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Isolation of Pigment-Producing Mutants from *Monascus* sp. KS2 and Optimization of Cultural Conditions

Hyung-Eun Park, Chon-Ho Kimand Kyung-Hee Min**

Department of Biology, College of Sciences, Sookmyung Women's University , Seoul 140-742, and

***Department of Food and Nutrition, Hanyang Junior Women's College, Seoul 133-791, Korea*

Monascus sp. KS 2 로 부터 색소 생산 변이주의 분리와 배양조건의 최적화

朴亨恩 · 金天浩** · 閔庚喜

淑明女子大學校 理科大學 生物學科

**漢陽女子專門大學 食品營養學科

ABSTRACT: Several isolates producing the red-pigment were isolated from the Korean and Brazilian soils. The pigment producton by the molds belongs to a genus *Monascus* was investigated under the submerged culture. Mutagenesis of *Monascus* sp. KS 2 as the highest pigment production by NTG was made to increase pigment production. This mutant was examined to produce red and yellow pigment with 2.4 and 1.6 times higher than the parental isolates, respectively. The optimal cultural conditions for the pigment production by this mutant were: pH 6.0 , temperature 30℃, rice powder 5%, and monosodium glutamate 0.15%.

KEYWORDS: *Monascus* pigments, Mutagenesis, High production of yellow and red pigment

The mold belonging to a species of *Monascus* has been traditionally employed for red wine and for red soybean cheese in China, Japan and Indonesia as inoculated in the steamed hulled-rice.

Recently, microbial pigment from *Monascus* sp. is gradually used for the coloring agent replaced with synthetic one in the food industry. Numerous studies have been made on composition and structure of the constituent molecules of these pigments, and on the growth of the microoganisms themselves. The molecular structure and chemical properties of these pigments are figured out (Su, 1983). The main components of pigments are known as rubropunctatin ($C_{21}H_{22}O_5$-red color) (Haws *et al.*,1961), monascorubrin ($C_{23}H_{26}O_5$-red color) (Kurono *et al.*, 1963), monascin ($C_{21}H_{26}O_5$-yellow color) (Birch *et al.*,1962; Fielding *et al.*, 1961), ankaflavin ($C_{23}H_{30}O_5$-yellow color) (Manchand *et al.*,1961), rubropunctamine ($C_{21}H_{23}NO_4$-purple color), and monascorubramine ($C_{23}H_{27}NO_4$-purple color).

For the production of the pigments, this mold has been cultivated by the solid culture methods (中澤亮治 *et al.*, 1930), using rice or bread as substrate in fermentation industry. However, the submerged culture methods by Lin (1973) have been improved extensively. Also there is very limited information regarding the production of *Monascus*-pigment by submerged culture, even using the wild type. Mutagens and X-ray or fast-neutron irradiation were used to improve the yield of *Monascus*-pigment production (Lin *et al.*, 1973; Su, 1983; Wong *et al.*, 1977).

This studies were subjected to isolate high pigment-producing mutant of *Monascus* sp.,and to investigate the optimal cultural conditions for red

Table I. List of Type Cultures and strains.

Strains	Sources
Monascus anka 30873	* IFO
Monascus araneosus 12152	** IAM
Monascus pilosus 4520	IFO
Monascus ruber 9203	IFO
Monascus sp. KS 2	Korean soil
Monascus sp. BS 621	Brazil soil
Monascus sp. BS 622	Brazil soil
Monascus sp. SUW 11	*Monascus* sp. KS 2
Monascus sp. SUP 12	*Monascus* sp. KS 2
Monascus sp. SUG 24	*Monascus* sp. KS 2
Monascus sp. SUG 25	*Monascus* sp. KS 2
Monascus sp. SUR 34	*Monascus* sp. KS 2
Monascus sp. SUR 37	*Monascus* sp. KS 2

* IFO : Institute Fermentation Osaka, Japan
** IAM : Institute Applied Microbiology, Japan

pigment production, and the properties of the pigment produced .

Material and Methods

Microoganisms

Type cultures and isolates used in this paper were shown in Table I. The isolates for pigment-producing strain were selected from Korean and Brazilian soils.

The isolates of *Monascus* sp. SUW or SUR series were obtained from KS 2 by NTG treatment, which would be mentioned later.

Culture media and cultivation

Fermentation medium for pigment production by submerged culture was composed of rice powder 3%, $NaNO_3$ 0.15%, $MgSO_4 \cdot 7H_2O$ 0.1%, KH_2PO_4 0.25%. and pH 6.0 (Lin, 1973). The medium was autoclaved at 120℃ for 15 minutes.

YM agar composed of yeast extract 0.3%, malt extract 0.3%, peptone 0.5%, glucose 1.0%, and agar 2% (pH 6.0) was used for isolation of mutant (Su, 1983). Malt extract agar (MEA) was used for seed culture, which was composed of 10g peptone, 5g malt extract, 20g glucose, and 20g agar in one liter of distilled water.

Twentyfive m*l* of culture broth in 250 m*l* Elenmeyer flask were inoculated with a loopfuls of vegetative mycelia grown on MEA for 6 days at 30℃. Fermentation was carried out on a reciprocal shaker for 5 days cultivation.

Mutagenesis

Monascus sp. KS 2 was cultured at 30℃ for 7 days on MEA media. The ascospore and conidia formed were collected and suspended in sterile distilled water. The suspension was filterd through cotton filter and sedimented by centrifugation at 7,000 rpm for 10 minutes. The suspension was adjusted to about 10^7 spore per m*l* of sterile distilled water.

N-Methyl-N'-nitro-N-nitrosoguanidine (NTG) was dissolved in 0.2 M citrate buffer, pH 5.0 and its concentration was adjusted to 3.0 mg per m*l*. Spore suspention was added to NTG solution, and the mixure was allowed to stand for 90 minutes without shaking. After NTG treatment the mixure was centrifuged, supernatant was discared and the spores was filtered with the strilized water. The ungerminated spore suspension was plated on YM agar. The colonies producing high pigment production were collected under the naked eyes.

Analysis and extration of *Monascus*-pigment

The mycelia of cultural broth were harvested by centrifugation at 7,000 rpm for 15 minutes and the supernatant was filtered with the filter paper (Milipore;0.45 μm). After diluting with the distilled water, the optical density at 400 nm (yellow pigment) and 500 nm (red pigment) of the filtrate was measured by spectrophotometer (Su, 1983). The values of absorbance multiplicated by the dilution factor are expressed as the amount of extracellular pigment.

The harvested mycelia were washed with the centrifugation and were mixed with two volumes of 70% ethanol in 250 m*l* flask, and the flask was shaken at 30℃ for one hour in order to extract the pigment from the mycelia. The extract was filtered and after proper dilution with 70% ethhol, the absorbance at 400 nm and 500 nm of the filtrate was read again. The values of absorbance multiplied by dilution factor are expressed as the amount of intracellular pigment (Su, 1983).

Table II. The pigment productivity of type culture and isolates.

Strain	Production of Pigment		Sources
	$O.D_{500}$	$O.D_{400}$	
Monascus anka	7.40	9.30	IFO
Monascus araneosus	0.62	0.74	IAM
Monascus pilosus	1.03	1.66	IFO
Monascus ruber	0.06	0.16	IFO
Monascus sp.KS 2	11.10	15.60	Korean soil
Monascus sp.BS 621	4.80	6.90	Brazil soil
Monascus sp.BS 622	5.54	7.44	Brazil soil

Determination of the amount of cell yield

The pigment-extracted-cell residues were weighed after drying at 105℃ for 3 hr.

Results and Discussion

Pigment Productivity of the Isolates

In screening the microoganisms with high pigment productivity, four strains of type cultures and three strains isolated from soils were examined with the pigment productivity in submerged culture with pigment producing medium. Among the strains, *Monascus* sp.KS 2 showed the highest pigment production (Table II). Hence, this strain was used for the subsequent experiments. Description of *Monascus* sp. identification will discuss later.

Isolation of hyperpigment-productive mutants

Six strains, *Monascus* sp. SUW 11, SUP 12, SUG 24, SUG 25, SUR 34, and SUR 37 were selected on the basis of pigment productivity from about 12,000 isolates. Poor growth of the mutant caused by mutagenesis was generally considered as a normal phenomenon. The growth rate of hyperproductive mutants SUR 34 and SUR 37 was much increased compared with that of their parent.

The effect of NTG concentration for the mutagenesis is quite different from that on other genera, such as *Rhizopus, Aspergillus* and others. The NTG concentration required to produce a 90 % killing rate with the genus *Monascus* was high, up to 3.0 mg per m*l* for 60 to 90 minutes treatment.

Pigment productivity of the mutants are summarized in Table III. Among mutants, SUR 37 produced large amounts of red-and yellow-colored substance: maximum absorption was at 500 nm and 400 nm. The highest yields of red and yellow pigment were 31.00 of OD at 500 and 27.80 of DO at 400. Pigment production compared with the parent strain, *Monascus* sp. KS 2 was over 2.4 and 1.6 times more in the red and yellow pigments, respectively (Fig. 1). Strains SUW 11 and SUP 12 were defective in production of red pigment in both agar slant and submerged cultures.

Lin & Suen (1973) and Su (1983) described that the productivities of red and yellow pigments from the mutants were over 4.9 and 7.0 times for *Monascus* sp. S-11, and 2.6 and 1.9 times for *M. anka* V-204, repectively, that of the original parent st-

Table III. Pigment productivity of the mutants derived from *Monascus* sp. KS 2.

Strains	Red pigment		Yellow pigment	
	Productivity (O.D.500nm)	Relative productivity (%)	Productivity (O.D.400nm)	Relative productivity (%)
Monascus sp.				
KS 2	11.10	100	15.60	100
SUW 11	0.19	1.7	0.48	3.0
SUP 12	0.10	0.9	0.45	2.8
SUG 24	3.90	35.1	6.30	40.3
SUG 25	7.20	64.8	10.40	66.6
SUR 34	13.10	118.0	19.70	126.3
SUR 37	26.60	239.6	25.30	162.1

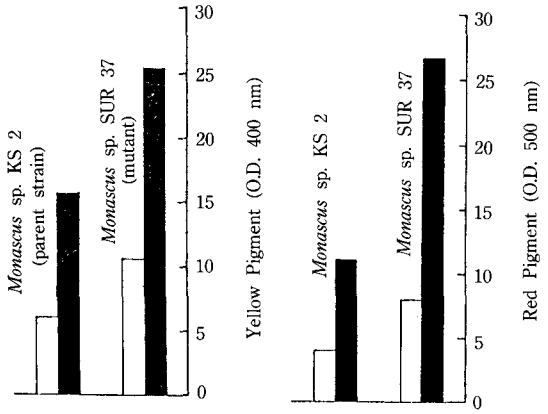

Fig. 1. Comparison of the pigment productivity between *Monascus* sp. KS 2 as parental strain and mutant SUR 37. ■; intracellular pigment, □; Extracellular pigment

Table IV. Effect of temperature on the pigment production by *Monascus* sp. SUR 37.

Temperature (℃)	Red pigment prodution (O.D.at 500 nm)	
	Extracellular	Intracellular
15	1.20	15.10
30	2.58	19.90
35	1.05	9.52
40	0.12	5.15

rain. Also, X-ray and neutron irradiation were used to induce several mutants of *M. purpureus,* which differed from the parental one in pigmentation and morphology. This fungus also produces effective antibiotics against *Bacillus, Pseudomonas and Streptococus* (Wong, 1977).

The conditions of pigment production by *Monascus* sp. SUR 37

1) Effect of temperature and pH

Table IV shows the influence of cultivation temperature on pigment production. Cultivation was carried out on a reciprocal shaker at various temperatures. Although cell growth was not affected, pigment production was greatly influenced by temperature, and the optimum temperature was 30 ℃, agreed with the result of Manandhar and Apinis (1971). However, pigment production showed a decrease remarkably at higher temperature than 30℃.

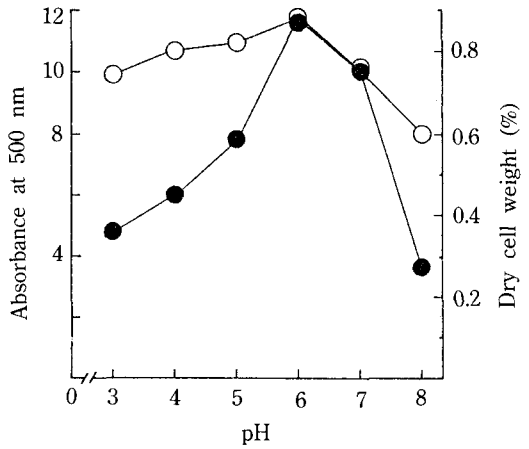

Fig. 2. Effect of the initial pH of the fermentation media on the pigment production by *Monascus* sp. SUR 37. Extracellular pigment(●) and dry cell weight (○) were determined by the Methods & Materials in this experiment.

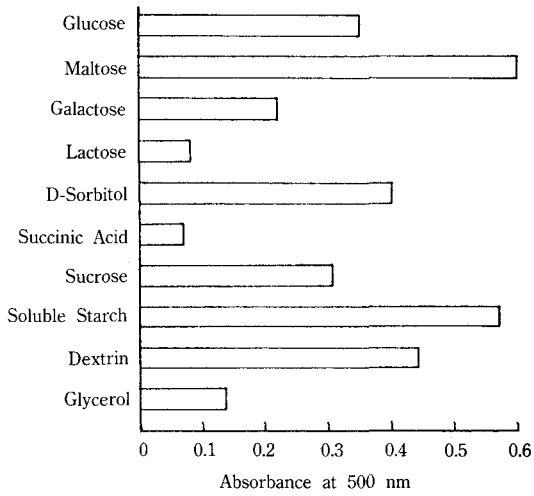

Fig. 3. Effect of carbohydrates on the pigment production in fermentation media by *Monascus* sp. SUR 37. Extracellular pigment was measured by Methods and Materals in this experiment.

Fig. 2 shows the influence of initial pH of broth medium on cell growth and pigment production in a shaking culture. Pigment production was influenced by initial pH, while growth was not affected. The highest productivity of pigment was observed at pH 6.0, but at pH higher than 7.0 pigment production was decreased noticeably.

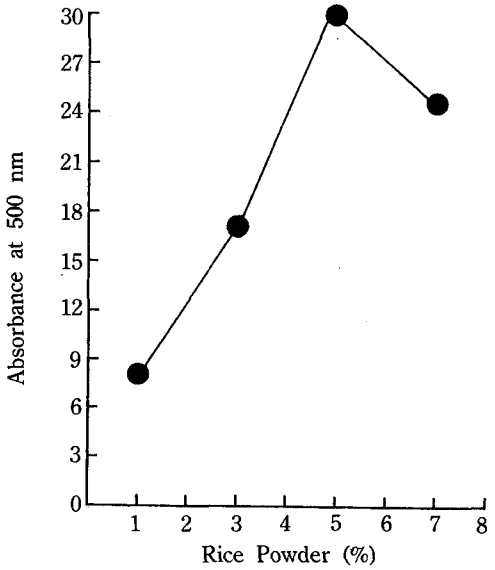

Fig. 4. Effect of rice powder concentration in fermentation media for the pigment production by *Monascus* sp. SUR 37.

2) Effect of the carbon sources

Soluble starch, maltose, and dextrin were found to be the most suitable substrate for the pigment production (Fig. 3). As reported by Lin (1973), the utilization of carbon sources of *Monascus* sp. showed similar result that soluble starch showed higher pigment productivity than monocarbohydrate. The pigment productivity was increased along with the increase of the rice powder concentration, from 1 to 5%, but when it exceed over 5%, the productivity was decreased noticeably as shown in Fig. 4. Thus, the pigment production with 5% of rice powder showed the highest value.

Table VI. Effect of the concentration of monosodium glutamate (MSG) on the pigment production by *Monascus* sp. SUR 37.

MSG (%)	D.C.W. (%)	Red Pigment (Absorbance at 500 nm)	
		Extracellular	Intracellular
0.05	0.64	0.43	24.10
0.10	0.80	0.74	26.20
0.15	0.85	1.18	30.80
0.20	0.79	1.62	28.80
0.30	0.78	2.82	26.90

3) Effect of nitrogen sources

Table V showed the effect of various nitrogen sources on the pigment production by *Monascus* sp. SUR 37. Monosodium glutamate (MSG), yeast extract, KNO_3, $NaNO_3$ and casamino acid were found to be suitable nitrogen sources for pigment production. Among them, MSG, 0.15% gave the highest yield of pigment (Table VI).

In the present study, the result of nitrogen sources utilization was similar to that of the other studies (Lin, 1973; Su, 1983; Kim *et al.*, 1979; Yoshimura *et al.*, 1975). The red pigments were produced in yeast extract or nitrate media at pH 6.5 and orange pigments were formed in ammonium sulfate or $(NH_4)_2HPO_4$ media, as reported by Carels and Shepherd (1977). Also, the greater amou-

Table V. Effect of nitrogen sources on the pigment production by *Monascus* sp. SUR 37.

Nitrogen Sources	*D.C.W (%)	Final pH	Red Pigment (O.D. 500nm)		Color
			Extracellular	Intracellular	
NH_4NO_3	0.96	5.2	0.49	8.30	red
Yeast extract	0.91	4.1	0.52	20.90	red
KNO_2	0.61	7.3	5.03	7.20	red
Casamino acid	0.72	5.6	0.86	10.70	red
$(NH_4)_2SO_4$	0.74	1.1	0.42	4.78	orange
$NaNO_3$	0.61	6.7	5.28	12.90	purplish-red
MSG	0.80	6.2	1.64	23.30	red
KNO_3	0.79	7.2	3.95	18.20	purplish-red
$(NH_4)_2HPO_4$	0.86	2.0	0.24	7.09	orange

*D.C.W. represents dry cell weight (g) per 100 m*l* of culture broth

Table VII. Effect of cereal sources on the pigment production by *Monascus* sp. SUR 37.

Cereals (3%)	D.C.W. (%)	Red Pigment (Absorbance at 500 nm)	
		Extracellular	Intracellular
Corn meal	1.18	3.50	25.40
Potato starch	0.60	1.73	33.40
Soybean flour	1.96	0.55	2.95
Barley flour	0.94	5.42	20.60
Glutinous rice	0.70	6.92	21.100
Wheat meal	0.78	8.75	23.30
Corn starch	0.65	3.08	34.90

Several kinds of cereals were substituted for rice powder.

Table VIII. Effect of amino acid on the pigment production by *Monascus* sp. SUR 37.

Amino acid	Red Pigment (Absorbance at 500 nm)	
	Extracellular	Intracellular
Control	4.88	11.70
L-Lysine	0.18	0.59
DL-Serine	2.12	19.50
L-Methionine	1.00	0.99
L-Glycine	0.61	12.70
L-Alanine	4.67	16.40
L-Arginine	3.50	10.60
L-Glutamic acid	5.42	4.80
L-Histidine	2.90	3.17
L-Tyrosine	8.75	3.90
DL-Valine	6.64	4.20
L-Cystine	1.46	2.71
DL-Threonine	20.24	7.60
L-Tryptophan	2.59	3.30
DL-Isoleucine	13.04	7.60

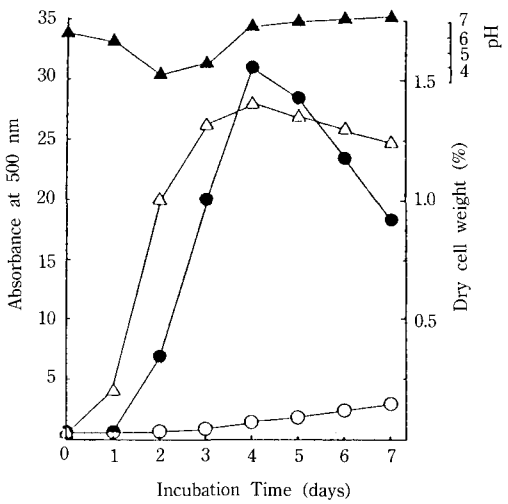

Fig. 5. Time courses of the pigment production by *Monascus* sp. KS 2.
Cultivation was carried out in a rotary shaker at 30℃ with the broth medium containing 5% of rice powder, 0.15% of monosodium glutamate, 0.1% of $MgSO_4$. $7H_2O$, and 0.25% of KH_2PO_4. ○-○; Extracellular pigment, ●-●; Intracellular pigment, ▲-▲; pH, △-△; Dry cell weight

nts of red pigments were formed in the media containing high level of ammonium nitrate and low glucose (Wong *et al.*,1981).

4) Effect of various kind of cereals as carbon sources

Corn starch, potato starch, and corn meal were found to be the best carbon sources for the pigment production by strain SUR 37 (Table VII).

5) Effect of Amino acid

The effect of amino acids on the pigment production was investigated by *Monascus* sp. SUR37.

Threonine, isoleucine and tyrosine were found to be the most effective substrate for the extracellular pigment production, while serine and alanine

Table IX. Effect of pH on soluble *Monascus*-pigment in water and 70% ethanol.

Solvent	pH						
	3	4	5	6	7	8	9
Water	reddish orange	reddish orange	reddish orange	reddish orange	reddish orange	reddish orange	reddish orange
Ethanol (70%)	reddish red	reddish red	red	red	purplish red	purplish red	purplish red

for the intracellular pigment production (Table VIII).

In addition to the result, it was found that glycine and arginine were effective substrate for intracellular pigment production. This result is good agree with that of Kim *et al.* (1979) and Yoshimura *et al.* (1975).

6) Time courses of the pigment production

Fig. 5 shows the time courses of pigment fermentation with broth medium and optimum cultural conditions in which the initial pH was adjusted to 6.0. The pigment productivity in broth culture was increased along with the cell growth, and maximum pigment production reached a peak at the four days cultivation. The pH value began to decrease at the early stage of cultivation. When carbon source was exhaused, pH commenced to rise up to about 7.0 correlated with pigment production.

After 4 days fermentation, the yeild of red pigment (O.D.500 nm) or yellow pigment (O.D.400 nm) was 31.00 or 27.80, respectively. Most of the pigment was an intracellular one and its intensity was about 20-fold that of extracellular pigment. *Monascus* pigment has also been considered as one of the most hopeful pigments which are now under development.

Properties of the pigment

The pigment was readily soluble in ethanol and only slightly soluble in water. Table IX shows the hue of *Monascus*-pigment in 70% ethanol at various pH values. *Monascus*-pigment can provide color huges ranging from reddish orange (pH 3-4) to red (pH 5-6) or purplish red (pH 7-9).

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摘 要

식품용 색소로 사용되고 있는 *Monascus*-pigment (홍국색소) 를 산업적으로 생산하기 위하여 공기, 토양등으로부터 균주를 순수분리하여 색소생산용 배지에서 30℃로 5일 동안 액체배양한 결과 색소생산이 가장 높은 *Monascus* sp. KS 2 를 분리하였다. 이균주를 모균주로 하여 NTG로 돌연변이를 유발한 결과 6개의 돌연변이주를 얻었으며, 색소생산성을 비교한 결과 가장 강력한 색소생산성 돌연변이주인 *Monascus* sp. SUR 37 을 선별하였으며, 모균주에 비해 적색색소와 황색색소가 각각 2.4배, 1.6배 높았다. 색소생산의 최적배양 조건은 온도 30℃, pH 6.0, 탄소원으로는 rice powder 5%, 질소원으로는 monosodium glutamate 0.15%, 그리고 아미노산으로는 DL-threonine, 배양기간은 4일이 가장 효과적이었다.

References

Birch, A. J., Cassera, A., Fitton, P., Holker, J. S. E., Smith, H., Thompson, G. A. and Whalley, W. B. (1962): Studies in relation to biosynthesis. Part XXX. rotiorin, monascin, and rubropunctatin. *J. Chem. Soc.* 3583-3586.

Carels, M., and Shepherd, D. (1977): The effect of different nitrogen sources on pigment production and sporulation of *Monascus* species in submerged, shaking culture. *Can. J. Microbiol.* **23**: 1360-1372.

Fielding, B. C., Holker, J. S. E., Jones, D. F., Powell, A. D. G., Richmond, K. W., Robertson, A. and Whalley, W. B. (1961): The chemistry of fungi. Part XXXIX. The structure of monacin. *J. Chem. Soc.* 4579-4589.

Haws, E. J., and Holker, J. S. E. (1961): The chemistry of fungi. Part XXXVIII. Further evidence for the structure of rubropunctatin. *J. Chem. Soc.* 3820-38 22.

Kim, H. S., Kim, D. H., Yong, H. S., Pyun, Y. R. and Yu, J. H. (1979): Studies on the red pigment produced by *Monascus* sp. in submerged culture. *Korean J. Appl. Microbiol. Bioeng.* 7(1): 23-30.

Kurono, M., Nakanish, K., Shinde, K. and Tada, M. (1963): Biosynthesis of monascorubrin and monascoflavin. *Chem. Pharm. Bull. Tokyo* **11**(3): 359-362.

Lin, C. F. (1973): Isolation and cultural conditions of *Monascus* sp. for the production of pigment in a submerged culture. *J. Ferment. Technol.* **51**(6): 407-414.

Lin, C. F. and Suen, S. J. T. (1973): Isolation of hyper-pigment-productive mutants of *Monascus* sp. F-2. *J. Ferment. Technol.* **51**(10): 757-759.

Manchand, P. S., Whalley, W. B. and Chen, F. C.

(1973): Isolation and structure of ankaflavin: a new pigment from *Monascus Anka*. *Phytochem.* **12**: 2531-2532.

Manandhar, K. L. and Apinis, A. E. (1971): Temperature relations in *Monascus*. *Trans. Brit. Mycol. Soc.* **57**(3): 456-472.

Su, Y. C. (1983): Fermentative production of *Anka*-pigments (*Monascus*-pigments). *J. Appl. Microbiol. Bioeng.* **11**(4): 325-337.

Wong, H.C. and Bau, Y.S. (1977): Pigmentation and antibacterial activity of fast neutron-and X-ray-induced strains of *Monasus purpureus* Went. *Plant Physiol.* **60**: 578-581.

Wong, H. C., Lin, Y. C. and Koehler, P. E. (1981): Regulation of growth and pigmentation of *Monascus purpureus* by carbon and nitrogen concentrations. *Mycologia* **73**: 649-654.

Yoshimura, M., Yamanaka, S., Miisugi, K. and Hirose, Y. (1975): Production of *Monasus*-pigment in a submerged culture. *Agr. Biol. Chem.* **39**(9): 1789-1795.

中澤亮治 and 佐藤喜吉 (1930): 臺灣産紅穀中の *Monasus* に就て. *Arg. Chem. Soc. Japan* **6**: 352-358.

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