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Contributed Paper

Utilization of Agro-industrial Products for Increasing Red Pigment Production of *Monascus Purpureus* AHK12

Pongrawee Nimnoi*[a], Neelawan Pongsilp [b] and Saisamorn Lumyong [c]

[a] Department of Microbiology, Faculty of Liberal Arts and Sciences, Kasetsart University, Nakhon Pathom 73140, Thailand.

[b] Department of Microbiology, Faculty of Science, Silpakorn University, Nakhon Pathom 73000, Thailand.

[c] Microbiology Division, Department of Biology, Faculty of Science, Chiang Mai University, Chiang Mai 50200, Thailand.

*Author for correspondence; e-mail: umco_perra@hotmail.com

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ABSTRACT

Natural pigments have been recently focused as an important alternative to harmful synthetic dyes. As the manufacturing process of natural pigments that is mainly based on the extraction from plant materials makes the market prices high, the present study aims to evaluate the potential of low-cost agricultural products as substrates for red pigment production of *Monascus purpureus* AHK12 by using solid-state fermentation for reducing the production cost. The result showed that corn meal was the best substrate for pigment production followed by bagasse and coconut residue, respectively, whereas soybean meal was not a suitable substrate. Additions of each of 4%, 6% and 8% glucose and molasses at the same concentrations potently improved the pigment yields. Corn meal supplemented with either 8% glucose or 8% molasses achieved the highest pigment yields up to 1.7-fold when compared with controls. Co-supplementations of either glucose or molasses with each of whey and soybean milk as nitrogen sources were also evaluated. Among all substrates tested, a co-supplementation of molasses and soybean milk conferred the highest pigment yield. Corn meal co-supplemented with 8% molasses and 1% soybean milk provided the highest pigment yields up to 5.94-fold when compared with the mere additions of 8% molasses and 8% glucose. The produced pigments were stable under high temperatures and long-time sunlight exposure, whereas they were degraded after long-time UV exposure. These properties suggest that *Monascus* pigments are applicably useful for thermal process that is significant for an industrial scale.

Keywords: *Monascus purpureus*, pigment production, agricultural products, solid-state fermentation

1. INTRODUCTION

The development of products with an attractive appearance has been an important goal in the food industry. Attractive colors of foods usually contribute to the increasing consumption. Nowadays, synthetic dyes have almost completely replaced natural dyes. However, some synthetic dyes contain potential carcinogens for humans. This therefore supports the growing demand for non-toxic colorants, especially for food applications. At the present, the production of natural dyes is mainly based on the extraction from plant materials that makes the current market prices still high. To solve this problem, other biological sources, especially microbial colorants, have been adopted to improve the yields of pigment production.

Among various pigment-producing microorganisms, *Monascus* has been reported to be a promising fungus for non-toxic pigments which can be used as food colorants. *Monascus* pigments are extracellular and water soluble. These properties facilitate the use for food applications including red coloring in meat, fish and ketchup. Some compounds from *Monascus* have an application as pharmaceuticals such as monacolin K that was found to reduce cholesterol as well as lovastatin that was found to reduce triglyceride [1-2]. *Monascus* also produces citrinin (mycotoxin) which becomes toxic for humans when accumulated in the bodies. However, Gheith et al. [3] and Lin et al. [4] treated hyperlipidemia patients by ingestion of *M. purpureus* Went rice (600 mg twice/day). They found that cholesterol, triglycerides and apolipoprotein were reduced after 6 months to 1 year of treatment and the liver and kidney systems were not affected by *M. purpureus* Went rice.

Despite the valuable properties mentioned above, the current production of pigments at an industrial scale is not economical. Therefore the development of low-cost processes is needed. In this study we introduce alternative practice to use of polypropylene plastic bags instead of Erlenmeyer flasks for solid state fermentation (SSF) for reducing the production cost. This introduced method is more efficient and easily applicable but less cost-consuming than those of traditional SSFs published so far. We evaluate the potential of various agricultural materials, including bagasse, coconut residue, soybean meal and corn meal, as substrates for growth and pigment production as previous reports found that additions of carbon and nitrogen sources were the factors affecting pigment production. Glucose and polysaccharides were better than other carbon sources for both growth and pigment production [5]. We therefore compare 2 sugars, including glucose and molasses, as supplements for the potential of increasing pigment production. Since there is no report regarding the utilization of whey as a nitrogen source for *Monascus* pigment production, we also evaluate the potential of co-supplementations of either whey or soybean milk together with each of sugars for pigment production. The effects of temperature, sunlight and UV light on pigment stability were also determined. These experiments are motivated in the hope to promote the utilization of agro-industrial materials for the production of natural pigments.

2. MATERIAL AND METHODS

2.1 Culture

M. purpureus AHK12 from the SDBR culture collection, Microbiology Division, Faculty of Science, Chiang Mai University, Thailand was used for SSF. The culture was maintained on potato dextrose agar (PDA) slants, preserved at 4°C and sub-cultured every 3 weeks.

2.2 Inoculum Preparation

Inoculum preparation was performed as described by Nimnoi and Lumyong [5].

The obtained inoculum was diluted to contain 1×10^6 spores/mL and used as the inoculum.

2.3 Substrate and Solid-state Fermentation

The low-cost agricultural products and residues which are abundant in Thailand, including bagasse, coconut residue, soybean meal and corn meal, were selected in the hope to raise their value and reduce their waste management. These experiments were focused on the evaluation of the best suitable substrates and supplements for the maximum yield of pigment production. Substrates obtained from the local markets were dried at 60°C for 12 h and then ground thoroughly. Five grams of dried substrate was placed in a 6×10-inch 100% polypropylene plastic bag. Then, autoclavable plastic tubing was inserted into the bag and plugged with cotton wool. The contents in the bag were mixed thoroughly and then autoclaved at 121°C for 15 min, and cooled to room temperature. The bag was inoculated with *M. purpureus* AHK12 spore suspension and incubated at 30°C for 14 days. Unless otherwise mentioned, these conditions were maintained throughout the experiments. To compare the impact of the additions of different sugars, including glucose and molasses, on growth and pigment production of *M. purpureus* AHK12, each of 4%, 6% and 8% (w/w) of glucose, 4%, 6% and 8% (v/w) of molasses was added into the substrates before autoclaving. Moreover, the experiments were also performed to evaluate the influence of the additions of either 1% (v/w) whey or 1% (v/w) soybean milk as nitrogen sources together with those sugars on pigment production.

2.4 Pigment Extraction

Five grams of fermented solid substrate was taken for pigment extraction as described by Carvalho et al. [6]. The extract was allowed to settle at room temperature and then filtered through Whatman No.1 filter paper.

2.5 Red Pigment Estimation

Estimation of the extracted pigments was done by measuring the OD value at 500 nm. The ethanol extracts of unfermented substrates were served as blank controls. After measuring, the concentration of red pigments was determined by comparison with a standard curve generated using pigment extracted from commercial Chinese red rice, to express the pigment yield as milligram per gram of dry substrate (mg/gds).

2.6 Biomass Estimation

Growth of *M. purpureus* AHK12 was estimated by determining *N*-acetyl glucosamine (NAG) released by the acid hydrolysis of chitin in the fungal cell walls as described by Babitha et al. [7].

2.7 Red Pigment Stability

The aqueous phase of extract solution was stored and used to investigate red pigment stability. Briefly, glass tubes containing 10 mL of the samples were incubated in a water bath at 40°C, 60°C, and 80°C for 1 h. The samples were also tested for pigment stability after pasteurization at 70°C for 10 sec. and sterilization at 15 psi, at 121°C for 15 min. The heated samples were cooled to room temperature. Another set of plastic plates containing 10 mL of the samples were exposed to sunlight for 12 h and UV light for 5 h. The OD values of the heated samples, pasteurized samples, sterilized samples, sunlight-exposed samples and UV-exposed samples (with a 1-h interval) were measured at 500 nm against water as a blank.

2.8 Thin Layer Chromatography (TLC) Analysis of The Extracted Pigments

Ten μL of ethanol extracts were applied to Silica Gel 60 F245 plates (Merck, Germany). The TLC plates were developed with the solvent mixture as described by Babitha et al. [7].

2.9 Statistical Analysis

All experiments were conducted in triplicate samples and all data were analyzed with SPSS program version 16.0 (SPSS Inc, Chicago, IL).

3. RESULTS AND DISCUSSION

3.1 Substrate Selection and Effect of Sugar Supplementations on Growth and Red Pigment Production

The aim of substrate selection was to evaluate the low-cost agricultural products that are most suitable for the maximum yield of red pigment production. Bagasse, coconut residue, soybean meal and corn meal without any supplementation were used as substrates for cultivations of *M. purpureus* AHK12. As shown in Table 1, the fungus produced the highest yield of red pigments when cultivated in corn meal (12.56±0.40 mg/gds), followed by bagasse (3.70±0.17 mg/gds), and coconut residue (3.61±0.20 mg/gds), respectively, whereas the lowest yield of pigment production was observed from soybean meal (1.60±0.01 mg/gds). The result is similar to that of Nimnoi and Lumyong [5] who concluded that corn meal was the best substrate for pigment production of *M. purpureus* CMU001.

Table 1. Pigment production of *M. purpureus* AK12 obtained from cultivations in each substrate with sugar supplementations.

Substrates	Pigment yields[a] (mg/gds)						
	controls	supplemented with glucose			supplemented with molasses		
	no sugar added	4% (w/w)	6% (w/w)	8% (w/w)	4% (w/w)	6% (w/w)	8% (w/w)
bagasse	3.70±0.17 b	4.69±0.11 c	7.50±0.05 ef	8.13±0.47 f	6.99±0.04 e	10.88±0.05 hi	11.39±0.04 i
coconut residue	3.61±0.20 b	4.53±0.07 c	5.65±0.05 d	7.24±0.01 e	5.77±0.06 d	9.23±0.10 g	10.39±0.12 h
soybean meal	1.60±0.01 a	1.58±0.10 a	1.65±0.02 a	1.68±0.01 a	1.59±0.02 a	1.64±0.05 a	1.66±0.04 a
corn meal	12.56±0.40 j	17.92±0.58 k	20.86±0.17 m	22.55±0.50 n	19.21±0.20 l	21.05±0.15 m	22.53±0.72 n

[a] Average ± standard deviation error from triplicate samples

Values with the same letters are not significantly different ($P > 0.05$) according to Tukey test

In the experiments, agricultural materials used for SSFs were not supplemented with salt solution but molasses was used instead for increasing pigment production. Molasses contains many minerals such as N, K, Cl, Mg, and S as well as also contains high amounts of sugars such as sucrose, dextrose, fructose and protein [8]. As previous report found that glucose is one of the best supplementary factors that confer the highest amounts of pigment production [5], we therefore conducted the experiments to compare the effect of glucose and molasses on pigment production.

For SSFs with glucose supplementations, all substrates (except soybean meal) supplemented with glucose (at every concentration tested) significantly increased the amounts of pigments (Table 1) when compared with each control (no glucose added). Corn meal supplemented with 8% glucose conferred the highest amount of pigments (22.50±0.50 mg/gds) that was significantly different from the other treatments, followed by corn meal supplemented with 6% and 4% glucose, bagasse supplemented with 8% glucose, coconut residue supplemented with 8% glucose, bagasse supplemented with 6% glucose, coconut residue supplemented with 6% glucose, bagasse supplemented with 4% glucose and coconut residue supplemented with 4% glucose, respectively. On the contrary, pigment production obtained from soybean meal supplemented with glucose at every concentration tested was not significantly different from that of control. The similar trends were observed for SSFs with molasses supplementations (Table 1).

Moreover, we found that corn meal supplemented with 4% molasses, bagasse and coconut residue supplemented with each of 4%, 6% and 8% molasses increased pigment production more than such substrates supplemented with glucose at the same concentrations. On the contrary, the amounts of produced pigments obtained from corn meal supplemented with each of 6% and 8% molasses were not significantly different from those obtained from corn meal supplemented with glucose at the same concentrations. These results are consistent with previous published papers [5] which showed that cultivation of *M. purpureus* with the additions of sugars as supplementary carbon sources increased red pigment production when compared to controls with no sugar added. According to Babitha et al. [7] who used jackfruit seed as a substrate for pigment production of *M. purpureus*, the addition of 4% lactose conferred the highest yield of pigments (16 Unit per gram of dry substrate, U/gds), followed by 4% sucrose (15 U/gds) and 4% sorbitol (13 U/gds), respectively. In our experiments, corn meal supplemented with either glucose or molasses (at every concentration tested) showed the potential to increase the pigment yields (16.78±0.60 U/gds to 21.51±0.51 U/gds; data in term of U/gds not shown) better than those previously reported sugars. Our result is the first report that shows the advantage of using molasses instead of glucose as a supplementary material for *Monascus* pigment production via SSF, offering lower cost than using other sugars. This demonstration is possibly beneficial for pigment production in food industry.

3.2 Biomass Estimation

Growth of *M. purpureus* AHK12 in substrates including bagasse, coconut residue, soybean meal and corn meal were evaluated. In SSFs of controls (no sugar added), corn meal conferred the highest concentration of NAG (12.80±0.40 mg/gds), indicating the maximum growth, and the concentrations of NAG obtained from bagasse (3.39±0.37 mg/gds) and coconut residue (3.05±0.34 mg/gds) were not significantly different. While the concentration of NAG obtained from soybean meal was undetectable. All substrates (except soybean meal) supplemented with either glucose or molasses (at every concentration tested) significantly increased fungal growth when compared with each control with no sugar added (data not shown).

3.3 Effect of Sugars Together with Nitrogen Source Supplementations on Red Pigment Production

Besides sugar supplementation, previous studies [7-8] reported that SSFs of *Monascus* supplemented with nitrogen sources were capable of increasing pigment production. Therefore, in this study, we also attempted to determine the effect of nitrogen supplementations on pigment production of *M. purpureus* AHK12. The selected nitrogen sources include whey and soybean milk. To evaluate the effect of sugars together with nitrogen supplementations, substrates including bagasse, coconut residue and corn meal were selected, whereas soybean meal was excluded because it provided low pigment production and did not support fungal growth. Each of the selected substrates was supplemented with the following combinations: (i) 6% and 8% glucose together with 1% whey, (ii) 6% and 8% glucose together with 1% soybean milk, (iii) 6% and 8% molasses together with 1% whey, and (iv) 6% and 8% molasses together with 1% soybean milk.

As shown in Table 2, co-supplementations of sugars and nitrogen sources significantly increased pigment production when compared with the mere additions of each sugar. Corn meal supplemented with 8% molasses together with soybean milk was the best treatment for achieving the highest yield of pigments. In addition, the runner-up co-supplementation was corn meal supplemented with 6% molasses together with soybean milk. The pigment yields obtained from corn meal supplemented with each of 8% glucose and 8% molasses together with soybean milk were up to 4.13-fold and 5.94-fold when compared with the additions of only 8% glucose and only 8% molasses, respectively. Our results introduced the new potential supplements for increasing of *Monascus* pigment production via SSF.

Table 2. Pigment production of *M. purpureus* AK12 obtained from cultivations in each substrate with sugar and nitrogen source supplementations.

Substrates	Pigment yields[a] (mg/gds)					
	supplemented with glucose					
	6% (w/w) and			8% (w/w) and		
	no nitrogen source added	1% (v/w) soybean milk	1% (v/w) whey	no nitrogen source added	1% (v/w) soybean milk	1% (v/w) whey
bagasse	7.50±0.05ab	54.98±2.29fg	44.85±1.43de	8.13±0.47ab	90.51±0.81p	76.79±3.88mn
coconut residue	5.65±0.05a	42.83±0.97d	41.11±1.37d	7.24±0.01ab	58.52±1.02gh	53.33±0.63f
corn meal	20.86±0.17c	58.12±3.03g	47.42±2.06e	22.55±0.50c	93.31±0.67p	73.10±2.64klm
Substrates	**Pigment yields[a] (mg/gds)**					
	supplemented with molasses					
	6% (w/w) and			8% (w/w) and		
	no nitrogen source added	1% (v/w) soybean milk	1% (v/w) whey	no nitrogen source added	1% (v/w) soybean milk	1% (v/w) whey
bagasse	10.88±0.05b	79.98±0.61n	65.55±0.94ij	11.39±0.04b	98.68±0.43q	73.78±1.65klm
coconut residue	9.23±0.10ab	72.89±0.98klm	62.66±0.28hi	10.39±0.12b	94.49±0.69pq	70.67±1.22kl
corn meal	21.05±0.15c	111.33±0.23r	68.89±0.68jk	22.53±0.72c	133.95±3.21s	85.20±1.17o

[a]Average ± standard deviation error from triplicate samples
Values with the same letters are not significantly different ($P > 0.05$) according to Tukey test

3.4 TLC Analysis of The Extracted Pigments

The extractants obtained from each substrate supplemented with each of only 8% glucose, only 8% molasses, 8% of each sugar together with either of 2 nitrogen sources were applied to TLC plates to confirm pigment production (Figure 1). TLC patterns revealed that yellow and red pigments were produced in the fermentation and the supplementations of sugars and nitrogen sources affected pigment production. The more intense bands of yellow and red pigments were generated from corn meal supplemented with each of 8% glucose and 8% molasses (lane 1 and 2, respectively) when compared with those of bagasse with the same supplementations (lane 7 and 8, respectively) and those of coconut residue with the same supplementations (lane 13 and 14, respectively). These results confirmed that corn meal is the best substrate for pigment production of *Monascus*.

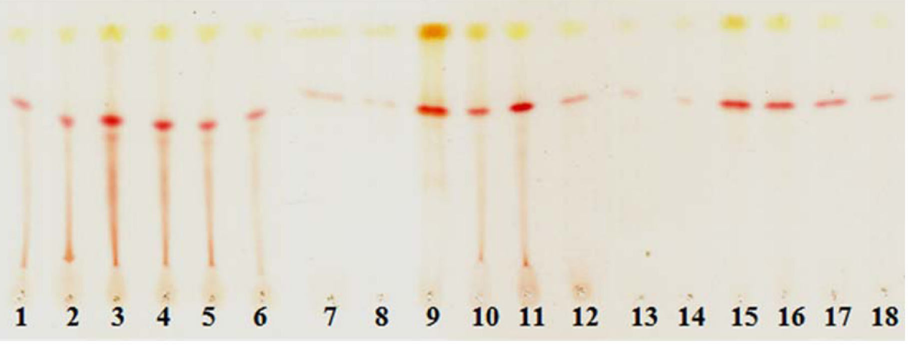

Figure 1. TLC analysis of pigments extracted from fermented substrates.

Lane 1, 2, 3, 4, 5, and 6: corn meal supplemented with 8% glucose, 8% molasses, 8% molasses together with 1% soybean milk, 8% glucose together with 1% soybean milk, 8% molasses together with 1% whey, and 8% glucose together with 1% whey, respectively.
Lane 7, 8, 9, 10, 11, and 12: bagasse supplemented with 8% glucose, 8% molasses, 8% molasses together with 1% soybean milk, 8% glucose together with 1% soybean milk, 8% molasses together with 1% whey, and 8% glucose together with 1% whey, respectively.
Lane 13, 14, 15, 16, 17, and 18: coconut residue supplemented with 8% glucose, 8% molasses, 8% molasses together with 1% soybean milk, 8% glucose together with 1% soybean milk, 8% molasses together with 1% whey, and 8% glucose together with 1% whey, respectively.

3.5 Red Pigment Stability

The extracted red pigments of *M. purpureus* AHK12 were tested for thermostability as well as tolerance to sunlight and UV light. For the thermostability tests, pigments were subjected to 40°C, 60°C, and 80°C for 1 h, pasteurization as well as sterilization. The results showed that pigments were tolerant to high temperatures as 9.79% of pigments were decayed after a 1-h incubation at 80°C. Moreover, 3.10% and 20.22% of the pigment intensities were decayed after pasteurization and sterilization, respectively. In addition, it was found that pigments were retained under sunlight as 6.62% of the pigment intensity was decayed after a 12-h sunlight exposure. For the tolerance to UV light test, the pigment intensities were measured using the relatives of residual absorbance after being exposed under UV light for 5 h (with a 1-h interval). The result showed that pigments were intolerant to UV. Pigments were decayed gradually along the exposure time and 82.63% of pigments were decayed after a 5-h UV exposure.

4. Conclusion

This is the first report that shows the advantage of using molasses and whey as supplementary materials for increasing red pigment production in *M. purpureus*. SSF supplemented with only 8% molasses is the best alternative practice which can be used instead of glucose for increasing the pigment yield and reducing the production cost simultaneously. The highest yield of pigments obtained from any substrate was achieved when supplemented with 8% molasses together with soybean milk. Moreover, we suggest that *Monascus* pigments are applicably useful for thermal process of food industry due to their thermostability.

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