

# Solvent Stability of Ultrasonic Mutants of *Monascus purpureus* Pigments

hantraporn Wongsorn, Issara Wongjewboot, and Sasithorn Kongruang

**Abstract**—The stability to solvent is one of crucial parameters to apply *Monascus* pigment in food and textile industries. Ultrasonic induced mutants from *Monascus purpureus* TISTR 3002 were tested for the solvent stability in order to select the best pigment producer with the wide range of solvents. Reports of pigment stability percentages of all generations were shown. Estimation of *Monascus* pigments can be expressed as the total pigment yield (y) from the results of the significant parameters (solvents, yellow, orange and red pigments) on multiple linear regression. The color yield value equations can be represented as  $y = 0.132 (\pm 0.35) + 1.278 (\pm 0.35) *[\text{yellow}] + 1.235(\pm 0.35)*[\text{red}]$ . The maximum total pigment yield gained from these strains was the results of the contribution of yellow and red pigments. Optimization analysis indicated that G4 was the best pigment producer that could sustained the wide range of solvents with the highest residual solvent stability.

**Index Terms**—*Monascus purpureus*, colorant, stability, kinetic, yellow pigment, orange pigment, red pigment, mutant.

## I. INTRODUCTION

The increasing interest in the beneficial ingredients of *Monascus* derived products and the attempt of decreasing of the toxic components for natural colorants in food and textile industries make the most of progress of the research in the exploitation of fungal biotechnology. To improve the advantages over the productivity from *Monascus* sp., many approaches including traditional mutagenesis and metabolic engineering methods have been investigated for example  $\gamma$ -Irradiation, UV irradiation, diethyl sulfate treatment, LiCl treatment, electron beam irradiation and ultrasonic induced mutation [1-5]. Not only an attempt of random mutation to gain more colors but also using a genetically modified species and using the gene manipulation techniques to enhance other nutraceutical values (higher monacolin K productivity and lower citrinin content) has been performed [6-10]. Under the safety regulation concerning the health issue, the physical treatment by induced mutation via ultrasound is consider as simple, safe and convenient method to produce all the beneficial compounds from *Monascus* derived products.

The stability of natural pigments is also an important issue to apply in food and textile industries. There are some reports [11-13], that *monascus* pigments are sensitive to pH, UV, and temperature. Since the tendency of degradation to light, temperature, pH, solvent and the concentration of oxygen are concerned, economical users have to monitor and control these parameters properly in order to achieve the successful

application. We have previously shown that the stabilities of color derivatives of *Monascus* pigments under various pH are greatly enhanced once the ultrasonic induced up to G4, compared to an original red pigment, and various pH affecting the stability of pigments were kinetically analyzed as the first-order rates of decay [14]. In this study, the extracellular pigments from ultrasonic mutants were used as model compounds for testing for the solvent stability, the Gibbs energy and first order rates of degradation were evaluated. The possible models for total pigment yield under accounting for generations, yellow, orange and red pigments were reported.

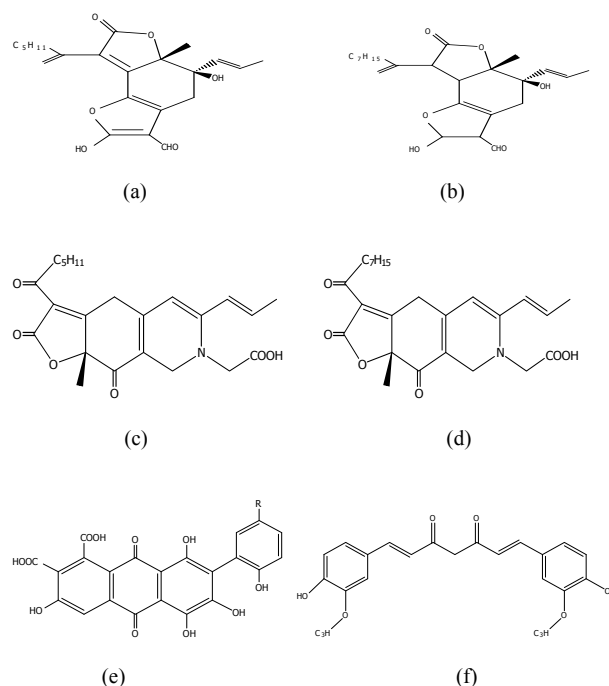


Fig. 1. Structures of six major pigments produced by *Monascus* spp.: Xanthomonasin A (a), Xanthomonasin B (b) Glycyl-rubropunctatin (c), Glycyl-monascorubrin (d), Laccacia acid A, B, C as the side chain of R<sub>A</sub>, R<sub>B</sub>, R<sub>C</sub> in that order (e), Curcumin (f)

## II. MATERIALS AND METHODS

### A. Chemicals and Culture Media

Solvents as hexane, ethanol, propanol, methanol, ethylether and double distilled water were commercially obtained from Labscan Co.,Ltd.,Thailand.

### B. Strains and Culturing Conditions

*Monascus 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 °C for 2 days and transferred to

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PDA and further incubated for 7 days. The fermentation was carried out in 500 ml Erlenmeyer flasks containing 300 ml of a modify yeast malt broth and 30 ml of inoculums. The batch fermentation was run in rotary shaking incubator at 200 rpm under 30 °C for 7 days. Details of cultivation method were experimented according to previously reported method [15]. Filtrated 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.

### C. Ultrasonic Induced Mutation Treatment

The culture medium of seed culture of *M. purpureus* wild types were filtrated and the seed suspensions in 7% saline solution were then treated by ultrasonic wave at 45 kHz for 2 min at 28 °C. After the ultrasound, the mycelia were washed with double distilled water before using as a seed for the radial growth determination and the cultivation for pigment production by follows the same procedure as previously mentioned. The mycelium grown in the PDA was used as the generation 1 (G1). This physical induced mutation of ultrasonic treatment was applied with the next generation until the G4 mutant was obtained.

### D. Effect of Solvent Pigment Stability

To investigate the effect of solvents on pigment degradation, samples were dissolved in six solvents with different polarity indices, including hexane (0.1), diethyl ether (2.8), propanol (3.9), methanol (5.1), ethanol (8.8), and distilled water (10.2). The solutions were mixed thoroughly with pigment solutions at 1:1 and sample solutions for yellow, orange and red pigments were measured using a spectrophotometer. The residual concentrations of all pigment solutions were measured after the solutions were exposed to solvents and incubated in the darkness at 30°C for 7 days.

### E. Multiple Linear Regression Analysis

All the experiments were done in triplicate. Experimental data were tested for the best fit by using a multiple linear regression. The second order polynomial coefficients were calculated and analyzed by using SPSS Version 7. A mathematical model, describing the relationships between the total pigment yield (Y)

$$y = \beta_0 + \sum \beta_i X_i + \sum \beta_{ij} X_i X_j + \sum \beta_{ii} X_i^2 \quad (1)$$

where Y is the predicted response variable as total pigment yield;  $\beta_0$ ,  $\beta_i$ ,  $\beta_{ij}$ ,  $\beta_{ii}$  are constant regression coefficients of the model, and  $X_i$  as  $X_1, X_2, X_3, X_4, X_5, X_6$  represent the independent variables (solvents) as distilled water, methanol, ethanol, propanol, hexane and diethyl ether. The accuracy and general ability of the above polynomial model could be evaluated by the coefficient of determination ( $R^2 > 0.80$ ). The standard errors associated with the model equations were also reported. The model equations were then replotted in 3D contour graphs using the Design Expert software (Version 7.1.5, Stat-Ease Inc., Minneapolis, USA) statistical package.

### F. Procedures for Kinetic Analysis

First-order degradation rate 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 ( $\text{min}^{-1}$ ), and t the storage time (min)). The solvents stability of pigments was tested after 7 days of incubation at temperatures of 30 °C with no light irradiation. The pigment amounts remaining after treatment under various conditions were measured using spectrophotometer. The Gibbs free energy of the stability in solvent system is calculated from the rate of degradation.

### G. Statistical Analysis.

The application of statistically based experimental designs to optimize fermentation media is an efficient approach to studying the effects of several factors and to improve product yields. Statistical analysis was performed according to the repeated measurements of one-way analysis of variance (ANOVA) followed by the LSD test. A possibility of  $p < 0.05$  was taken to indicate a significant difference between means. Values are expressed as means (SEM).

## III. RESULTS AND DISCUSSION

### A. Cultivation of *Monascus* Pigments

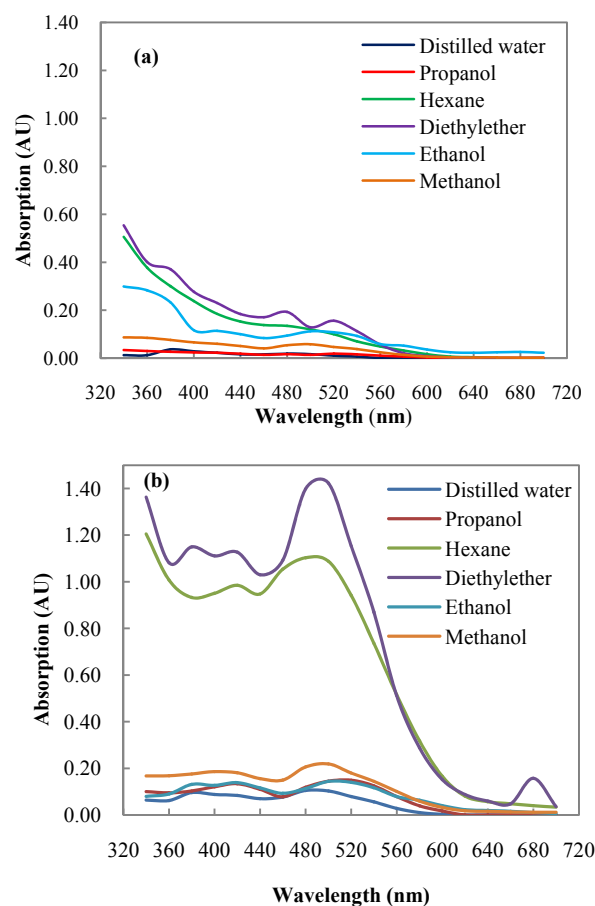


Fig. 2. Spectral changes of all pigment derivatives in various solvents; (a) wild type (G0) and (b) generation 4 (G4)

*Monascus* pigments from ultrasonic induced mutation of both wild type and four generations were cultured on a batch fermentation in modified yeast malt medium supplemented with cassava starch as the carbon source and monosodium glutamate. Fermentation broths were then extracted and analyzed for extracellular pigment concentrations. These pigments were tested for the stability in order to select the

appropriate generation, which its stability can sustain the wide range of solvents. Changes in the intensity profile of derivatives spectrum in each solvent were varied as illustrated in Fig. 2a. Diethyl ether and hexane solutions in generation 4 showed significantly enhanced in intensity once compared with those of generations (Fig. 2b). Moreover, approximately 5 times lower absorptions were observed in the rest of solvents.

### B. Effect of Solvent on the Stability of Pigments.

Stabilities over several solvents of all generation were measured using a different in concentrations between the initial day and the final residual concentration after incubation 6 days incubation in the darkness. All values were reported in the percentage of pigment stability as shown in fig.3 a-e. The solvent stability percentage indicated that the pigment decayed over time. Results indicated that the generation which was sustained in most solvents and retained stability above 80% was G4. The pigment stabilities of the red pigment after they were dissolved in methanol, propanol, hexane and diethyl ether retained 85.12%, 100%, 85.90% and 100%, respectively As depicted in fig. 3 (e), G4 red pigment retained stability ration values of 1.40, 1.41, 3.06 and 1.15-fold, respectively when compared with those of wild type (G0). For orange pigment, values of 1.44, 1.27 and 1.03-fold were detected in methanol, hexane and diethyl ether, respectively. On the other hand, the pigment derivatives sustained stabilities in ethanol lower than that of wild type by 0.63. Ratios of 1.23 and 1.62 -fold found in methanol and hexane for yellow pigment.

Our results showed that ethyl ether was most favorable to the derivatives stability whereas propanol and methanol were the worst. These properties suggest that the pigments from *M. purpureus* TISTR 3002 G4 has a great potential to use in the textile industry since it was gained stability over a wide range of solvents. However, the solvent stability is dependent of strains and amount of derivatives.

As the report from Jung et al., 2005 [15] who studied the enhanced photostability of *Monascus* pigment derived with various amino acids via fermentation using *Monascus* strain KCCM 10093 from Korea Culture Center for Microorganism. They found that hexane was most favorable to the derivatives whereas this study found that acetonitrile and chloroform were the worth. They also detected the higher stability in water relatively the same as ethanol. On the other hand, the control red as the original red pigments (monascorubramine and rubropunctamine), which were produced by this Korean species under no adding condition of amino acids was most stable in water [13, 15]. However, it is difficult to directly correlate our results with the results obtained by them because the amino acid derivatives of *Monascus* pigments much more stable compared to their parent red pigment under UV light than under sunlight while we ran our under the darkness condition.

### C. Effect of the Derivative Pigments and Generation to the Total Pigment Yield

Based on the obtained variables, the responses were, respectively, expressed by Eqs. (2) and (3) for the total pigment yields:

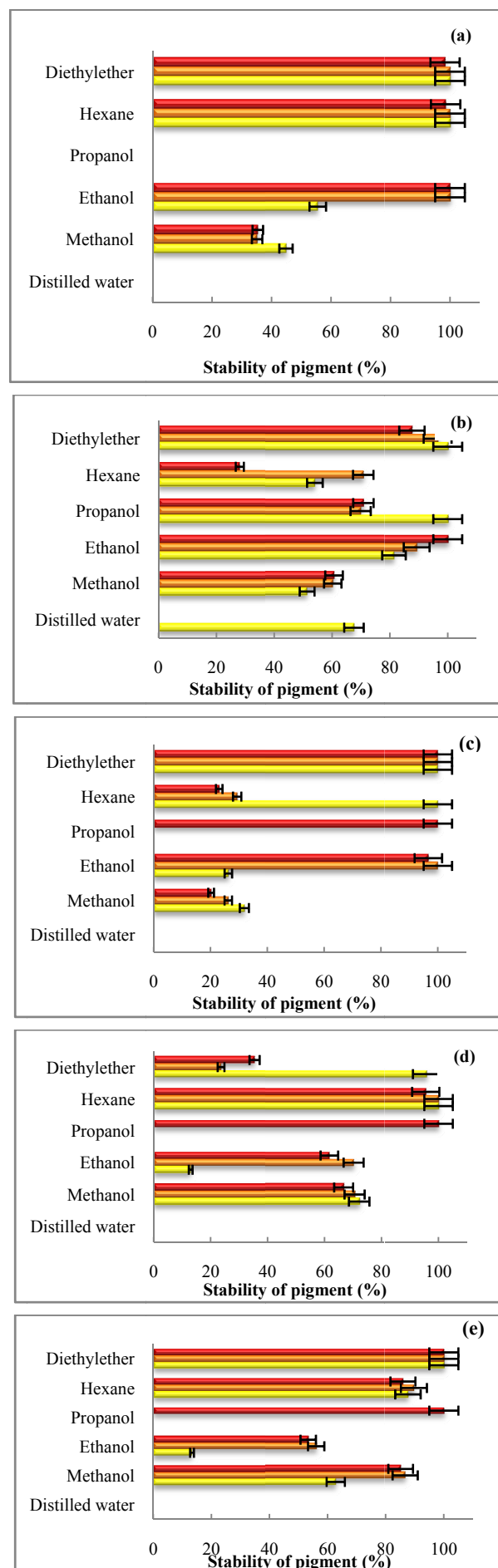


Fig. 3. Solvent stability of yellow (■), orange (■) and red (■) pigments for (a) G0, (b) G1, (c) G2, (d) G3 and (e) G4

$$\text{Model 1; } y = 0.101 (\pm 0.41) + 2.135 (\pm 0.41) *[\text{yellow}] \quad (2)$$

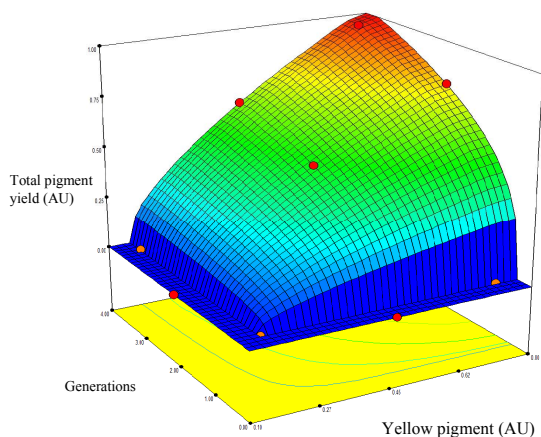
$$R^2 = 0.814$$

$$\text{Model 2; } y = 0.132 (\pm 0.35) + 1.278 (\pm 0.35) *[\text{yellow}] \quad (3)$$

$$+ 1.235 (\pm 0.35) *[\text{red}]$$

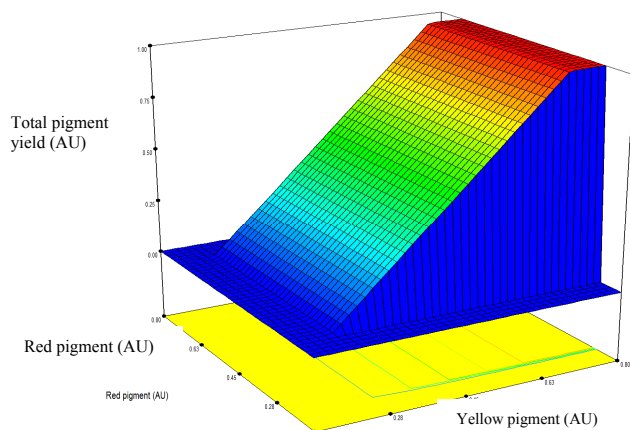
$$R^2 = 0.895$$

In order to find the generations which is appropriate to use as producer, the properties of that generation had to gain more pigment concentrations under various solvent exposure. Generations, yellow, orange and red pigments was accounted as important parameter at which potentially influenced the total pigment yield, Results found that total pigment yield of all generations can be represented as the equation model 1 as shown above. The contour plot of this relationship was illustrated in fig 4 a. In model 1 case, there was only contribution from yellow but no red and orange pigments to enhance the total pigment yield. From the regression analysis which was also performed to fit the response of total pigment yield from the experimental data, the better model can be views as a model 2 as well as the contour plot in fig. 4b. These overall results indicated that G4 was an excellent producers compared with wild type and the rest of generations. The total pigment yield gained from model 2 equation explains that total pigment yield was a result of yellow and red pigments contribution.



(a)

Fig. 4.a. Simulated response of total pigment yield generated using the best fit from multiple linear regression on experimental data.



(b)

Fig. 4.b. Simulated response of total pigment yield generated using the best fit from multiple linear regression on experimental data (continue).

TABLE I: THE RATE CONSTANTS AND HALF-LIFE VALUES ( $T_{1/2}$ ) AND GIBB'S ENERGY FOR PIGMENTS

Generation	Solvent	$k \times 10^{-2} (\text{min}^{-1})$			$t_{1/2} (\text{days})$			$\Delta G (\text{kJ} \cdot \text{K}^{-1} \cdot \text{mol}^{-1} \cdot \text{min})$		
		Yellow	Orange	Red	Yellow	Orange	Red	Yellow	Orange	Red
0	Distilled water	-	-	-	-	-	-	-	-	-
	Methanol	6.65	5.07	4.96	7.24	9.50	9.71	24.23	24.91	24.97
	Ethanol	2.03	1.12	0	23.68	42.81	0	27.22	28.71	0
	Propanol	-	3.85	3.42	-	12.51	14.07	-	25.61	25.90
	Hexane	6.11	3.42	12.61	7.87	14.07	3.82	24.44	25.90	22.62
	Diethylether	0	0.32	1.34	0	151.21	35.82	-	31.89	28.26
1	Distilled water	-	-	-	-	-	-	-	-	-
	Methanol	7.96	10.38	10.31	6.04	4.64	4.67	23.78	23.11	23.13
	Ethanol	5.84	0	0	8.25	0	0	24.56	0	0
	Propanol	-	-	-	-	-	-	-	-	-
	Hexane	0	0	0.15	0	*	328	0	114.02	33.83
	Diethylether	0	0	0.17	*	*	288.75	115.77	115.77	33.52
2	Distilled water	-	-	-	-	-	-	-	-	-
	Methanol	11.34	13.27	15.86	4.25	3.63	3.03	22.89	22.49	22.04
	Ethanol	13.26	0	0.34	3.63	0	142.31	22.49	0.00	31.73
	Propanol	-	-	-	-	-	*	-	-	0
	Hexane	0	12.16	14.55	0	3.96	3.31	0	22.71	22.26
	Diethylether	0	0	0	0	0	0	0	0	0
3	Distilled water	-	-	-	-	-	-	-	-	-
	Methanol	3.24	3.47	4.02	14.85	13.89	11.97	26.04	25.87	25.50
	Ethanol	20.26	3.51	4.80	2.38	13.70	10.03	21.42	25.84	25.05
	Propanol	-	-	0	-	-	0	-	-	0
	Hexane	0	0	0.46	0	0	105.51	0	0	30.98
	Diethylether	0.43	14.31	10.30	112.65	3.36	4.67	31.14	22.30	23.13
4	Distilled water	-	-	-	-	-	-	-	-	-
	Methanol	4.62	1.42	1.60	10.42	33.91	30.13	25.15	28.12	27.82
	Ethanol	20.01	5.75	6.24	2.41	8.38	7.71	21.46	24.60	24.39
	Propanol	-	-	0	-	*	*	-	-	114.02
	Hexane	1.31	1.08	1.51	36.75	44.58	31.93	28.32	28.81	27.97
	Diethylether	0	0	0	0	0	0	0	0	0

-Not detectable, \* Not determined

#### D. Kinetic Analysis of the Solvent Stability of *Monascus* Derivatives.

The degradation of pigments of all generations followed a first-order reaction. On the basis of this, the rate constants, half-life values ( $t_{1/2}$ ) and Gibb's energy for pigments were reported in Table I. All of the generations showed the associated rates of yellow, orange and red degradations differently and not dependent upon the solvents.

## IV. CONCLUSION

The stabilities to solvent and pH were elucidated from the ultrasonic induced mutation of *M. purpureus* TISTR 3002 both wild type and all mutant generations. However, the stability towards temperature, light and a combination of stability conditions are needed to be more investigations. This is because currently these pigments are limit to apply to certain types of products that fit the stability requirements of the colorant. Moreover, the fungal production of natural food colorants would need more substantial fundamental research to gain more productivity and stability in a wide range. More applications to food and textile system will then be overcome all drawbacks associated with the existing natural colorant production system.

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