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# Recovery of Red Pigments from *Monascus purpureus* FTC 5357 by Extraction of Fermented Solids: Operational Conditions and Kinetics

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## HIGHLIGHTS

- Red pigment of *Monascus purpureus* effectively extracted from dried fermented oil palm frond (OPF) using 60% ethanol.
- Maximum pigment recovery was obtained at 30 °C, 180 rpm agitation speed, 16 h, ratio of 1:60 solid to solvent and pH 6 of 60% ethanol.
- Peleg's model proved to be the best for describing solid-liquid extraction of the pigments under the above specified conditions.

**Abstract:** Solvent extraction of red pigments from fermented solids is reported. The pigments were produced by solid-state fermentation of oil palm frond (OPF) biomass with the food-safe fungus *Monascus purpureus* FTC 5357. The effects of extraction solvent and other operational conditions (pH, temperature, agitation rate, contact time) on the recovery of pigments are discussed. The recovery was maximized using aqueous ethanol (60% ethanol by vol) as solvent at pH 6, 30 °C, with an extraction time of 16 h and an agitation rate of 180 rpm. A fermented solids dry mass of 1 g was used for each 160 mL of solvent during extraction. The kinetics of extraction were assessed by fitting the experimental data to different models. Peleg's model proved to be the best for describing solid-liquid extraction of the pigments under the above specified conditions. The highest extraction yield of red pigments under the above specified optimal conditions was  $207 \pm 6.08$  AU g<sup>-1</sup> dry fermented solids.

**Keywords:** fungal pigments; food colorants; *Monascus purpureus*; solid-state fermentation; *Monascus* pigment.

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## INTRODUCTION

Natural colorants extracted from plants, animals and microorganisms are in demand as substitutes for synthetic pigments used as food colorants. Although most natural colorants are extracted from plants [1–5], microorganisms [6–11] offer an attractive alternative for several reasons. Unlike plants, microorganisms can be grown anywhere, anytime and under controlled culture conditions to provide a high and consistent productivity of the target compound. This work is concerned with solvent extraction of red pigments produced by a *Monascus* fungus grown on an agroindustrial solid substrate. Solid-state fermentation (SSF) has been used to produce many fungal products [12,13] including *Monascus* pigments [10,14,15-17]. Oil palm fronds are an agricultural residue of the palm oil industry [18], which can be effectively used for growing *Monascus purpureus* [19,20]. Although extraction of the red pigments from such fermented solids has not been demonstrated. Pigments and other metabolites produced by SSF are generally recovered by solvent extraction of the fermented solids [17,21]. Extraction is expensive and attaining a high recovery yield of the target compound requires an optimally designed recovery scheme. A suitable nontoxic extraction solvent must be selected to recover the pigments for use as food colorants. Extraction may be affected by diverse factors including pH, contact time, solvent-to-solids ratio and temperature.

A simple solvent extraction requires less sophisticated equipment compared to other methods such as Soxhlet extraction [22], ultrasound-assisted extraction [23] and microwave-assisted extraction [24]. Extraction solvent penetrates the solids being extracted and dissolves the target compound, preferably preferentially relative to the many other compounds that may be present. The dissolved solute is transported from the interior of the solid particles to their surface by pore diffusion and from there to the bulk liquid phase [25]. Mass transfer of the solute is influenced by temperature as the diffusion coefficient generally increases with temperature [3,25]. In addition, turbulence at the solid-liquid interface and in the bulk solvent influences mass transfer [3,25].

Extraction of diverse natural products has been studied, but mainly from plant materials [1, 3, 4, 26-28], not fermented solids. Thus, this work reports on solvent extraction of *Monascus* red pigments from fermented solids. Optimal solvent and other extraction conditions (pH, temperature, solid-to-solvent ratio, solid-solvent contact time, mixing regimen) are established and extraction kinetics are quantified. Kinetics study quantified in the study helps in understanding conditions that affect the extraction rate, especially when designing extraction unit for industrial scale.

## MATERIAL AND METHODS

### Culture and Solid-State Fermentation

*Monascus purpureus* FTC 5357 was purchased from the culture collection of Malaysian Agricultural Research and Development Institute (MARDI), Malaysia. The fungus was maintained on potato dextrose agar (PDA) plates at 4 °C and subcultured every month. Oil palm fronds (OPF) were collected from Felda Lepar Hilir oil palm plantation, Gambang, Pahang, Malaysia. The fresh fronds were cut into 5 cm long pieces and dried in an oven at 60 °C for 1 day, ground and sieved (Retsch AS 200 Basic, Germany) to obtain 1 mm particles [29]. These OPF solids were stored in plastic bags at room temperature until needed. When needed, the dry OPF powder was mixed with distilled water such that the resulting slurry contained 1 g of OPF powder for each 18 mL of water, autoclaved at 121 °C for 15 min, and cooled to room temperature [29]. The heat treated OPF solids were recovered by filtration, washed with the same volume of distilled water as the original slurry sample, recovered again and oven dried at 60 °C for 24 h [29]. The pretreated OPF solids were mixed with peptone (4 g peptone per 100 g dry pretreated solids), sufficient distilled water was added to obtain a moisture content of approximately 75% (wt/wt) and the pH was adjusted to 8 using HCl (0.5 M) or NaOH (0.5 M). This moist solid medium was autoclaved at 121 °C for 20 min, cooled to room temperature, inoculated with a standardized spore suspension (20 mL suspension per 100 g dry OPF solids) of *M. purpureus*, and mixed well aseptically. The inoculated solids were incubated in an Erlenmeyer flask at 30 °C for 8 days.

The spores for inoculation had been grown on PDA slants at 30 °C for 8 days. Sterile distilled water was added to the slant and the spores were gently scraped off from the agar. The spores were counted using a Neubauer haemocytometer and the suspension was standardized by adding distilled water to a concentration of  $1.0 \times 10^7$  spores mL<sup>-1</sup>.

## Extraction Process

Fermented OPF solids were dried in an oven at 60 °C for 24 h. In different experiments, the same original batch of fermented dried solids was extracted with different solvents and under different conditions. Three solvents were evaluated: distilled water as control, 60% ethanol (60:40 by volume ethanol and water) and 95% ethanol (95:5 by volume ethanol and water). All extraction experiments used 1 g of dry fermented solids per 160 mL of fresh solvent [29]. Dried fermented solids (0.5 g) were placed in a 250 mL Erlenmeyer flask and mixed with the solvent and agitated at 180 rpm in an incubator-shaker at 30 °C, for 1 h. This procedure was used to identify the most effective extraction solvent.

Using the best identified extraction solvent, further separate experiments were carried out at different extraction temperatures (i.e., 30, 40, 50, and 60 °C). The other conditions remained fixed (agitation speed = 180 rpm, extraction time = 2 h). Once the most effective extraction temperature had been identified, all future experiments used this temperature. Further separate experiments were carried out using different pH values (i.e., pH 2, 4, 6, and 8) for the extraction solvent while keeping the agitation speed and extraction time, as specified above. The solvent pH was adjusted as specified earlier for the fermentation step.

Using the optimal extraction solvent, temperature and pH identified in earlier experiments, further separate experiments were conducted at different agitation speeds during extraction (i.e., 100, 140, 180, and 220 rpm) while the extraction time remained fixed at 2 h. Once a suitable extraction speed had been identified, further separate experiments were carried out to assess the effect of extraction time (i.e., 4, 8, 12, 16, 20, and 24 h). The above sequential scheme of experiments identified an optimal extraction solvent and the other conditions (temperature, pH, agitation speed, contact time). All extractions were carried out in triplicate experiments.

## Pigments Assay

On completion of the extraction period, the suspension was set aside for 15 min under static conditions at room temperature and then filtered using a Whatman No. 1 filter paper (GE Healthcare, Pittsburgh, PA, USA). A UV/VIS spectrophotometer (Thermo Scientific Genesys™ 10S UV-Vis Spectrophotometer, USA) was used to measure the absorbance of the extract at a wavelength of 500 nm [14]. The blank was an extract of uninoculated sterilized OPF solids prepared using the same solvent as the extract of fermented solids [20]. The measured absorbance of the sample was corrected for any dilution required prior to measurement [20]. The corrected absorbance (AU, absorbance units) was used to express the concentration of the pigments as AU g<sup>-1</sup> of the fermented dry solids extracted.

## Kinetics of Extraction

Three widely used models of extraction kinetics [28,30] were evaluated for extraction of the red pigments from the fermented solids. The models were the first-order kinetic model [31], Peleg's model [4,26,28,30,32,33] and logarithmic model [4,26,28,30]. The equations of the model were as follows:

$$\frac{dC_t}{dt} = k_1(C_s - C_t) \quad (1)$$

for the first-order model;

$$C_t = C_0 + \frac{t}{k_2 + k_3 t} \quad (2)$$

for Peleg's model; and

$$C_t = a \log t + b \quad (3)$$

for logarithmic model.

In the above equations,  $C_t$  (AU g<sup>-1</sup>) was the concentration of pigments extracted at time  $t$  (min),  $k_1$  (min<sup>-1</sup>) was the first-order extraction rate constant,  $C_s$  (AU g<sup>-1</sup>) was the maximum concentration of the pigments that could be extracted,  $C_0$  (AU g<sup>-1</sup>) was the initial pigments concentration (i.e. pigments concentration at  $t = 0$  min),  $k_2$  (g min AU<sup>-1</sup>) was Peleg's rate constant,  $k_3$  (g AU<sup>-1</sup>) was Peleg's capacity constant [32], and  $a$  (AU g<sup>-1</sup> min<sup>-1</sup>) and  $b$  (AU g<sup>-1</sup>) were constants in Equation (3).

## RESULTS AND DISCUSSION

### Effect of extraction solvent

Choice of extraction solvent is important. A solvent must be able to effectively solubilize the targeted metabolite. In addition, it should be cheap for large scale use and easily separable from the solute after extraction. A solvent used in extraction of food products must be safe and nontoxic. Therefore, water and aqueous ethanol solutions were assessed (Table 1) as preferred solvents. In separate experiments, the polarity of extraction solvent was varied by using different proportions of ethanol in water (Table 1). All solvents proved capable of extracting the red pigments, but 60% ethanol was by far the most effective solvent and water was the least effective (Table 1). For otherwise identical conditions, extraction with 60% ethanol, the optimal solvent, recovered nearly 17-fold more pigments compared to extraction with water. Also, 60% ethanol was nearly 5-fold more effective in extraction compared to 95% ethanol (Table 1). In earlier work on extraction of pigments from *Monascus*-fermented solid substrates, 60% ethanol was reported to be the most effective solvent [15], but the substrates used (various grains, cassava starch, potato) were all noncellulosic and morphologically quite different compared to the pretreated oil palm fronds used in the present work.

**Table 1.** Maximum concentration of red pigments extracted using different solvents<sup>a</sup>

Solvent <sup>b</sup>	Concentration (AU g <sup>-1</sup> )
Distilled water	3.23
60% ethanol	53.73
95% ethanol	10.32

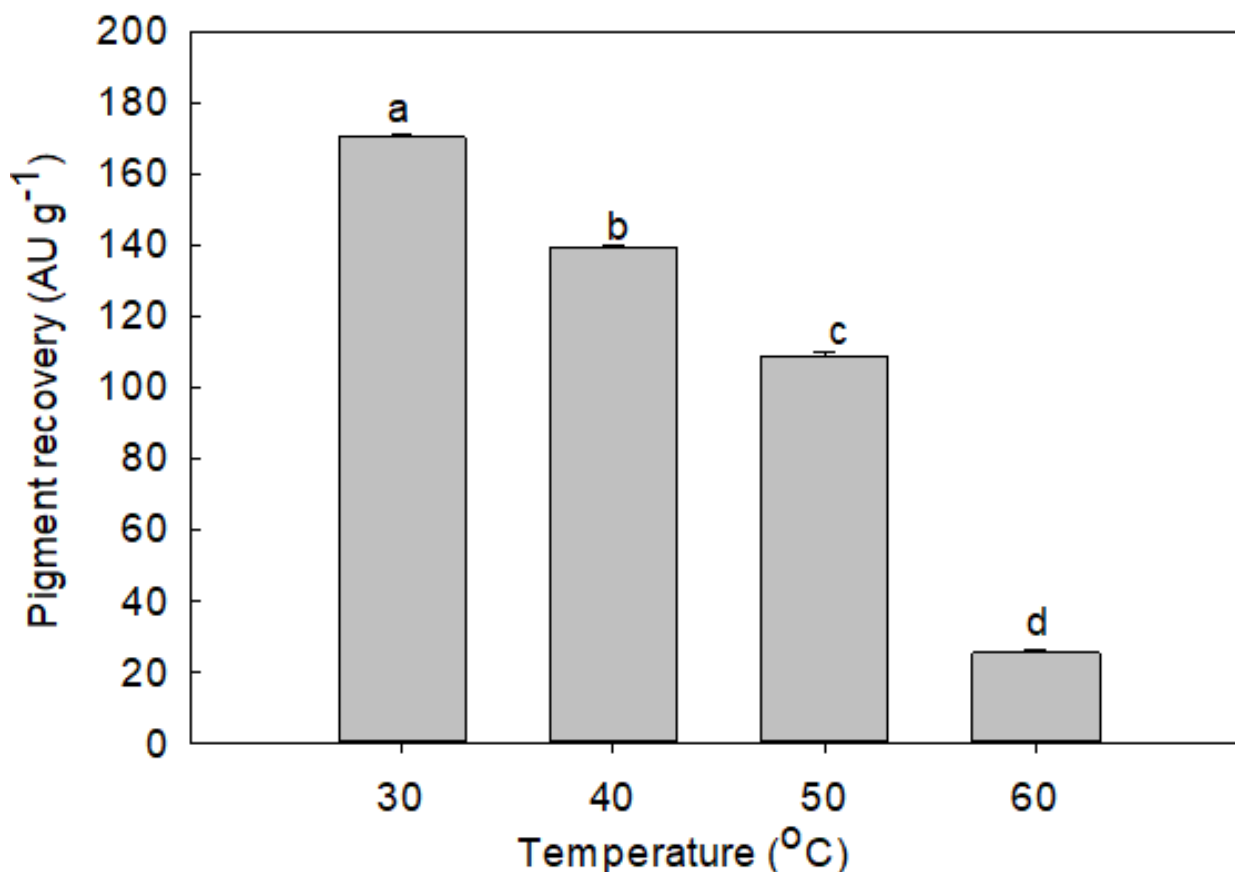
<sup>a</sup> Extraction conditions were: 1 g fermented solids per 160 mL solvent; agitation speed = 180 rpm; temperature = 30 °C; extraction time = 1 h

<sup>b</sup> Solvents are tabulated in decreasing order of polarity.

Dielectric constant of a solvent is an indicator of its polarity. A higher dielectric constant means a higher polarity. At 20 °C, the dielectric constant of pure water and ethanol are 80.1 and 25.2, respectively. For both pure solvents, dielectric constants decrease with increasing temperature and the same is expected for a mixture of water and ethanol. Increasing concentration of ethanol in water reduces the polarity of mixture. At a suitable polarity of solvent, the solubility of the red pigments is maximized, that is the pigments are most compatible with the solvent in terms of polarity. In addition, the pore diffusion of a solvent in a solid matrix such as the fermented solids can be affected by solvent polarity. A solvent less compatible with the solid matrix may have a reduced potential to penetrate the solids and recover the pigments [34]. Similar finding was found by Srinta and coauthors [35], where high *Monascus* pigment was extracted at 7:3 ethanol to water ratio. In view of Table 1, 60% ethanol was the most effective solvent and it was used in all subsequent work.

### Effect of temperature on extraction

For otherwise fixed conditions, temperature affected recovery of the pigments as shown in Figure 1. Extraction was most effective at 30 °C compared to higher temperatures, which was supported by earlier work [35]. Generally, a higher temperature enhances the solubility of a solute and, therefore, should increase recovery. Furthermore, an increasing temperature reduces solvent viscosity and increases solute diffusivity and both these effects are expected to improve diffusive mass transfer of the pigments within the solid pores and in the bulk solvent [25] to increase the observed rate of extraction. The results in Figure 1 were therefore contrary to expectations.

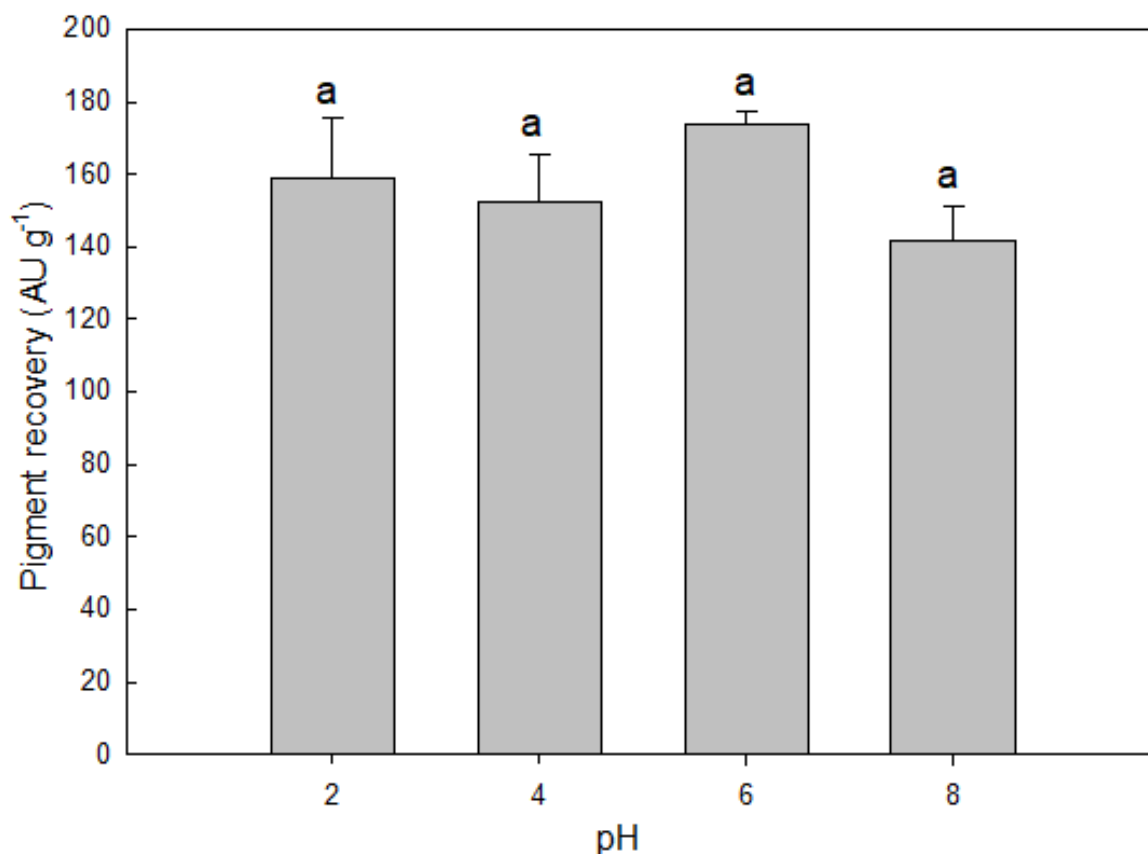


**Figure 1.** Amount of pigments extracted from dry fermented solids at different temperatures. The extraction solvent was 60% ethanol. The other extraction conditions were: 1 g dry solids in 160 mL solvent; agitation speed = 180 rpm; extraction time = 2 h. Columns with different superscript letter are significantly different ( $P < 0.05$ ) via Duncan's Multiple Range Test.

*Monascus* pigments are known to be susceptible to thermal degradation at temperatures  $>60$  °C although slow degradation does occur at lower temperatures (30 to 50 °C) in a neutral (pH 6 to 8) medium [36]. For the measurements in Figure 1, the pH was not adjusted but was around 7 to 8, the typical range for *Monascus*-fermented solids [11], and therefore the observed reduced recovery at 40–50 °C must have other contributing factors. For example, the solvent may impact thermal stability. For the data in Figure 1, the solvent was 60% ethanol whereas in the earlier work [36] it was a buffered aqueous solution. In the present work, pigments recovery was highest at 30 °C and therefore this extraction temperature was used in all subsequent work.

### Effect of pH on extraction

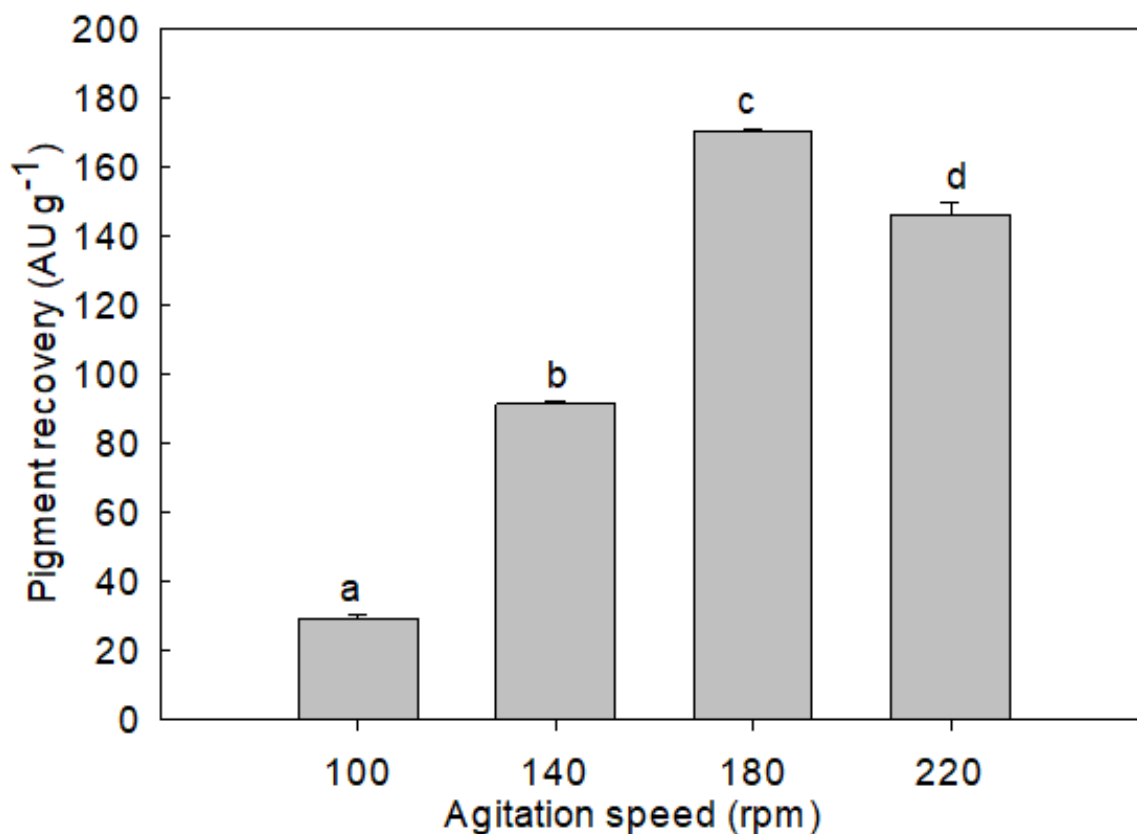
Extraction is known to be strongly affected by pH especially if the solute being extracted changes ionization status with pH. In view of their chemical structure [14], the red pigments of *Monascus* are unlikely to be affected by changes in pH. Nonetheless, extractions were carried out at different pH values in the range of pH 2 to 8. The results are shown in Figure 2. A maximum amount of pigments ( $172 \text{ AU g}^{-1}$ ) was extracted at pH 6, although at pH 4 extraction was reduced by only 17% and at pH 8 it was reduced by 22% relative to the maximum. Therefore, extraction was not highly sensitive to pH, suggesting that the chemical structure of the red pigments was mostly unaffected by changes in pH. As pH 6 was most effective for extraction, all subsequent experiments used this extraction pH.



**Figure 2.** Effect of pH on extraction of red pigments. Amounts of red pigments extracted from dry fermented solids at various pH values are shown. The extraction conditions were: Extraction solvent = 60% ethanol; 1 g dry solids in 160 mL solvent; agitation speed = 180 rpm; temperature = 30 °C; extraction time = 2 h. Columns with similar superscript letter are not significantly different ( $P < 0.05$ ) via Duncan's Multiple Range Test.

### Effect of agitation rate on extraction

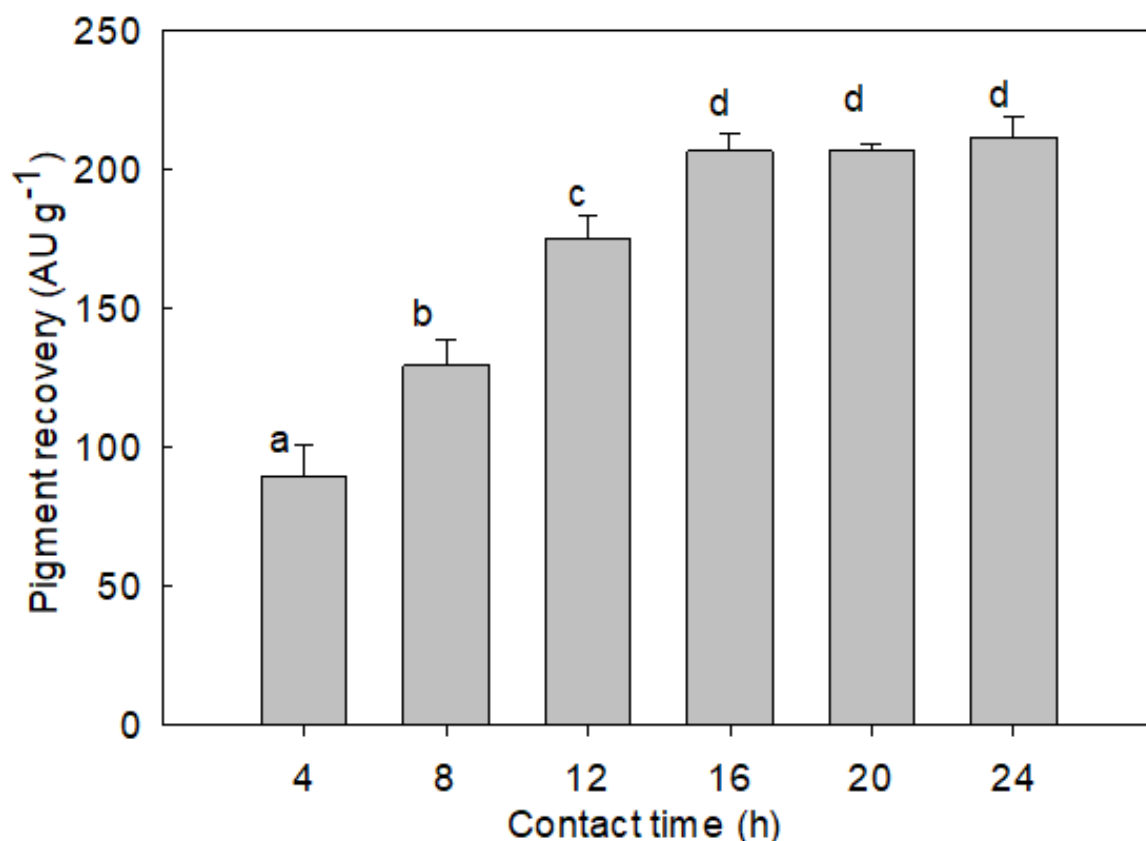
Effect of different agitation speeds on pigments recovery is shown Figure 3. The pigments recovery increased as the agitation speed was increased to 180 rpm, but the recovery decreased distinctly as the agitation speed increased further to 220 rpm. The maximum pigments recovery at 180 rpm was 170 AU g<sup>-1</sup> dry fermented solids. As agitation intensity increases, more of the solid material is brought into suspension and this improves solid-liquid contacting to enhance extraction. In addition, mass transport of a solute through pore diffusion within the substrate and the exterior of solid particles is improved with increased agitation and mixing in the slurry [25,27] and this contributes to enhanced extraction. Increasing turbulence at the external surface of a particle causes pressure fluctuations that induce mixing also within the pores connected to the surface to enhance intraparticle mass transport of solute [25]. Once the solid particles are fully suspended, further increase in agitation speed has little effect on solid-liquid mass transfer which is governed by relative motion between the phases [25] as determined by differences in densities of the phases [25]. This explains an absence of further increase in pigments recovery once the agitation speed increased beyond 180 rpm (Figure 3). Unbaffled Erlenmeyer flasks placed on an orbital shaker platform were used in extraction. In such devices, the slurry simply swirls instead of mixing if the agitation speed is too high. This effect likely explained the reduced pigments recovery once the agitation speed was further raised to 220 rpm (Figure 3). As pigments recovery was highest at 180 rpm, all future experiments used this agitation speed.



**Figure 3.** Effect of agitation rate on pigments extraction. The extraction conditions were: Extraction solvent = 60% ethanol; 1 g dry solids in 160 mL solvent; pH = 6; temperature = 30 °C; extraction time = 2 h. Columns with different superscript letter are significantly different ( $P < 0.05$ ) via Duncan's Multiple Range Test.

#### Effect of solid-liquid contact time on extraction.

The contact time between the extraction solvent and the solids being extracted affects recovery of the solute. Solute recovery progressively increases with time until an equilibrium is reached. At equilibrium further extraction ceases either because the solvent has become saturated with the solute, or the maximum possible amount of solute has been extracted. The previously identified optimal extraction conditions (60% ethanol used as extraction solvent; 1 g dry solids in 160 mL solvent; 180 rpm; pH 6; 30 °C) were used to establish the time needed to maximize pigments recovery. Samples collected at 4 h intervals for 24 h were used to measure the pigments recovery. The results are shown in Figure 4. Pigments recovery progressively increased with contract time until equilibrium was reached at 16 h (Figure 4). The maximum pigments recovery at 16 h was  $207 \pm 6.08$  AU g<sup>-1</sup> dry fermented solids. Thus, under the specified operational conditions, extraction was complete at 16 h. At 16 h, nearly 2.3-fold more pigments was extracted compared to the situation at 4 h (Figure 4). An extraction period of up to 16 h at the above specified operational conditions was used to assess the extraction kinetics discussed in the following section.



**Figure 4.** Effect of contact time on extraction. The extraction conditions were: Extraction solvent = 60% ethanol; 1 g dry solids in 160 mL solvent; agitation speed = 180 rpm; pH = 6; temperature = 30 °C. Columns sharing the same superscript letter are not significantly different ( $P < 0.05$ ) via Duncan's Multiple Range Test.

### Comparison of kinetic models of pigments extraction.

The pigments concentration versus contact time data were plotted as shown in Figure 5. Straight line plots were expected for the kinetic models that most closely matched the experimental data. Regression lines were calculated for the data plotted according to the requirements of the first-order model, Peleg's model and the logarithmic model (Figure 5). The coefficient of determination ( $R^2$ ) of the straight lines (Figure 5) and root mean square error (RMSE) for model-predicted pigments concentration compared to the measured data, are shown in Table 2. From the plots (Figure 5) and the data in Table 2, extraction clearly did not follow first-order kinetics. Both Peleg's model and logarithmic model fitted well the experimental data (Figure 5 B, C), but the RMSE value for logarithmic model was more than 40-fold higher compared to RMSE for Peleg's model (Table 2). For product recovery from some plant materials, both Peleg's and logarithmic models have been reported to equally well describe the extraction kinetics [4,28,30]. In extraction of pigments from fermented solids, Peleg's model best fitted the experimental observations.

**Table 2.** Kinetic constants, coefficient of determination ( $R^2$ ) and root mean square error (RMSE) for the extraction models<sup>a</sup>

Model	Model constant	$R^2$	RMSE <sup>b</sup>
First-order kinetics	$k_1 = 0.0153 \text{ min}^{-1}$	0.8183	2.447
Peleg's model	$k_2 = 1.8793 \text{ g min AU}^{-1}$ , $k_3 = 3.3 \times 10^{-3} \text{ g AU}^{-1}$	0.9758	0.213
Logarithmic model	$a = 170.87 \text{ AU g}^{-1} \text{ min}^{-1}$ , $b = -318.24 \text{ AU g}^{-1}$	0.9628	8.821

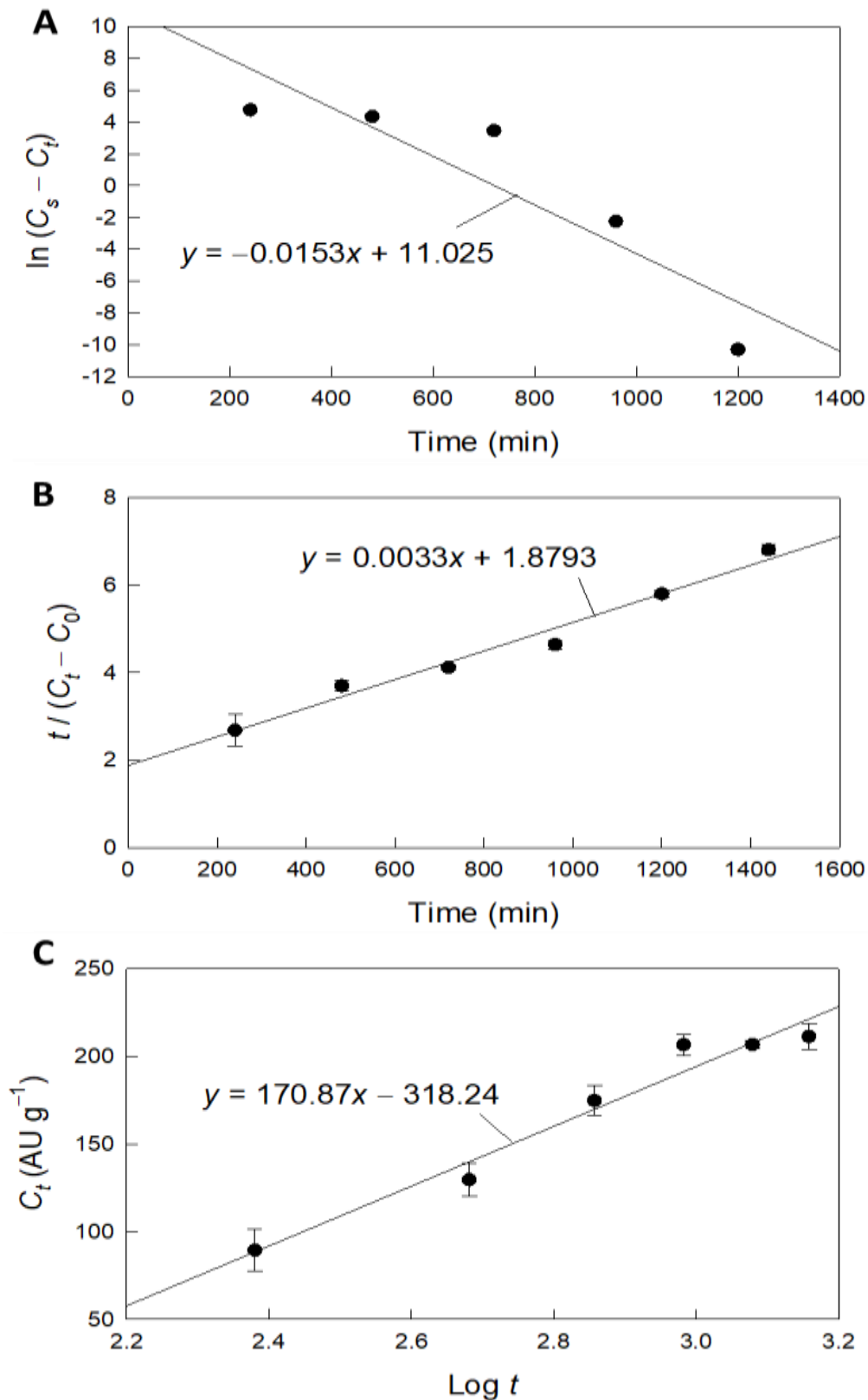
<sup>a</sup> The extraction conditions were: 1 g fermented solids per 160 mL solvent (60% v/v ethanol); agitation speed = 180 rpm; temperature = 30 °C; pH = 6

<sup>b</sup> Calculated using the following equation:

$$\text{RMSE} = \sqrt{\frac{1}{n} \sum_{i=1}^n (C_{t,\text{measured}} - C_{t,\text{calculated}})^2}$$

where  $n$  is the number of measurements,  $C_{t,\text{measured}}$  is the measured concentration of the pigments at time  $t$ , and  $C_{t,\text{calculated}}$  is the pigments concentration calculated using the model at time  $t$ .





**Figure 5.** Pigments concentration versus time data plotted using linearized forms of the equations for: (A) first-order kinetics; (B) Peleg's model; and (C) logarithmic model. All data were obtained for extraction with 60% ethanol at the following conditions: 1g dry fermented solids for 160 mL solvent, temperature = 30 °C, pH = 6, and agitation speed = 180 rpm.  $C_t$  ( $\text{AU g}^{-1}$ ) is the concentration of pigments extracted at time  $t$  (min),  $C_s$  ( $\text{AU g}^{-1}$ ) is the maximum concentration of the pigments that could be extracted and  $C_0$  ( $\text{AU g}^{-1}$ ) is the initial pigments concentration (i.e. pigments concentration at  $t = 0$  min).

## CONCLUSION

The solubility of the red pigment of *Monascus purpureus* is maximized at a suitable solvent polarity, which is 60% ethanol. The yield recovery was maximum at pH 6, temperature of 30 °C, agitation speed of 180 rpm, and contact time of 16 h. Kinetics models of red pigment extraction constructed were in conformity to Peleg's model and logarithmic model. However, Peleg's model best fitted the experimental observations with lower RMSE. Under the above specified optimal extraction conditions, Peleg's rate constant  $k_2$  was 1.8793 g min AU<sup>-1</sup> and the capacity constant  $k_3$  was 3.3×10<sup>-3</sup> g AU<sup>-1</sup>.

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