 0.1, 0.3, and 0.5% methionine were slightly pink coloured, but the one added with 1% methionine was not. The colour then developed gradually from faint pink to red and finally dark red on Day 14. These research findings are consistent with Dikshit and Tallapragada (2011) in that both studies recorded colour production starting on Day 4 that intensified along with the incubation period. Yang *et al*. (2015) suggested that, in the major pigment biosynthesis phase (four to eight days), a high level of pyruvate in rice (culture medium) can be converted to acetyl-CoA. The growth process of *M. purpureus* in methionine-added cheese as a solid substrate was similar to the one in jackfruit seed (Subhasree *et al*., 2011).

The study revealed that *M. purpureus JK2* grew well in cheese substrate without the addition of methionine at room temperature (25-30^{o}C). The addition of 0.1, 0.3 and 0.5% methionine slowly decreased the growth, while 1% methionine inhibited it. The mycelia formation of *M. purpureus* JK2 decreased as the concentrations of the added methionine increased, i.e. 0.1, 0.3, and 0.5%. Meanwhile, with the addition of 1% methionine, neither mycelial growth nor red pigmentation was detected for up to 14 days of fermentation. These results are similar to Wong *et al*. (1981). The high nitrogen (ammonium nitrate) inhibits not only growth but also pigmentation of *M. purpureus.* The growth of *M. purpureus* JK2 in cheese substrate during the 14-day incubation at room temperature (25–30^{o}C) is shown in Table 2.

In general, cheese substrates provide nutrients for the growth of *M. purpureus*, including lactose (carbon source)–as confirmed by Omamor *et al.* (2008), proteins (nitrogen source), and minerals. *M. purpureus* can grow not only on starch but also on protein from natural substrates (Rasheva *et al*., 1998) and produce proteinase (Nilantha Lakshman *et al*., 2011). In the presence of lactose, the protease activity increases significantly (da Costa and Vendruscolo, 2017). Nitrogen sources play a substantial role in increasing sporulation, spore germination leads to enhanced mycelia growth (Tallapragada *et al*., 2017). In addition, cheese substrates might contain organic nitrogen in the form of amino acids with suitable types and quantities for producing the red pigment. Agboyibor *et al*. (2018) suggested that *Monascus* red pigments particularly depend on the amino acids or related proteins. Ammonia reaction between

Table 2. The growth of *Monascus purpureus* JK2 in methionine-added cheese as a solid substrate during 14-day incubation at room temperature (25–30^{o}C)

Incubation	Methionine levels (%)				
(Day *i-th)*	0	0.1	0.3	0.5	1
1	-	-	-	-	-
2	-	-	-	-	-
3	-	-	-	-	-
4	+	+	+	+	-
5	++	+	+	+	-
6	+++	++	+	+	-
7	+++	++	+	+	-
8	++++	+++	+	+	-
9	++++	+++	++	+	-
10	++++	+++	++	+	-
11	++++	+++	++	++	-
12	+++++	++++	+++	++	-
13	+++++	++++	+++	++	-
14	+++++	++++	+++	++	-

- No growth; + slight growth, light pink ; ++ moderate growth, pink; +++ dense growth, red; ++++ dense growth, dark red

Monascus orange pigment and amino acids, resulting in deep red colour (Monascorubramine and rubropunctamine) as *Monascus* culture ages.

The results indicated that *M. purpureus* JK2 requires an adequate methionine concentration in a substrate to support its growth and red pigment synthesis. However, among the tested methionine levels in the substrate, 1% appeared to inhibit red pigment production. A low concentration of methionine can offer sufficient S-adenosyl methionine (SAM), which is synthesized from methionine and ATP as a precursor of secondary metabolites, including pigments. Meanwhile, its high presence in a substrate (1%) leads to the proliferation of toxic hydrogen sulfide in the cell and, consequently, inhibits pigment production (Jirasatid *et al*., 2013).

3.3 Colour characteristics of Monascus purpureus-fermented cheese

Monascus purpureus JK2 grew in cheese substrates at room temperature (25–30^{o}C) for 14 days. Based on visual observation of the produced pigments, this mould strain can generate a red pigment on cheese as a solid substrate (Figure 1). The colour characteristics of the pigment were measured in a colourimeter using the

Table 3. Colourimetric values of the pigments of cheese substrates added with different methionine concentrations and fermented by *Monascus purpureus* JK2 after 14 days of incubation

Items	L*	a*	b*	°hue (h)	Chroma (C*)
Cheese (raw materials)	74.24±0.297^{a}	2.425±0.106^{b}	21.16±0.269^{a}	83.46±0.212^{ab}	21.30±0.276^{b}
Cheese JK2, Day 0	72.05±2.107^{a}	0.975±0,375^{b}	20.08±1.156^{a}	87.26±0.863^{a}	20.11±1.570^{b}
Methionine (0%)	32.65±5.459^{d}	21.560±0,990^{a}	9.80±1.004^{d}	24.48±3.203^{d}	23.71±0.488^{ab}
Methionine (0.1%)	36.01±0.983^{cd}	22.805±0.417^{a}	10.03±0.983^{cd}	23.70±1.670^{d}	24.92±0.778^{ab}
Methionine (0.3%)	40.58±3.804^{bc}	24.865±0.488^{a}	13.80±1.556^{bc}	28.99±2.263^{d}	28.45±1.181^{a}
Methionine (0.5%)	44.69±0.997^{b}	22.075±3.868^{a}	15.21±2.786^{b}	34.55±0.226^{c}	26.81±4.773^{a}
Methionine (1%)	66.83±3.670^{a}	3.88±1.796^{b}	23.22±1.874^{a}	80.34±5.063^{b}	23.58±1.556^{ab}

Cheese JK2 denotes cheese fermented by *M. purpureus* JK2.
Values are presented as mean±SD. Values with different superscript within the same column are significantly different

CIELAB colour system (Table 3). The L*, a*, and b* values were all positive, indicating redness and yellowness. The L* (lightness) and b*values (yellowness) of methionine-added cheese showed a decreasing trend throughout the 14 days of incubation, whereas a* values (redness) increased. Hue (h),

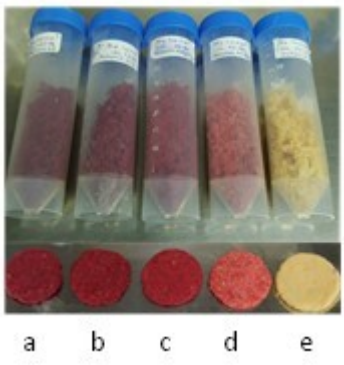

Figure 1. Pigmentation in *Monascus purpureus*-fermented cheese added with different methionine levels: a. 0%, b. 0.1%, c. 0.3%, d. 0.5%, e. 1%

calculated from L*, a*, and b* values, defines red at close to 0. The cheese without methionine and with 0.1–0.5% methionine had the h value lower than other treatments.

After 14 days of incubation, the cheese colour was compared with that of Day 0 and before inoculation with *M. purpureus* JK2 (raw materials). Colour differences indicate variation in pigment synthesis caused by the addition of methionine. Significant differences were found in all colour measurement parameter values: L*, a*, b*, h, and C*. The L* values of cheese added with 0.1–0.5% methionine ranged from 32.65±5.459 to 44.69±0.997, indicating dark colours or different from the bright colours of raw materials, cheese inoculated with *M. purpureus* JK2 on Day 0, and cheese added with 1% methionine (from 66.83±3.670 to 74.24±0.297). Hue (h) indicates a classification of colour, while chroma (C*) shows colour saturation (vivid against dull); both are a function of a* and b*. The results showed that cheese without the addition of methionine and with 0.1 and 0.3% methionine, the pigment's h values on Day 14 varied from 23.70±1.670 to 28.99±2.263, and the C* values were between 23.71±0.488 and 28.45±1.181, indicating dark red colours (based on the chromaticity diagram) and significant colour differences from the cheese substrates receiving other treatments. These results are in line with Ristarini *et al*. (2017) in that the hue angles of *Monascus products* are indeed in the range of 0–30° or red-purple to red. Similarly, Jung *et al.* (2003) reported that red pigments of amino acid derivatives generally have hue angles of 0-30° and are categorized into deep red or rose. Cheese added with 0.5% methionine had dull colour with a red hue (h = 34.55±0.226 and C* = 26.81±4.773), whereas raw materials cheese, cheese on 0 Day and cheese added with 1% methionine had pale colour with a yellow hue (h = 80.34±5.063 to 87.26±0.863 and C* = 20.11±1.570 to 23.58±1.556).

The results showed that *M. purpureus* JK2 could produce red pigments in cheese substrate without the addition of methionine. Its red colour was similar to the pigments produced in cheese substrates added with 0.1 and 0.3% methionine and relatively darker than with 0.5% methionine. When 1% methionine was added to the cheese substrate, the growth and red pigmentation were substantially inhibited. Jirasatid *et al*. (2013) reported that methionine is a significant factor and has a proven adverse effect on the biosynthesis of metabolites, including pigments. At 0.01–0.5%, methionine suppresses the formation of monacolin K and yellow pigments. Moreover, Wang *et al*. (2003) suggested that the addition of methionine (0.5% and 1%) as a nitrogen source suppresses monacolin K and citrinin formation, which tends to decrease red pigment production. As previously mentioned in section 3.2, appropriate methionine concentration provides an adequate amount of S-adenosyl methionine (SAM) as the precursor of red pigment synthesis. Presumably, the amino acid enters the *Monascus* cell, and the nitrogen in its amino groups substitutes the oxygen in the orange pigment and yields a

red colour instead (Tallapragada *et al*., 2017).

The production of red pigments failed when 1% methionine was added to the substrate due to the proliferation of toxic hydrogen sulfide in the cell. Bouillaud and Blachier (2011) suggested that high sulfide contents severely disrupt cellular bioenergetics. Sulfide inhibits cytochrome oxidase (mitochondrial complex IV), which transfers electrons from cytochrome c to oxygen to form water. When electron transfer in mitochondrial is hindered, the phosphorylation of ADP into ATP is disturbed, impeding the provision of energy required for cellular activity and increasing cell exposure to sulfide poisoning that can be fatal for the organism.

4. Conclusion

Monascus purpureus JK2 can grow and produce red pigments in cheese (a solid substrate) at room temperature (25–30^{o}C) without the addition of methionine, provided that favourable conditions of incubation exist, such as high water activity (aw) (0.93±0.009), low water content (42.59±1.070), and acidic pH (5.50±0.007). The addition of methionine to cheese substrates decreased the growth and red pigment production and, even tended to inhibit growth at a concentration of 1%. These results highlight the significance of applying simple methods of generating culture products for *Monascus*-ripened cheese manufacture development.

Conflict of interest

The authors declare no conflict of interest.

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