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ORIGINAL RESEARCH ARTICLE

Production of biopigment by *Monascus ruber* AUMC 245 under submerged fermentation and its application as colorants of some food products

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ABSTRACT

The goal of the current investigation was to examine the production of red biopigment by *Monascus ruber* AUMC 245 during submerged fermentation and assess its safety as a bio-colorant in the food sector due to due to only sensory evaluation in food products. The various factors used for the production of red pigments, citrinin, and biomass yield were follows: 20 ℃–40 ℃ of temperature, 4.5–8.5 of pH, carbon and nitrogen sources. The optimum conditions were 30 ℃, pH 6.5, rice, peptone and incubation time for 10 days. The produced biopegment was without mycotoxin (citrinin). So, it is suitable for application in food industry. Kids jelly cola, ice sherbets and luncheon meat were prepared using the red pigments as natural colorant. The tested foods colored by nature biopigment appeared sensory results (90%) as acceptable consuming evaluation. It was not far from the applied industrial chemical one, but it is advantages by safe conditions. In conclusion, we can state that the obtained red pigments are safe to use in food products instead of chemical one.

Keywords: biopigment; *Monascus ruber*; submerged fermentation; food products

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1. Introduction

One of the first sensory characteristics utilized to select and purchase food is color. Although subjective, it is essential since it affects how other features, such fragrance, taste, and texture, are experienced[1]. The color can be used to convey meaning, contrast or novelty in packaging by conveying information about a category of goods and individual brands[2]. Food manufacturers have tried to use more natural and fewer synthetic dyes as a response to consumers' growing perception that natural dyes are safer. This is also because it is required to include a warning that there are natural dyes in the main food packaging panel, which contributes to unfavorable product advertising. Utilizing bio-pigments, often known as natural pigments made by microorganisms, is an excellent substitute for the food industry[3].

There are several benefits to producing pigments through microbial fermentation, including lower costs, simpler extraction, larger yields, no lack of raw materials, and no seasonal changes[4]. Microorganisms are commonly linked to food deterioration and contamination, but they also produce ingredients including amino acids, vitamins, organic acids, enzymes, and colors[5,6]. From these ingredients, Yongsmith et al.[7] discovery the *Monascus* genus as a

producer for biopigments. In Asia, particularly in southern China, Japan, and Southeast Asia, this filamentous fungus has been extensively used in the manufacturing of fermented dishes, i.e., Chinese red rice[5]. Natural *Monascus*-fermented pigments have attracted interest as a coloring agent due to their high economic value and numerous benefits, including ease of production on inexpensive substrates, good solubility in water and ethanol, a large number of bioactivity and complete safety when produced under certain conditions[8].

A complex mixture of six chemically distinct colored chemicals is produced by *Monascus* sp. These compounds are rubropunctatine, monascorubrine, rubropunctamine, monascorubramine, monascine and ankaflavine[9]. The red pigment (monascorubramine) is produced by *Monascus* as a high demand[10].

Though red pigments are often produced on solid support for use as a coloring agent, most laboratory investigations have been done utilizing submerged fermentation, which presents easily regulated conditions. However, solid state fermentation typically yields and produces more pigment than submerged fermentation, but typically requires complicated conditions that are challenging to employ for illuminating metabolic pathways and other growth-related aspects[11]. A number of factors, including the culture medium's composition (carbon and nitrogen sources), temperature, the presence of oxygen, humidity, light intensity and pH, influence the formation of pigments[12]. Citrinin, as a human hepato-nephrotoxic chemical is generated by the *Monascus* sp. as a food fermentation microorganism[13], for a safety it should be not detected in the produced pigments.

Therefore, in this study, *Monascus ruber* AUMC 245 was applied as a producer of red pigments. The optimum conditions that produce high amounts of biopigment were studies. Also, citrinin was conducted to ensure getting a safe natural pigment. Finally, we add pigment to enhance the appearance and acceptability of food stuffs so, jelly, sharbate and lunchoan will be developed by adding *Monascus* pigment, then will be sensory evaluated.

2. Materials and methods

2.1. *Monascus* strain

In this investigation, *Monascus ruber* AUMC 245, which was received from the Assuit University Mycological Center (AUMC), Assuit, Egypt and used as a source of red pigments. It was kept on a rice medium as the following ingredients: 50 g/L rice powder; 2.5 g/L KH_2PO_4; 3 g/L $NaNO_3$; 0.5 g/L $MgSO_4.7H_2O$; and 1 L of distilled water[14]. The medium was autoclaved at 121 ℃ for 15 min and the fungus was then inoculated and incubated for 10 days at 28 ℃.

2.2. Preparation of inoculum

The *Monascus ruber* strain was activated under static circumstances at 28 ℃ on rice slants. A 10 mL of sterile distilled water was added to the fully sporulate (10-day-old) agar slope culture, and the spores were scraped in a strictly aseptic manner. Once the target concentration was prepared, the acquired spore suspension was employed as an inoculum (10^6 spores per mL).

2.3. Broth media preparation

Pigment was produced by media consists of; broken rice powder, 50 g/L; KH_2PO_4, 2.5 g/L; $NaNO_3$, 3 g/L; $MgSO_4.7H_2O$, 0.5 g/L; $FeSO_4.7H_2O$, 0.01 g/L; $ZnSO_4.7H_2O$, 0.01 g/L. The initial pH of the medium was adjusted to 6 with diluted phosphoric acid (H_3PO_4).

2.4. Optimization of pigment production conditions

From the environmental factors, temperature and pH values were studied. To assess the optimum incubation temperature for pigment production, 100 mL of broth fermentation medium were dispensed into

250 mL Erlenmeyer flask and autoclaved at 121 ℃ for 20 min, pH 6.5. After cooling at room temperature, each flask inoculated with 1 mL of fungal spore's suspension (10^6 spores per mL) and then incubated at 20, 25, 30, 35 or 40 ℃ for one week.

As for optimization the pH value, the same method with different pH values ranging 4.5, 5.5, 6.5, 7.5, or 8.5 was carried out with temperature of 30 ℃. From nutritional factors, effect of carbon and nitrogen sources was studied. Carbon sources like rice powder, wheat flour, corn flour, corn cob powder and glucose with $NaNO_3$ as nitrogen source. Nitrogen sources like $(NH_4)_2SO_4$, NH_4NO_3, KNO_3, peptone and urea were applied as a different nitrogen source with rice as carbon source and pH 6.5 for maximizing pigment production and incubated at 30 ℃.

2.5. Optimization of incubation period for pigments production

To detect the optimum incubation period for maximum production of red pigments, 100 mL of optimal medium containing were dispensed into 250 mL flask and autoclaved at 121 ℃ for 20 min. After cooling at room temperature, each flask was inoculated by *Monascus ruber* strain and then incubated for 4, 6, 8, 10, 12, 14, 16, 18 or 20 days. The optimal medium contained rice as carbon source, peptone as nitrogen source, pH 6.5 and incubated at 30 ℃. In all experiments, the biomass and pigments production were measured at the end of each incubation period.

2.6. Estimation of dry-mass weight and produced pigment

The mycelia were filtered through Whatman No.1 filter paper to remove the broth medium. They were then washed with distilled water, dried in an oven at 70 ℃ to a consistent weight, and weighed again[15].

Pigment concentration was determined by spectrophotometer. Firstly, the produced red pigments were scanned using UV-VIS spectrophotometer (200–800 nm) to select the maximum wavelength. For extracellular pigment, 100 mL of culture broth was filtered using Whatman No.1 filter paper and then measured by spectrophotometer at 500 nm. To make sure the absorbance measurement was between 0.3 and 0.7, the filtrate was, if necessary, diluted with distilled water. In case of intracellular pigment, the freshly harvested washed mycelia (0.5–1 g) from the above step were scraped off with spatula on filter paper and transferred to a 150 mL flask and 10 mL of 95 % ethanol was added and then sonicated for half hour. The supernatant was recovered by centrifugation at 10,000 xg for 10 min and measured at 500 nm[16–18].

2.7. Detection of citrinin mycotoxin

Monascus ruber strain was activated on PDA slopes for 7 days, and then the spore suspension was prepared in 2 mL of water. The strain was inoculated into 250 mL Erlenmeyer flasks each containing 50 mL of PDB medium, then incubated at 28 ℃ for 10 days. A culture of *Monascus ruber* growth was homogenized for 5 min in a high-speed blender (16,000 rpm for 10 min) with 150 mL of chloroform and then separated the chloroform phase by separating funnel. The extraction procedure was repeated three times. The chloroform extracts were combined, washed, filtered and concentrated to near dryness and the mycotoxin was detected using thin layer chromatography[3,18].

2.8. Application of the produced red pigments as foods colorant

Jelly cola was prepared by mixing the ingredients, gelatin (15%), sucrose (85%), citric acid (0.2%), flavoring agent and mix of sodium benzoate and potassium citrate (0.1%). Concentrated pigment was added after mixing the above mentioned ingredients. Ice sherbets was prepared in laboratory by adding *Monascus* pigments ranging from 0.1% to 0.5% (w/w) using the traditional procedure. The formulation of ice sherbets was shown as following: sugars (15%), water (84.2%), citric acid (0.2%), flavoring agent (0.1%) and red pigments. These contents should be mixed very well and heated at 90 ℃ for 15 min, cooled until 80 ℃ and

then put in polyethylene boughs and placed in deep freezer at −18 °C[19]. The control of ice sherbets was prepared with 0.10 % of synthetic colors (carmoisine). Preparation of luncheon meat was performed according to Egyptian Standard 1696 and 1114[20]. The luncheon was produced with 80% imported deep frozen beef chuck, 1.8% sodium chloride, 0.3% polyphosphates, 100 ppm sodium nitrite, 10% corn starch, and 7.5% water. The control of luncheon was prepared with paprika.

2.9. Sensory evaluation

Sensory evaluation was carried out by ten panelists. The panelists were asked to evaluate taste, color, texture, odor and over all acceptability for prepared foods. The value was calculated as 10 remarks is the highest value.

2.10. Statistical analyses

The represented results were expressed as the mean ± standard deviation (SD). Statistical significance was evaluated using analysis of variance (ANOVA, SAS software) test followed by the least significant difference (LSD) test at 0.05 levels.

3. Results and discussion

3.1. Induction and optimization of biopigment by *Monascus ruber*

Monascus ruber AUMC 245 strain was obtained from Assuit University Mycological Center (AUMC), Assuit, Egypt as a culture collection of fungal strains in Egypt. The fungal strain was maintained on the PDA plates and then inoculated into potatoes dextrose broth and rice broth media to select the best medium for producing the biopigment. After one week of incubation, the rice broth medium was chosen as production medium for red biopigment induction. Firstly, *Monascus ruber* AUMC 245 strain had moderate growth on the culture medium, the pigment was faint brick red color (0.44). This means that the pigment production was good but it was not the highest. Pigment productivity can be influenced by many factors like the *Monascus* strain, composition of the used medium and environmental fermentation conditions[11]. Therefore, this study was attempted to increase the production of *Monascus* pigment through enhancement of culture and environmental conditions.

3.2. Optimization of environmental conditions

Temperature as environmental factor affects the rate of all biochemical activities of microorganisms, such as nutrient uptake, enzyme synthesis and pigments production[21]. The optimum temperature for growth of *Monascus* species and pigment production was broad depending on species, thus, the optimal temperature could vary from 25 °C to 37 °C[22]. Therefore, an experiment was conducted to find out the effect of different incubation temperatures (20 °C, 25 °C, 30 °C, 35 °C and 40 °C) on biomass yield and red pigments production by the used *Monascus ruber*. The results represented in **Table 1** exposed that the optimal fermentation temperature for *M. ruber* was 30 °C due to affecting the highest biomass yield and red pigments production. By gradually raising the fermentation temperature from 20 °C to 30 °C, the production of red pigments was gradually increased, and then decreased with increasing the incubation temperature from 30 °C to 35 °C. This was in agreement with Park et al.[23] and Jeon et al.[24]. They found that, the optimum temperature for red pigments production by *Monascus purpureus* MMK2, *Monascus ruber* KCTC 6122 and *M. purpureus* P-57 was 30 °C. Also, Padmavathi and Prabhudessai[25] stated that mycelium growth and pigments production of *M. anguineus* and *M. purpureus* MTCC 410 were optimized at fermentation temperature of 30 °C. These results were slightly different compared with Kumari et al.[26] who found that maximum pigment production was around 32 °C to 35 °C and Hajjaj et al.[27] who stated that the optimal temperature for pigment production was 28 °C.

Table 1. Biomass yield of *Monascus ruber* AUMC 245 and red biopigment production after incubation at different temperature degrees.

Tempreture (℃)	Red pigments production (Ab_{500})	Biomass yield (g/100 mL)
20	0.30 ± 0.04^{c}	0.50 ± 0.06^{bc}
25	0.56 ± 0.04^{b}	0.63 ± 0.09^{b}
30	1.03 ± 0.02^{a}	0.95 ± 0.05^{a}
35	0.46 ± 0.04^{b}	0.47 ± 0.03^{bc}
40	0.23 ± 0.04^{c}	0.37 ± 0.07^{c}

The means ± standard errors are not significantly different at $P < 0.05$, according to Duncan's multiple range tests.

The initial pH of the medium is well recognized to have a significant impact on the development of enzymes and microbial metabolites like pigment. So, the experiment was created to look at how varying pH values (ranging from 4.5 to 8.5) affected the productivity of red pigments and biomass of *Monascus ruber*. Results represented in **Figure 1** showed that at pH 6.5, the highest pigment output was attained. However, the alkaline pH significantly impeded the pigment synthesis. The pH 7.5 significantly decreased pigment production, and pH 8.5 completely repressed it. The acquired results closely matched those established by Lee et al.[28] and Joshi et al.[29], who discovered that the generation of *Monascus* pigments optimized at the ideal pH value ranged from 5.5 to 6.5. In addition, Musaalbakri et al.[30] discovered that the ideal beginning pH for the synthesis of red pigments ranged from 6.5 to 9.0. Also, at pH 4.5 to 7.5, Babitha et al.[31] achieved the maximum pigment output from *M. purpureus*.

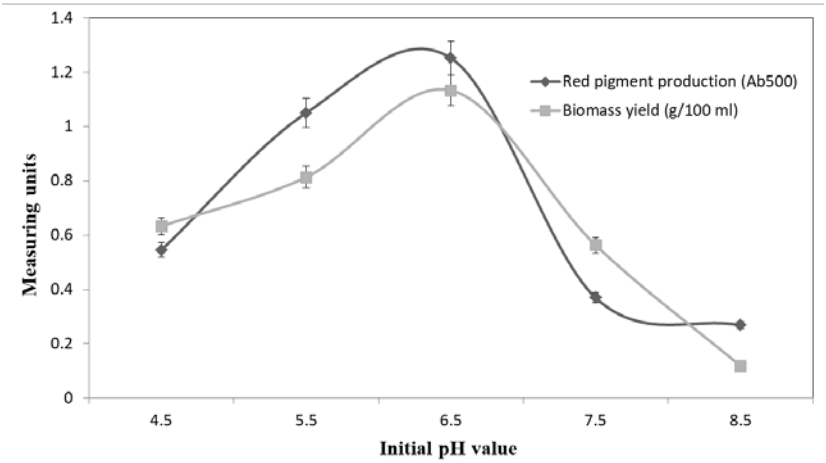

Figure 1. Biomass yield of *Monascus ruber* AUMC 245 and red biopigment production after incubation with different initial pH values.

3.3. Nutritional and cultural conditions optimization

Carbon as an essential element for organism's energy plays a critical role in cell metabolism and may affect the growth pigment production of *Monascus* sp. Carbon sources provide the carbon needed for the biosynthesis of cellular constituents, i.e., carbohydrates, proteins and lipids. Also their oxidation provides energy for the cell[32,33]. Therefore, in order to determine the optimal carbon source for the synthesis of biopigments, a variety of carbon sources (rice powder, wheat flour, maize flour, corncob powder, and glucose) were used in this study. Data shown in **Figure 2** revealed that rice powder was the best source for biomass. Glucose, wheat flour and corn flour came second, finally corn cob powder. As for pigment production, also rice powder and glucose were the best carbon source. Corncob powder was the worst source for pigment production. Upon the previous articles, the obtained results in this work were not far from these researches[22,34]. They recorded the highest pigment by rice powder.

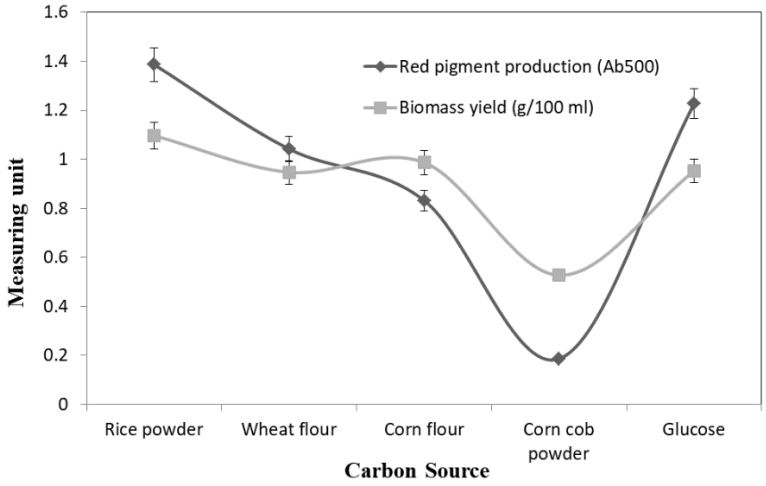

Figure 2. Biomass yield of *Monascus ruber* AUMC 245 and red biopigment production after incubation with different carbon sources.

Additionally, it was investigated how nitrogen sources affected *Monascus ruber* AUMC 245's ability to produce red biopigments. Microorganism growth requires a source of nitrogen, about 10% of the dry weight of fungus biomass is nitrogen, mostly in the forms of proteins and nitrogen-based substances[35]. Growth, sporulation, and pigment production are influenced by the kind of nitrogen supply. So, organic (peptone-urea) and inorganic (NH_4NO_3-NH_4SO_4-KNO_3) nitrogen sources were used to obtain higher pigment yield. Results shown in **Figure 3** illustrated the organic nitrogen sources were better than inorganic nitrogen sources because these sources stimulate *Monascus* growth and pigment production[36,37]. Our results also revealed that through the organic nitrogen sources, peptone was better than other nitrogen sources. Jůzlová et al.[38] reported that peptone favors the formation of red pigments and it is better for pigment production by *Monascus* sp. According to Zhao et al.[39], monosodium glutamate was found to be outstanding for red pigments production, followed by peptone.

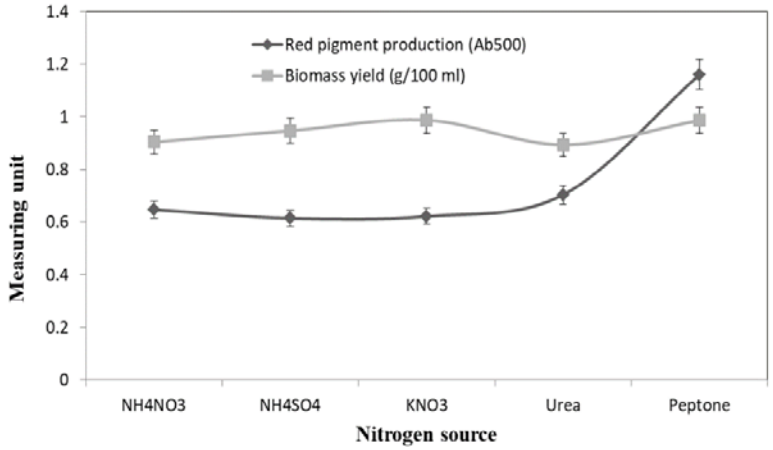

Figure 3. Biomass yield of *Monascus ruber* AUMC 245 and red biopigment production after incubation with different nitrogen sources.

The quick harvest of the product is crucial economically impact in industrial fermentation. In order to establish the best time to optimize the production of biopigment, an experiment was done to look at the rate of pigment creation throughout various fermentation periods. The pigments production broth medium was inoculated and incubated at 30 ℃ and pH 6.5. Culture samples were taken after 6, 8, 10, 12, 14, 16 and 18 days for the biomass, and pigments production at the end of each incubation period.

Data in **Figure 4** demonstrated that for the examined fungal strain, the rate of pigment synthesis rose progressively as the incubation time lengthened, reaching its highest value (2.32) after 10 days and subsequently fell until it reached its lowest value after 18 days. The maximum cell biomass concentration was

obtained after 16 and 18 days of incubation period. Biopigment is a fungal metabolite affected and/or affected by fungal growth through incubation periods[40].

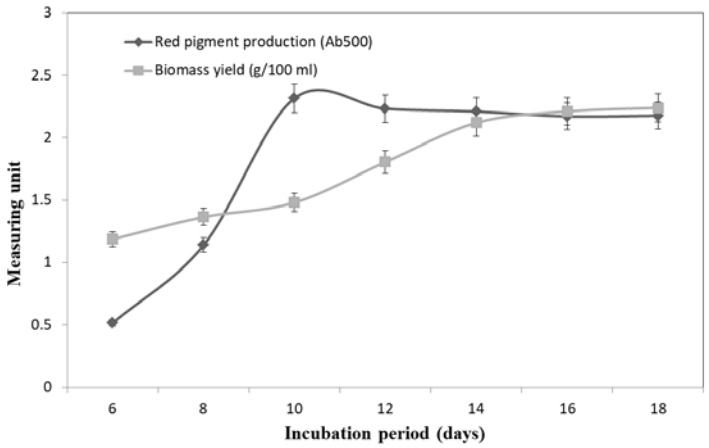

Figure 4. Biomass yield of *Monascus ruber* AUMC 245 and red biopigment production after incubation for different periods.

3.4. Production and extraction of red biopigment by *Monascus ruber* AUMC 245

The red biopigment was produced by large amounts after optimization of *Monascus ruber* AUMC 245 cultural and environmental conditions. As data illustrated in **Figure 5**, the produced red pigments were enhanced after optimization the conditions for *Monascus ruber* AUMC 245 growth and reached maximum absorption 3.154. Based on previous articles, the red biopegment can be produced by *Monascus ruber* without detecting any toxicity and producing cetrinin as mycotoxin[3,41]. This leads to use the produced red pigments instead of chemical colored in food industry.

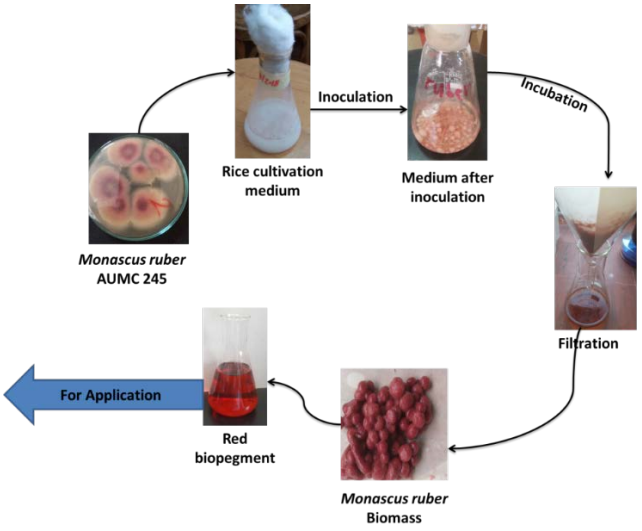

Figure 5. Flowchart for red biopegment production and extraction from *Monascus ruber* AUMC 245.

3.5. Safety of red biopigment and citrinin analysis

Monascus sp. was reported to produce a mycotoxin citrinin as fermentation byproduct along with coloured pigments and monacolins. Citrinin has been considered to have hepato-nephrotoxic properties[42,43]. It causes functional and structural kidney damage and alterations in liver metabolism. It inhibits several enzymes linked to the respiratory chain of the kidney cortex and liver mitochondria, as well as malate and glutamate dehydrogenases and the ATP-synthetase complex[44]. There are several studies on the toxicity of *Monascus* pigments showing that this biopigment is apparently safe in the quantities tested. On the other hand, there are several studies suggest that *Monascus* pigment are low or non-toxic[45]. Blanc et al.[46] stated that

Monascus could produce citrinin but that not all *Monascus* strains. It was also found that formation of citrinin depends on the culture conditions (the nitrogen source used could interfere in the citrinin production). So the present study was aimed at assessing the safety evaluation of red *Monascus* pigment by citrinin analysis to the strain (analyzed by AUMC). No citrinin (nephrotoxic or hepatotoxic) was found in the medium used to culture the *Monascus ruber* strain, according to analyses in the current study. These findings concur with those made by Chen et al.[13] and Moharram et al.[47], who looked at the location of genes involved in mycotoxin citrinin production in 18 *Monascus* strains. According to the findings, *M. purpureus*, *M. kaoliang*, and *M. sanguineus* all possess the acyltransferase and ketosynthase domains of the pksCT gene, which codes for citrinin polyketide synthase. Moreover, although *M. sanguineus* lacked the ctnA gene, which is a key stimulator of citrinin production, *M. purpureus* and *M. kaoliang* did. Both *M. purpureus* and *M. kaoliang* possessed the orf3 gene, which codes for oxygenase and is situated between pksCT and ctnA. While the ctnA and orf3 genes were shown to be largely similar in *M. purpureus* and *M. kaoliang*, the pksCT gene was highly conserved in *M. purpureus*, *M. kaoliang*, and *M. sanguineus*. PksCT, ctnA, and orf3 genes appear to be absent from or substantially different in *M. pilosus*, *M. ruber*, *M. barkeri*, *M. floridanus*, *M. lunisporas*, and *M. pallens*, according to the results of the PCR and Southern blot tests. Using high-performance liquid chromatography (HPLC), a citrinin-producing phenotype was only found in *M. purpureus* and *M. kaoliang*. These findings demonstrate unequivocally that citrinin production is carried out by the highly conserved citrinin gene cluster in *M. purpureus* and *M. kaoliang*. In particular, the citrinin gene cluster can classify the *Monascus* species in accordance with the evolutionary groupings defined using the b-tubulin gene. The TLC results mentioned no any citrinin in the *Monascus* extract. Citrinin production by the tested *Monascus ruber* strain was undetectable. As a result, it was determined that the *Monscus ruber* employed in the study may be used in food. We can infer that the *Monscus ruber* utilized in the study is safe for use in food since citrinin toxin is not heat-tolerant and, if present in a food item, may be destroyed by heat treatment (above 60 ℃).

3.6. Application of the produced red pigments as foods colorant

Three food products, jelly cola, sherbets and luncheon were manufactured by the concentrated pigment extracted and separated from submerged culture of *Monscus ruber*. The top evaluation score is 10 and the values calculated from that. The obtained results were shown in **Table 2** and **Figure 6**. We can conclude that food products gain more intense and stable red color and improved organoleptic characteristics when *Monascus* pigment was used. Lollipops and Jelly cola showed appealing color, appearance and were overall acceptable (**Figure 6**). The pigment distributed evenly in the food product giving a pleasing appearance. Moreover, application of the natural pigment promotes consumer's health protection and allows manufacturing fully natural food without any synthetic additives[48,49]. Red *Monscus* pigment is generally regarded as a safe product.

Table 2. Average sensory evaluations of food items with *Monascus ruber* AUMC 245 pigments.

Food products	Color (10)	Taste (10)	Texture (10)	Flavor (10)	Overall acceptability (10)
Jelly cola control	9.23 ± 0.12	9.266 ± 0.23	9.3 ± 0.208	9.6 ± 0.152	9.35 ± 0.025
Jelly cola *Monascus*	9.38 ± 0.07	9.03 ± 0.185	9.3 ± 0.264	9.6 ± 0.152	9.3 ± 0.064
Sherbets control	9.06 ± 0.06	8.83 ± 0.44	-	9.53 ± 0.032	9.144 ± 0.173
Sherbets *Monascus*	9.06 ± 0.08	8.83 ± 0.375	-	9.56 ± 0.062	9.155 ± 0.149
Luncheon control	8.4 ± 0.30	8.46 ± 0.46	8.63 ± 0.18	8.70 ± 0.057	8.55 ± 0.086
Luncheon *Monascus*	8.43 ± 0.26	8.5 ± 0.305	8.7 ± 0.11	8.66 ± 0.081	8.57 ± 0.01435

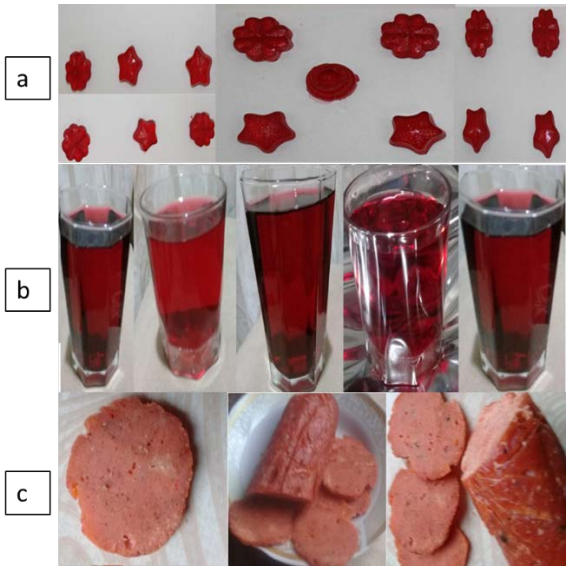

Figure 6. Three food products, **(a)** jelly cola; **(b)** sherbets; and **(c)** luncheon manufactured by the concentrated pigment separated from submerged culture of *Monscus ruber*.

4. Conclusions

From the obtained results, we can conclude that red pigments were produced by *Monascus ruber* AUMC 245. The optimum conditions for production high yield of red pigments were pH 6.5, rice as carbon source, peptone as nitrogen source and incubation time for 10 days at 30 °C. Also, citrinin was conducted to ensure getting a safe natural pigment. Finally, we add pigment to enhance the appearance and acceptability of food stuffs so, jelly, sharbate and lunchoan will be developed by adding *Monascus* pigment.

Author contributions

Conceptualization, methodology, validation, formal analysis, data curation, writing—original draft preparation, OMD and MRB; data analysis, writing—original draft preparation, MEE; supervision, MAS and MBA. All authors have read and agreed to the published version of the manuscript.

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Conflict of interest

The authors declare no conflict of interest.

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