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Development of New Edible Pigments using *Monascus* spp.

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Abstract

Carotene, xanthophyll, carotenoid anthocyan, phycopyrine, chlorophyll, and monascus pigments are used as natural coloring agents since they are more stable to human body than synthetic coloring agents. Among them, monascus pigments are a natural red pigment produced by the *Monascus purpureus*. For the development of edible paint using natural pigment, *Monascus purpureus* strain was cultured at a temperature of 35℃ for 15 days on a PDYA plate and liquid medium to produce a red pigment. In addition, a large amount of the red pigment was extracted from Hongkuk Koji in parallel with water extraction and ultrasonic wave extraction. At this time, the yield of ultrasonic extract was 2~4 times higher. Thus, *Monascus purpureus* strains, etc. were prepared by freeze-drying powder. In conclusion, natural paints made with red pigments have enabled the development of been edible paints that can be used as eco-friendly materials with good viscosity, enhanced spread ability and coloration.

Key words: *Monascus* pigment, *Monascus purpureus*, edible paints, natural paints, eco-friendly paints

Introduction

The pigment has natural or synthetic color and is used in various fields such as food, cosmetics and clothes dyeing. In general, food coloring agents are classified into synthetic colorants and natural colorants, which are chemical compounds. Synthetic colorants are widely used instead of natural pigments because of their high color ratio (Food and Drug Administration 2018). However, its use has been strictly regulated due to safety issues such as carcinogenicity and toxicity to the human body (Food and Drug Administration 2018). Due to the safety problems of these synthetic pigments and the health-oriented lifestyle patterns, natural product-oriented consumption trends have been increased more and more to natural pigment market, which is currently used for food coloring is greatly expanding.

Unlikely artificial synthetic pigments and natural pigments have high safety and reliability, and they can be combined in various ways due to many kinds of hues. Most of the ingredients of foods that can be edible and useful for coloring all foods. It is now a natural color, and natural pigments are attracting attention again and they are welcoming the renaissance.

The development of food and health functional products are expanding through stabilization technology that complements the disadvantages of natural coloring and search for new coloring. With the development of technologies to improve the functionalities by changing natural pigments into stable form, mass production of various food additives. Particularly, the coloring effect is good and the production and refining technologies are simpler than the synthetic coloring materials and the manufacturing cost can be reduced (Rural Development Administration 2014).

However, depending on the limitations and types of the coloring materials, it is expensive and can not be easily obtained. However, it is expected that new biological techniques will overcome this issue and develop more functional pigments (Lee KO 2006; Kim SJ 2007). In particular, it can be used as a natural dye having a stable condition that does not impair the quality

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of the food itself by reacting with other ingredients in the food to form a by-product (Song JC 1998). Therefore, in the field of food, some artificial coloring materials have been replaced with natural coloring materials and in fact, the development of this field is increasing (Lee et al. 2001; Griffiths JC 2005; Jeon et al. 2006).

Carotene, xanthophyll, carotenoid anthocyan, phycopyrine, chlorophyll, and Monascus pigment are used as natural coloring agents which are more stable to human body than synthetic coloring agents. Monascus pigments are a natural red pigment produced by the *Monascus* spp.. Monascin and ankaflavin are the yellow pigments, monascorubrin and rubropunctatin are the orange pigments, monascorubramine and rubropunctamine are the red pigments. In addition to natural pigments, they are involved in the synthesis of cholesterol. It inhibits the activity of HMG-CoA reductase, which is an enzyme, and produces various secondary metabolites such as monacolin K that effectively reduce blood cholesterol levels. They have nutritional and pharmacological properties related to vitamins, and have the advantage of being able to produce more natural colors than when used as food coloring agents. And the productivity is lower than that the synthetic coloring materials. Therefore, the production of natural coloring materials has been interested in microorganisms such as red pigments (Martinkova et al. 1995; Martinkova et al. 1999; Wu et al. 2000; Lee et al. 2001; Wild et al. 2002).

Nulug is with the history of mankind. It is often known as what comes to mind when it is leavened is used when making wine. The *red Nulug* is called *Hongkuk*, and the *Hongkuk* was recognized as a raw material for health functional foods in 2005 by the Food and Drug Administration (Seo et al. 2007; Bang et al. 2013). *Hongkuk* was used in processed foods such as alcohol, tofu, meat in China and Malaysia. The structure of the red and yellow pigment of *Hongkuk* was determined. The color components were red monascorubin, rubropunctatin, purple monascorubramine, rubropunctatamine, yellow monascin and ankaflavin (Choi et al. 2009). The red pigment is used for processed fish products and processed livestock products because of its good affinity for proteins. It is mainly used for coloring ham in China, Japan and Southeast Asia (Kim SJ 2007).

The safety of *Hongkuk* has already been recognized (Li et al. 1995). It is sold in Korea as cereal processed products, enzymatic foods in health functional foods, and functional rice. It is designated as a food additive to be used for coloring purposes. *Hongkuk* is obtained by inoculating rice with normal rice or glutinous rice and inoculating it with *Monascus purpureus*, *M. pilosus*, *M. anka*, and *M. ruber*, followed by fermentation (Seo et al. 2007). Some domestic companies also produces and sell *Hongkuk* in large quantities. In the future, the demand for the development of health functional foods and general foods in *Hongkuk* will increase gradually. In developed countries such as the EU and the United States, non-dye-based pollution-free ink and paint products are under development.

In particular, paint and ink that can be edible by using pigments extracted from *Monascus* spp. are not currently mass-marketed. By developing this paint or ink, it can be used for food, but it is also useful for improving the living environment exposed to environmental hormones or harmful substances. It can also produce all products that require food colors (non-toxic paints, body painting inks, camouflage creams, foods, etc.). Therefore, it is possible to use widely for development and related products as a material of the product which is used safely from infants to old people.

Therefore, this study aims to develop edible paint by substituting and fusing red pigment extracted from non-toxic pigment type ink or paint by cultivating *Monascus* spp. instead of botulinum toxin (BTX) type dye. In this study, isolates of *Monascus purpureus* and several species of *Monascus* were screened for their ability to produce pigments with excellent pigmentation ability, and red pigment of *Hongkuk koji*.

Materials and Methods

1. Colony size and culture characteristics of *Monascus* spp. strains

In this experiment, five strains, *Monascus perpureus* (3 species), *M. ruber* (1 species), and *M. pilosus* (1 species) were distributed from KCCM. Table 1 summarizes the strains provided by KCCM. The cultivars of *Monascus* spp. were cultured on a solid plate culture medium by inoculation with normal rice and inoculated with *M. pilosus, M. purpureus, M. anka*, and *M. ruber*. The medium used was PDYA (potato dextrose yeast extract agar; 2.4% potato dextrose Yeast broth (Difco), 2% bacto agar (Difco)), PDA (potato dextrose agar; 2.4% potato dextrose broth (Difco), 2% bacto agar (Difco)), MEA (malt extract agar; 1.5% malt extract, 2% bacto agar (Difco)).

Each strain was cultivated at 20℃, 25℃, 30℃, and 35℃ in PDYA, PDA, and MEA media for 7 days and subcultured at 1

Table 1. Strain purchased from KCCM

Microbe (strains)		Media	Temp.(℃)
① *Monascus perpureus*	KCCM 11832	MEA	26
② *Monascus perpureus*	KCCM 60462	PDYA	24
③ *Monascus perpureus*	KCCM 35473	MEA	26
④ *Monascus ruber*	KCCM 60142	PDA	24
⑤ *Monascus pilosus*	KCCM 60396	PDA	24

week intervals to maintain physiological activity. In order to compare the colonies of *Monascus* spp. cultured on a solid plate culture medium, the cultivation of *Monascus* spp. used in this experiment proceeded according to the strain summary information provided by KCCM, but the culture results were not good.

The culture conditions were examined in a solid plate culture medium. In order to measure the growth rate of each strain, the size of the annulus was measured on the day of incubation while culturing on a solid plate culture medium. That is, the growth rate of each strain was measured by measuring the size of the annulus for 18 days at the intervals of 2 days for each culture at 25℃, 30℃, and 35℃, As a result, culture temperature and incubation time were determined. Based on this, *Hongku koji* (*M. purpureus*) was extracted and a large amount of red pigment was extracted (Juzlova et al. 1996; Kim et al. 2012).

2. Red pigment production of *Hongkuk koji*

The production of *M. purpureus* spore suspension was carried out by subculturing *Monascus* spp. on the PDYA solid-phase plate medium at 35℃ for 3 days or more, based on the culture characteristics determined above. This spore was sprayed on rice *koji* prepared. In other words, 30 g of white rice (An Sung rice) was added to a 250 mL Erlenmeyer flask, and water was added and soaked overnight. This was autoclaved at 121℃ for 30 minutes, and the moisture content was adjusted to a content of 30% (5.0×10^5 spores/mL). was sprayed onto white rice grown with *M. purpureus* strain capable of producing *Monascus* spp. and excellent pigment production, and cultured at 35℃. and 85% humidity at 130 rpm for 15 days with shaking. *Hongkuk* rice *koji* was prepared (Kwak et al. 2003). The spore suspension was prepared by culturing the *M. purpureus* strain in a PDYA solid phase tube medium at 35℃ for 7 days, adding sterile distilled water, suspending the spores and mycelium with sterilized platinum beads, filtered to obtain spore suspension (Seo et al. 2007).

The red pigment, which is the raw material of the edible paint, was used as raw material for the rice *koji* which was mass cultured in the Erlenmeyer flask. The extraction of the red coloring matter was carried out using a vapor extraction device. In this experiment, distilled water (10 times as much as the weight of the sample) was used as the extraction solvent in the extraction flask with vertical reflux condenser, which is a common extraction device in the experimental room, for each sample weight. Extraction was carried out at 100℃ for 12 hours. This was again extracted with ultrasonic waves of 40 KHz (JINWOO Co. 40 kHz) at 60℃ for 20 minutes. The extracts of the obtained red pigment were filtered with a vacuum filter, concentrated, and freeze-dried. The yield of each red pigment powder was determined by weighing.

3. Comparative analysis of red pigment

The red pigment produced by *Hongkuk koji* was analyzed by TLC and HPLC instrument. For the TLC analysis of the red pigment, TLC analysis was carried out using a silica gel 60 TLC plate (Merck, Darmstadt, Germany). The samples were made from hexane extracts of commercial red pigment and red pigment of *Hongkuk koji*. For TLC analysis, and 5 μL of each pigment extract was applied onto a TLC plate and dried, and developed with chloroform: methanol: acetic acid (95: 7: 3) as a developing solvent. In order to identify the red pigment of *Hongkuk koji*, the strongest part of red was taken by TLC and analyzed by HPLC (Waters HPLC system). Conditions for HPLC analysis were as follows; column (Discovery® C18 (4.6 mm×250 mm, 5 μm)), column temp. (40℃), mobile phase (A solution : 0.1% Phosphoric acid, B solution : Acetonitrile, A:B = 65:35/Init→ 5:35/5 min→5:95/28 min→5:95/34 min→65:35/34.1 min→ 65:35/40 min), UV detector (390, 520 nm), flow rate (1.0 mL/ min), Inj. vol. (20 μL) (Morovjan et al. 1997; Food and Drug Administration 2010; Kim et al. 2012).

4. Edible paint manufacturing

Based on the patented technology (No. 10-1310713) of Eco-Gel Co., Ltd., we developed edible paint using food coloring material produced by *Hongkuk koji*. The edible paint manufacturing process changed the proportion of red pigment concentrate of by *Hongkuk koji* and the ratio of basic materials required for paint manufacture (Table 2) to produce edible paint. The red pigment was obtained by using the freeze-dried powder and the commercial red pigment in comparison with the pigment produced by *M. purpureus* (National Institute of Technology Quality 1999).

Table 2. Basic raw material mixing ratio of Echo-Gel Co., Ltd. Patent, Ink composition ratio for marking pen and edible paint using red pigment powder of *Hongkuk koji*

Blending source / Components	Echo-Gel Ink Amount of blending (w/w%)	Red pigment powder of *M. purpureus* 60462 (w/w%)	Remarks
Ethanol	62,89~24.98	100.0(g)	Solvent
TiO_2 / Red pigment powder	10.0(300 nmϕ) 20.0(250 nmϕ)	5.0	Pigment
Polyvinyl butyral resin	4.0~7.0	10.0	Polysaccharide, stickiness
Polyethylene glycol	12.0~22.0	6.0	Release agent
Liquid paraffin	1.0~2.0	-	Surfactants
Alkylamino fatty acid salt	2.0~5.0	2.0	Anti-sedimentation
Silica	0.01~0.02	0.05	Drying prevention
Ethylene glycol	1.0~2.0	-	Improvement of cleanness, prevention of cold freezing
Propylene glycol	1.0~2.0	2.0	Maintain moisturizing properties
Glycerin	1.0~2.0	5.0	Prevent drying
Distilled water	5.0~8.0	10.0	Air bubble removal corrector
Glyceryl paraben	0.1~5.0	-	Sunscreen

5. Viscosity, color development, degree of erosion, fade in and out, and prospects of edible paint

Monascus spp. using the red pigment of *M. purpureus* KCCM 60462, which has the clearest red color, the ratio of the substances required for concentrate and paint production was varied with the four mixing methods shown in Table 3.

At this time, pigments, glycerin, PG, BYK 185 and BYK 420 were added according to the blending ratio, and the mixture was mixed at a predetermined temperature (150℃) for 5 minutes at 50 rpm. In order to observe the viscosity, coloring, erasure, fade and drop of the painted paint, it was tested on a stainless steel plate for 5 days.

Results and Discussion

1. Colony size and culture characteristics of *Monascus* spp. strain

The cultivation of *Monascus* spp. in this experiment proceeded according to the summary information provided by the KCCM, but the culture results were not good and the cultivation conditions were examined in a solid plate culture medium. The optimal culture conditions for pigment production by solid phase culture of *Monascus* spp. were investigated. The size of *Monascus* spp. was significantly smaller than those cultivated at 35℃ and 25℃, and it began to be distinctive from 7th to 9th day. The

Table 3. Raw material blend ratio for characteristic comparison of edible paint using red pigment powder of *M. purpureus* KCCM 60462 strain (Unit: g)

Blending / Source components	Compound (Data 1)	Compound (Data 2)	Compound (Data 3)	Compound (Data 4)	Remarks
Ethanol	100	100	100	100	Solvent
Polysaccharide (resin)	8	8	12	12	Stickiness
KONASIL (silica)	0.05	0.1	0.05	0.1	Drying prevention
BYK185	10	6	-	-	Surfactants
BYK420	-	-	10	6	Surfactants
Polyethylene glycol	2	2	2	2	Release
Glycerin	5	5	5	5	Prevent drying
Distilled water	10	10	10	10	Air bubble removal
Red pigment powder	5	5	5	5	Pigment

size of the microorganisms grown to 30℃ at the observation day (about 18 days) was similar to that of the *Monascus* spp. cultured at 25℃.

However, the culture at 35℃ grew to be a large circle with a red pigment remarkably higher than that cultured at 25℃ and 30℃, and the red pigment specific to *Monascus* spp. also showed a dark red color. The most representative strain was *M. perpereus* KCCM 60462, which grows very rapidly. From this day on, the strain of colony began to appear and grew rapidly until the 3rd day of culture. On the 3rd day, it began to show a deep red color. The size of the final pear grew to be large enough to fill the Petri dish. The colony of *M. perpureus* strains with good culture conditions is shown in Fig. 1. In order to measure the growth rate of each strain, the ring size was measured for each culturing date in the solid plate culture medium. The growth rate of each strain was measured by measuring the size of the rings for 18 days at 2 days intervals at 25℃, 30℃ and 35℃. For comparison with the strain *M. perpureus* KCCM 60462, *M. ruber* KCCM 60142 was cultivated at 25℃, 30℃ and 35℃ in PDYA medium and the colony diameter (cm) was measured (Table 4). As a result, it was found that the culture temperature of the *Monascus* spp. was the best at the growth rate when at 35℃.

It was confirmed that not only the size but also the color and morphological characteristics of the same strain were different according to the kind of medium. In the case of *M. ruber* strain, it was red in PDYA or PDA medium, but not in MEA medium. However, *M. perpureus* strains showed colony which showed red color as incubation days became longer. The incubation time was also examined. In other words, the incubation temperature increased from 13 days at 35℃, and it remained similar after 15 days. Therefore, the *M. perpureus* strain was cultured at a culture temperature of 35℃ for up to 15 days on PDYA plate and liquid

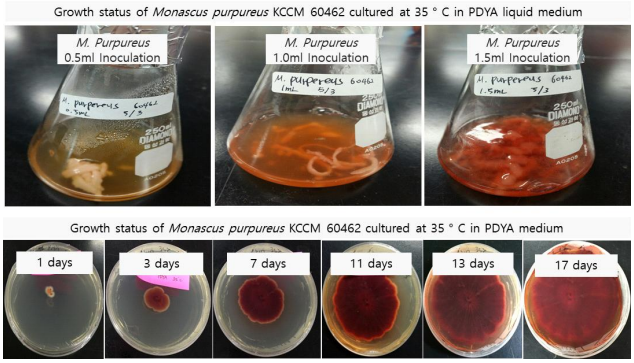

Fig. 1. Colony shape of *M. perpureus* cultured at 35℃ in PDYA medium.

Table 4. The size of the circle of cultured *Monascus* spp.
(Unit: cm)

Strain / Culture (day)	Circle size of *M. perpureus*			Circle size of *M. ruber*		
	25℃	30℃	35℃	25℃	30℃	35℃
0	0.5	0.5	0.5	0.5	0.5	0.5
3	2	1.6	2	1	1	1.5
5	4	3.5	3.5	1.5	1.5	2.5
7	6	5.5	5	2	2	2.5
9	8	7	6.5	2.5	2.5	3
11	8.5	8	8	4	3	3.5
13	9	8.5	9	5	3.5	4
15	9.5	9	9.5	5.5	4	4
17	9	9	9.5	5	3.5	4
18	8	8	9.5	5	3.5	4

medium to produce red pigment (Fig. 1).

As reported by Kim et al. (2012), it was important that the color of the mycelium changed to red in order to produce *Hongkuk* rice *koji*. Therefore, *M. ruber* KCCM 60141, *M. perpureus* KCCM 60016, *M. perpureus* KCCM 60344, *M. ruber* KCCM 60401 and *M. perpureus* KCCM 60397 were found to be useful in terms of color. In the liquid culture of Juzlova et al. (1996), the culture temperature range of *Monascus* strains was 25~37℃, and the most suitable temperature was 30℃.

2. Red pigment production of *Hongkuk koji*

Red pigment production of *Hongku koji* was from white rice (An Sung rice). The *M. purpureus* strain with excellent pigment production was sprayed with spore suspension (5.0×10^5 spores/mL). *Hongkuk rice koji* was prepared by shaking at 35℃ and 85% humidity at 130 rpm for 14 days. About 50% of the microbial cell constituents are carbon, which is used as an energy source for growth, as well as its constituents in microorganisms. However, there are many differences in their availability depending on the microbial strain (Jeon et al. 2006).

These results suggest that the growth of *Monascus* spp. is highly dependent on the type of carbon source. Therefore, the effect of *M. purpureus* MMK2 on pigment production was investigated. The degree of pigment production was measured by adding 3.0% sucrose, maltose, glucose, rice flour, barley flour, soybean flour, wheat flour and buckwheat flour. And the degree of pigment formation was measured by shaking culture at 30℃ at 130 rpm for 9 days. As a result, when the wheat flour was added

3.0% as a carbon source, the pigment productivity was the highest at 15.51 unit. Followed by buckwheat flour, barley flour and rice flour. These results indicate that Kim et al. (1977) and Park et al. (2005), *Monascus* sp. and *M. purpureus* showed different results from 4.0% rice flour reported as the optimum carbon source.

In addition, Seo et al. (2007), The optimal concentration of rice powder, which is a good carbon source for cell production and pigment production, was best in 5% rice powder production. At the concentration of rice powder of 4%, 38.52 units of yellow pigment, 39.53 units of orange pigment, and 37.45 μnits of red pigment were produced, and the pigment production was the highest. Based on the above results, the preparation of *Hongkuk koji* was made using rice (Kwak et al. 2003).

The red coloring matter extract of *Hongkuk koji*, which was cultivated in an Erlenmeyer flask, was combined with steam extraction and ultrasonic extraction. The extracts of the obtained red pigment were filtered and concentrated by using a vacuum filter, and then freeze-dried to produce a red pigment powder (Fig. 2). The yields of each red pigment powder were calculated as shown in Table 5 (Jeon et al. 2006).

In general, the stability of pigments such as anthocyanin (Cho et al. 2003), chlorophyll (Han et al. 1984), and carotenoid (Lee & Kim 1989; Kim et al. 2002) is also known as to decrease with heating temperature and storage time. Therefore, ultrasonic extraction was performed in order to suppress the degradation of pigment due to heat treatment as much as possible. Park et al. (2002) reported that the ethanol-soluble red pigment of *M. pilosus* has a very high thermal stability at 60~80℃. Min KH (1992) and Kim et al. (1997), the pigment was stable at 60~100℃ for 1 hour. In this study, it is expected that slight chromaticity

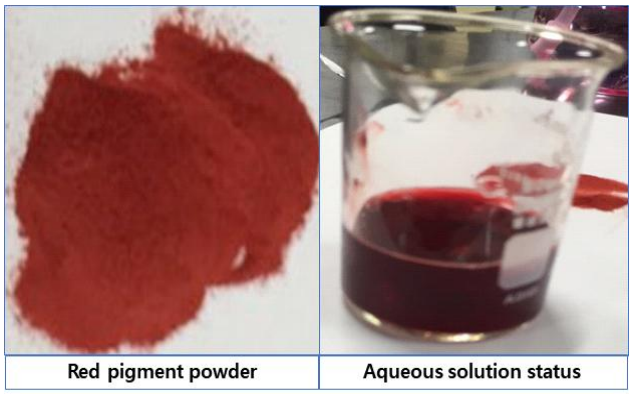

Fig. 2. Red pigment powder and aqueous solution status of *Hongkuk koji*

Table 5. Extraction yield of red pigment from *M. purpureus* culture

Sample \ Item	Raw material (g)	Weight after drying (g)	Extraction yield (%)
(1) *M. perpureus* (ultrasound)	1,500	102.332	**6.82**
(2) *M. perpureus* (water+steam)	1,316	43.832	**3.33**
(3) *M. perpureus* (water)	752	21.232	**2.82**

changes due to heat treatment is expected but, not enough to degrade tastiness and quality. As shown in Table 5, the extraction yield of the steam extractor was about 1.3 times higher than that of the ordinary water extract, and the extraction yield of the ultrasonic parallel extract was 1.2 times higher than that of the steam extractor. It is considered that the yield of ultrasonic concurrent extracts is 2~4 times higher than conventional water extracts because of the deaeration phenomenon. It releases dissolved oxygen or bubbles out of the oven during ultrasonic irradiation and causes synergy by interacting with liquids with each other (Kim et al. 1997; Lim & Kwak 2004).

3. Identification of red pigment ingredients

For the TLC analysis was developed as shown in Fig. 3. Red

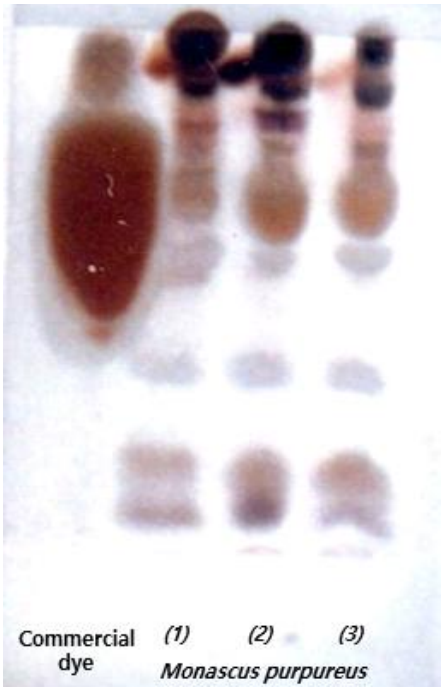

Fig. 3. TLC chromatogram of red pigment.

pigment can be seen in the upper part of the concentration. Therefore, the red pigment of the red pigment was identified by taking a portion having the same Rf value as the commercial pigment. For comparison, 20 μL of each dye was analyzed by HPLC (Waters HPLC system). The results of HPLC analysis are shown in Fig. 4. The red pigment of *Monascus* spp. was measured by HPLC and showed a large peak and a small peak. However, it was observed that there was little difference in the size or area of peaks and it was confirmed as a single material.

4. Manufacture of edible paint

Edible paint was prepared and using red pigment powder of *Monascus* spp. The powder was used to prepare an edible paint at the blending ratio shown in Table 3. Based on the patented technology of Eco-Gel Co., Ltd., which produces eco, non-polluting, non-toxic technology, edible paint was produced using edible red pigment produced by *Monascus* spp.. The concentration of the concentrate is shown in Fig. 5. When the non-sedimented paint is developed by using the red pigment of *Monascus* spp., the storage period becomes longer, and there is no reason to dispose of the sediment because the sediment is not solidified, and waste and environment can be reduced. It extracts pigment from eco natural materials to make powdered pigment, which can be used as a raw material for food, and can be painted directly on food or made into applicable edible paint. *Hongkuk*'s red pigment is also expected to be highly utilized for natural eco pigments.

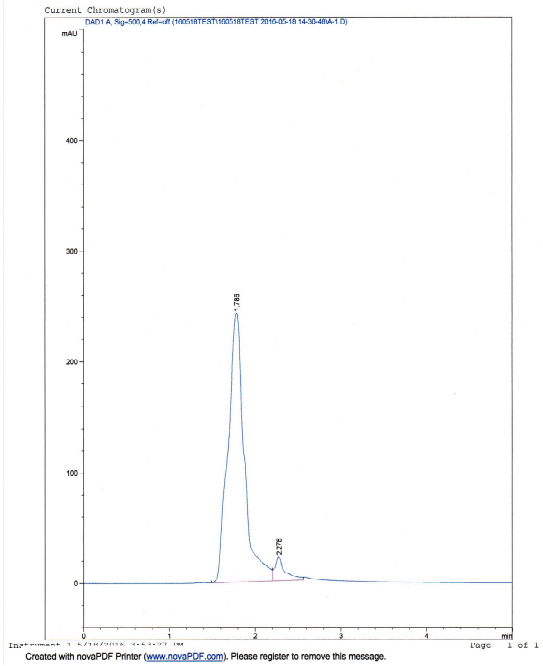

Fig. 4. HPLC chromatogram of red pigment.

5. Characteristic comparison of edible paint and prospects of edible paint

The red pigment of *M. purpureus* KCCM 60462, which has the clearest red color, was used to make edible paint using four different mixing methods, and the characteristics were compared. The results of the investigation of the characteristics of each angle are shown in Fig. 6.

In the case of compound 1 (data 1), the best condition was observed, while in compound 2 (data 2), the oil film was present on the surface after application. In the case of compound 3 (data 3), the viscosity was kept high as in the case of the sweet red-bean paste in the hardened state, and the state showed relatively poor falling without spreading. In the case of formulation 4 (data 4), the back side of the paper was wetted with water, but the best result was seen.

Natural red pigment of *Monascus* spp. can be used to make natural paint, and as shown in the red part of Fig. 7, this edible

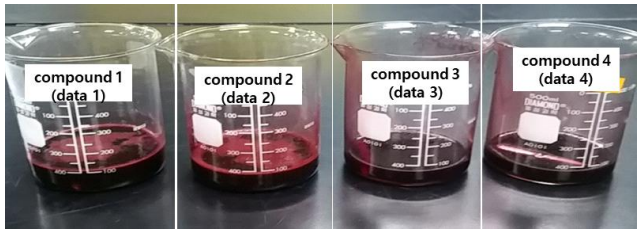

Fig. 5. Edible paint solution prepared by adding coloring matter and based on the determined temperature and rpm.

Fig. 6. Edible paint characteristics of red pigment of *M. purpureus* KCCM 60462 strain.

Fig. 7. Chocolate pie cake with an edible paint (red part above).

paint is applied to the choco pie cake to check the spreadability (red part above). It was found that it is possible to develop an edible paint which can be used for foods such as breads and confectioneries because of its high chromaticity and good spreadability. Currently, paint is used domestically and globally as the main material of paint, so pigment is precipitated by particles, difficult to use for a long time, and dye is non-eco because it is extracted from petrochemical. To solve problems of these dyes and pigments, and to be used in various foods, it was possible to develop edible paints and edible inks by using natural edible pigments extracted from nature. In the future, further basic experiments will be needed to supplement the mass production technology and develop food additive or eco materials for industrial use.

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