pH Stability of Ultrasonic Thai Isolated Monascus Purpureus Pigments

Issara Wongjewboot, Sasithorn Kongruang

***Abstract*—The ability of *Monascus* species to produce many different types of pigments and secondary metabolites, such as monacolin K and citrinin, is remarkable. Mutations of Thai strains of *Monascus purpureus* in TISTR 3002 were induced by using an ultrasonic wave of 45 kHz for 2 min at 28°C. The mutations were carried out for 4 generations in order to develop efficient food colorants and to study the enhancement of secondary metabolites. The results showed that monacolin K could not be detected in fermented broths from any of the 4 generations. Mutant generations from TISTR3002, contained citrinin at concentrations in G_0 (wild type), G_1, G_2, G_3 and G_4 of 18.48, 0.01, 64.98, 2.34 and 110.96 µg/ml, respectively. A quantitative analysis of pigments was performed on all generations and results indicated that the stability of derivatives of yellow, orange and red pigments was greatest at pH 8. A significant increase in pigment stability was gained when TISTR 3002 was ultrasonically induced up to G4. Pigment derivatives of all generations of TISTR 3002 exhibited a greater stability in the basic pH range when compared with an acidic pH range.**

***Index Terms*—*Monascus purpureus*, yellow pigment, orange pigment, red pigment, mutant, ultrasound**

I. Introduction

At present, one of the most popular natural color-producing fungus strains for food application is *Monascus purpureus* because of its remarkable ability to produce many different types of pigments and secondary metabolites of polyketidic structure. It has recently become a popular dietary supplement because of the discovery that it contains many bioactive constituents which have pharmaceutical benefits. These constituents include monacolins, pigments, dihydromonacolins, citrinin, γ-Aminobutyric acid and dimerumic acid [1-5]. However, under certain conditions some strains of *Monascus* produce a secondary metabolite citrinin which is toxic and which can cause a safety problem for consumption [6]. Chen et al. [6] have shown that the acyl-transferase and ketosynthase domains of the *pksCT* gene that encode for citrinin polyketide synthase are found in *M. purpureus*, *M. kaoliang*, and *M. sanguineus*. Furthermore, the *ctnA* gene, a major activator for citrinin biosynthesis, was found in *M. purpureus* and *M. kaoliang*, but was absent in *M. sanguineus*.

As shown in Figure 1, there are eight major colorants produced by the *Monascus* fungus. The identification of these eight colorants was made through HPLC and mass spectrometry [7].Rubropunctamine and monascorubramine are the richest sources of red colorants, while monascin and ankaflavin are yellowish colorants and rubropunctatin and monascorubrin are orange colorants. An additional yellowish colorant named Xanthomonasin A in the mutant of *M. anka* has also been identified [8]. In addition to their use as colorants, all eight pigments have been found to have biomedical applications. Among the pigments, monascorubrin is the most effective for coloring foods and textiles, and it also has a therapeutic function because it has anti-inflammatory activity [9]. The orange pigments, rubropunctatin and monascorubrin have been found to possess antibiotic activity against bacteria, yeast, and filamentous fungi [10]. Rubropunctatin and monascorubrin can inhibit the growth of *Bacillus subtilis* and *Candida pseudotropicalis*. The yellow pigments, monascin and ankaflavin show immunosuppressive activity on mouse T splenocyte cells [8]. Because of their possible applications it is important to set up quality control standards for the eight *Monascus* pigments and for citrinin.

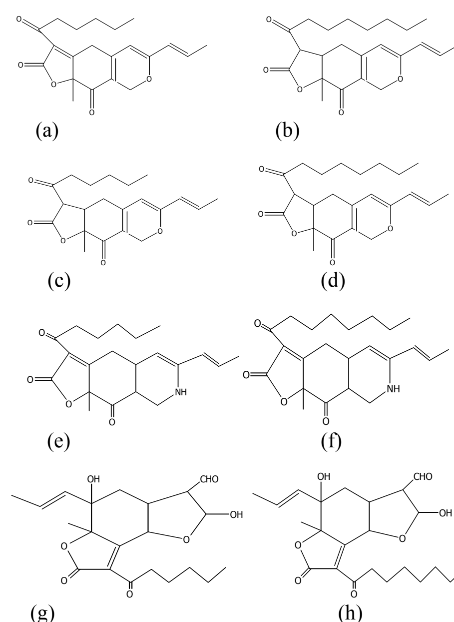


Fig. 1 Structures of eight major pigments produced by *Monascus spp.* : (a) rubropunctatin, (b) monascorubrin, (c) monascin, (d) ankaflavin, (e) rubropunctamine, (f) monascorubramine, (g) xanthomonasinA, and (h) xanthomonasin B

Growth and pigment production of *Monascus* species is usually carried out by fermentation. Fermentation can be carried out by several different approaches, for example, by solid-state submerged cultivation or by batch fermentation using either free or immobilized cells. In both approaches, the composition of the medium can be optimized to get high pigment yields. The fermentation approaches have been extensively investigated because *Monascus* pigments are

considered as excellent colorants of yellow to red with a variety of properties [11, 12].

There are large differences in the properties of the different colorants derived from *Monascus* strains. Many of the pigments are not hydrosoluble and can decolor under light. However, they are stable in a pH range of 2-10 and are heat-stable to autoclaving. Their low water solubility and decoloring under light have limited the use of *Monascus* pigments in the food industry [13,14].

In this paper, we study the stability of derivatives of *monascus* pigments obtained from ultrasonic induced mutation of wild Thai isolated types of *M. purpureus* TISTR3002. The mutants (generations 1-4) were obtained by fermentation of a modified yeast extract supplemented with cassava starch and monosodium glutamate. The pigments were analyzed for stability under varying conditions of pH.

II. Materials and Methods

A. Reagents

Culture medias (potato dextrose agar, yeast extract powder, peptone, malt extract powder) were purchased from Himedia Laboratories Pvt. Ltd., India. N-source-medium (monosodium glutamate) and C-source (cassava starch) were purchased commercially in Thailand. L-glucose was a product of Sigma-Aldrich Co. Pure citrinin was purchased from Sigma Chemical Company.

B. Strains and Culturing Conditions

M. purpureus TISTR 3002 was obtained from Thailand Institute of Scientific and Technological Research, Thailand. The stock of culture in freeze-dried ampoules was activated in YM broth at 30^0 C for 2 days and then transferred to PDA and further incubated for 7 days. The radial growth of each of the four generations was measured in duplicate by point inoculation at the center of 8 PDA petridishes. The cultures were then incubated at 30°C for 14 days. The radius of each colony was measured from the center of the dish along 2 perpendicular axes at intervals of 2 days over a period of 14 days. The growth rate of each generation was calculated by using linear regression.

C. Cultivation for pigment production

The stock of culture in freeze-dried ampoules was activated in YM broth at 28^0C for 2 days and transferred to PDA and further incubated for 7 days. A spore suspension of 10^6 spores/ml of 0.1% Tween 80 solvent was initially transferred to a 30 ml autoclaved liquid medium of modified YM broth (5% peptone, 3% yeast extract powder, 3% monosodium glutamate, 10% cassava starch in distilled water). An Erlenmeyer flask containing 30 ml of seed culture medium was then incubated in a shaking incubator at 200 rpm, 30^0C for 7 days. The culture was then transferred into a flask containing 300 ml of the same medium. The culture media was then separated by centrifugation at 4°C for 20 min (10,000 ×*g*). The mycelia were extracted and filtered again through a 0.45-μm membrane. The supernatant was kept for further analysis.

D. Ultrasonic induced mutation treatment

The culture medium of seed culture of wild type *M. purpureus* TISTR 3002 was filtrated and the seed suspensions in 7% saline solution were then treated by ultrasound at 45 kHz for 2 min at 28 ^{0}C. After the ultrasound, the mycelia were washed with double distilled water. They were then used as a seed for radial growth determination and for cultivation for pigment production by the procedures described above. The mycelium grown in the PDA was used as generation 1 (G1). This the ultrasonic treatment was repeated on G1 to obtain a second generation (G2). The ultrasound treatment was repeated to obtain generations G3 and G4.

E. Pigments, citrinin and monacolin K determination

Pigment concentrations were estimated using spectrophotometer (UV-vis spectrometer, Shimadzu Co., UV-1201, Tokyo) at 340, 440 and 480 nm for yellow, orange and red pigments. An HPLC system (Waters, Milford, MA, USA) was used to perform an HPLC analysis using the method previously described [15]. A C_{18} column, (250 x 4.6 mm, i.d.5μm) was used as the analytical column. Injector volume: 40 μl was used. The mobile phase consisted of acetonitrile /water/ trifluoroacetate (55:45:1, v:v:v). The flow rate was set at 1 ml/min and citrinin was detected using a UV detector set at 238 nm. Pure citrinin was used as the standard curve.

F. Effect of pH condition on the pigment stability

Sample solution concentrations at 340, 440 and 480 nm were measured using a spectrophotometer after the solutions were adjusted to various pH at 30°C. Buffer solutions (0.1M, potassium phosphate) at 8 different pH values (1, 3, 4, 5, 6, 7, 8, and 13) were prepared for pH stability tests. After 6h, the optical density of the pigment solutions was measured at 340-700 nm using a spectrophotometer.

G. Rate of stability degradation

The first-order degradation rates of the stability reactions were assumed for pigment decomposition as follows: $dC/dt = -kC$. The equation was integrated as follows: $\ln(C/C_0) = -kt$ (C is the pigment concentration at t, C_0 the pigment concentration at t = 0, k the rate constant (min^{-1}), and t the storage time (min).

H. Statistical Analysis.

Statistical analysis was performed on the repeated measurements by a one-way analysis of variance (ANOVA) followed by an LSD test. A probability of $p < 0.05$ was taken to indicate a significant difference between means. Values are expressed as means (SEM).

III. Results And Discussion

A. Morphological characterization of M. purpureus

The growth rates of Thai isolated ultrasonic mutant generations of *M. purpureus* TISTR 3002 grown in the PDA over 14 days at 30^0C were studied. The wild type (generation G0) and generations G1,G2,G3,G4 initially formed fluffy, yellow colored colonies with an orange spot in the middle of

a colony. The middle part of each colony turned to red as mycelia became mature from 10 days onward and then all of the mycelium in a colony turned reddish after 14 days of cultivation. The change in the growth appearance of this species over the cultivation is shown in Table I.

Under the microscope, the species of *Monascus* are normally characterised by nonostiolate ascomata arising singly at the tip of stalk-like hyphae scattered on the mycelium, and an ascomatal wall composed of two distinct layers, an inner layer which results from the swelling of the tips of the stalk-like hyphae forming a vesicle-like structure and an outer layer consisting of hyphal branches growing out from the base and fusing with the inner vesicle. Under the microscope, the *Monascus* species also show asci evanescent at an early stage; hyaline, 1-celled, ellipsoidal ascospores; and formation of a *Basipetospora* anamorph [17]. The morphological characters of colonies (including the size, color, shape, and aerial hypha) and microscopic characters (including hyphae, conidia, cleistothecia, and ascospore) were observed using the protocol described by Li and Guo, (2003) [16].

TABLE I. MORPHOLOGICAL APPEARANCE OF *M. PURPUREUS*

Time (days)	*Monascus purpureus* TISTR 3002			
	G1	G2	G3	G4
2				
4				
6				
8				
10				
12				
14				

Previous authors [18-20] have studied the dendograms of *Monascus* species. Based on dendrograms generated via sequence-related amplification polymorphism marker analysis, *M. purpureus*, *M. aurantiacus*, *M. serorubescens*, *M. anka*, and *M. ruber* were found to be clustered in the same clade and to produce red orange soluble pigments. However, *M. albidus*, *M. fuliginosus*, and *M. barkeri* were clustered with *M. pilosus* in a second clade and they produced non-red or orange pigments. Lastly, *M. lunisporas* and *M. argentinensis* occurred together in a third cluster distinct from the other *Monascus* species.

B. Effect of ultrasonic induction on the growth rate

The growth rates of each generation are shown in Table II. The 3002 wild type showed a faster growth rate of 0.5137 cm/day ($R^2 = 0.9860$) than those of mutants. Results showed that there was no significant difference among the growth rates of the 3002 generations G1-G4.

TABLE II. GROWTH RATES OF M. PURPUREUS ON PDA

Gen.	Equation	R^2	Growth rate (cm/day)
3002 G0	y = 0.5137x - 0.0093	0.9860	0.5137
3002 G1	y = 0.4468x + 0.2556	0.9962	0.4468
3002 G2	y = 0.4468x + 0.2556	0.9962	0.4468
3002 G3	y = 0.4155x + 0.4016	0.9950	0.4155
3002 G4	y = 0.4133x + 0.4651	0.9872	0.4133

C. Effect of ultrasonic induction on monacolin k and citrinin

Scanning spectra of each mutant generation showed that these fungi produced yellow, orange and red pigments at maximum wavelengths of 340, 440 and 480 nm in that order. A quantitative analysis by HPLC of the fermentation broths was carried out to determine citrinin and monacolin K contents. The results showed that monacolin K (a hypertensive agent) could not be detected in any of the 4 generations of fermented broths, whereas citrinin (an antibacterial compound) was found in all generations. The citrinin concentrations for G_0 (wild type), G_1, G_2, G_3 and G_4 were 18.48, 0.01, 64.98, 2.34 and 110.96 µg/ml, respectively.

D. Effect of pH on the stability of pigment

To analyze if there is a color shift of pigments with pH, the absorption spectra for the derivatives were observed at different pH values. The spectrum profile of the derivatives did not change with a changing pH, but the absorption intensity varied (Fig. 1). The highest absorption intensity was obtained at pH 8 for the yellow, orange and red colors. In the neutral and base ranges the absorption intensity changed more than in the acidic range. The absorption intensity also reached a lower minimum value in the acidic range than in the neutral and base ranges.

A quantitative analysis of pigments was performed on all generations of TISTR 3002 as follows. Pigment derivatives were dissolved in various pH buffers at a 1:1 ratio. The results for the stability of yellow, orange and red pigments over the acidic, neutral and basic ranges are shown in Fig. 2 a-e. The stability ratios are the ratios of pigment concentration after standing for 6 hours in the PH buffer to the initial pigment concentration. The derivatives of yellow, orange and red pigments were found to be most stable at pH 8. G4 yellow pigment retained stability ratio values of 1.47, 1.38, 1.44 and 1.47-fold, respectively when compared with those of wild type (G0). For orange pigment, values of 2.81, 2.63, 2.81 and 2.81-fold were observed. Stability ratio values of 3.82, 3.23, 3.52 and 3.52-fold were sustained for the red pigment (p-value < 0.001). The values of red stability in G4 were also significantly greater than the values seen for the

other generations. A significant increase of pigment stability was therefore obtained when *M.purpureus* TISTR 3002 was ultrasonically induced up to generation G4.

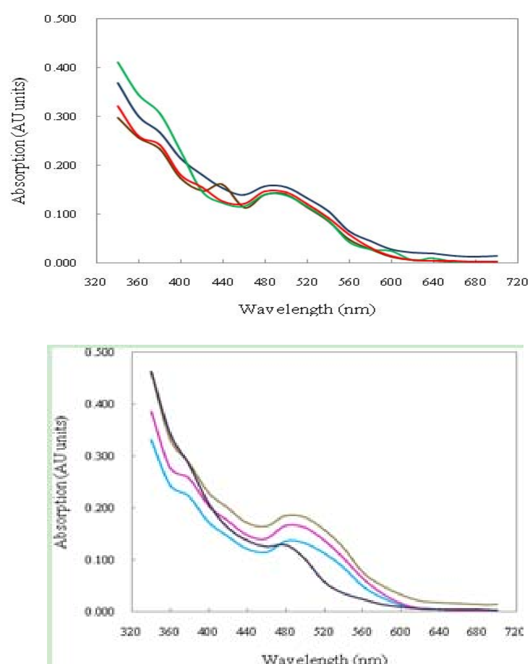
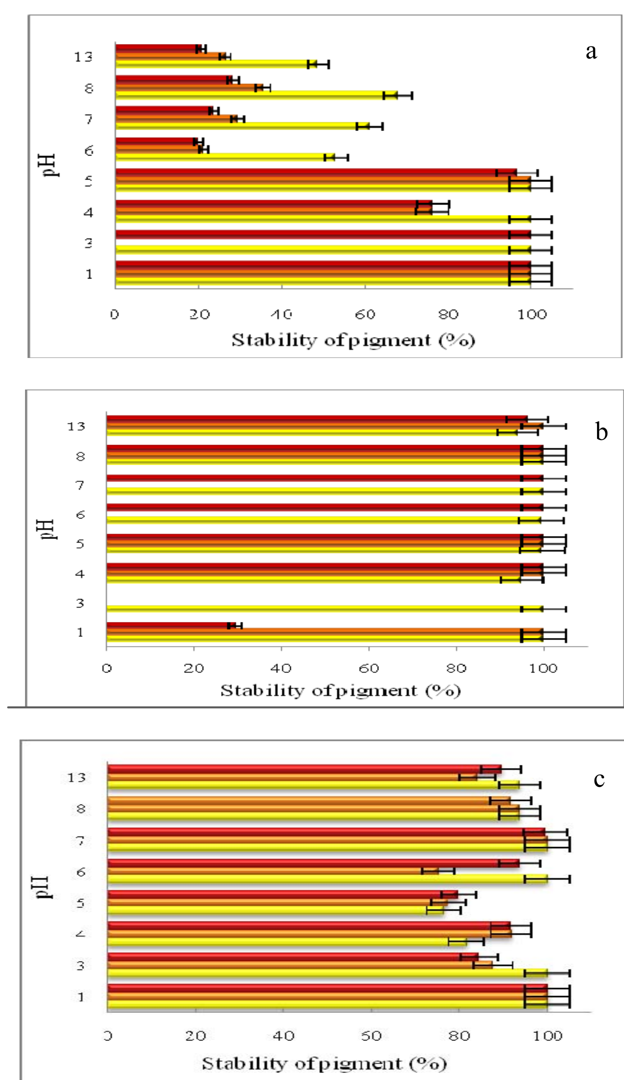


Fig. 1 Spectral changes of 3002 wild type (G0) pigment derivatives in various buffer solutions at (a) acidic and (b) neutral and base ranges.



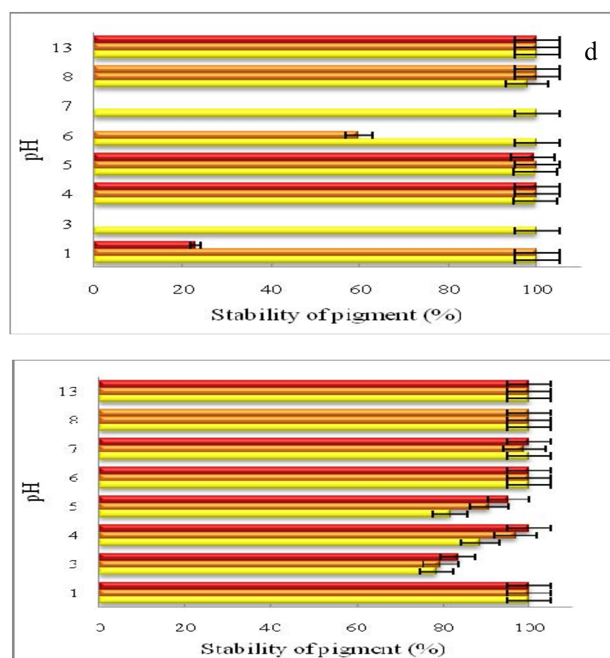


Fig. 2 pH Stability of yellow ( ), orange ( ) and red ( ) pigments for (a) G0 , (b) G1 , (c) G2 , (d) G3 and (e) G4

TABLE III. KINETIC RATES FOR DISCOLORATION OF VARIOUS PIGMENT DERIVATIVES UNDER VARIOUS PH (R^2> 0.99)

pH	Rate of degradation (min^{-1})					
	Red		Orange		Yellow	
	G0	G4	G0	G4	G0	G4
1	0	0	0	0	0	0
3	0	1.81E-05	-	2.29E-05	0	2.41E-05
4	2.66E-05	0	2.69E-05	3.18E-06	0	1.20E-05
5	3.34E-06	4.86E-06	0	9.56E-06	0	2.00E-05
6	1.59E-04	0	1.54E-04	0	6.29E-05	0
7	1.43E-04	0	1.21E-04	1.30E-06	4.88E-05	0
8	1.25E-04	0	1.02E-04	0	3.82E-05	2.20E-20
13	1.56E-04	2.20E-20	1.32E-04	0	7.15E-05	0

The pigment concentrations in the pH buffers were measured at intervals of 1 hour over a period of 6 hours. The rates of decay of the concentrations were modeled as a first-order reaction. A detailed comparison of the first-order decay rates between G0 and G4 was made and the rate constants for each pigment were estimated. The results are shown in Table III. We have compared our stability results with results reported in [23] for the stability of *Monascus* derivatives cultured in glutamic or amino acids. Our results agree with the glutamic acid results in showing that the pigments are more stable at pH 7.0 and 9.2 than at pH 3.0. Fabre [24] also reported that *Monascus* extracts show a red color at acidic pH values and form crystals at an extreme alkaline pH. These results are similar to our data. Our results also agree with results in [23-25] on the pH stability of *Monascus* and their derivatives, such as xanthomonasin A (yellow), monascorubrin (orange), and a threonine derivative of rubropunctatin (purple-red) These derivatives

were also found to be more stable in neutral and/or alkaline pH ranges than in an acidic range.

In conclusion, we have shown that fungal pigment derivatives of all generations of TISTR 3002 are more stable under basic conditions than under acid conditions. Ascomycetous fungi pigments may provide significant natural food colorants to the textile industry as they have excellent stability in all main color components in the basic pH ranges.

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