



# Optimization of Pigment Extraction from Quinoa Flour Fermented by *Monascus purpureus* Supplemented with Fish Hydrolysate and Sodium Chloride <sup>†</sup>

Franz Tucta-Huillca <sup>1,2,3</sup> , Evelyn Quispe-Rivera <sup>1,2,3</sup> , Marcial Silva-Jaimes <sup>3</sup> , Vasco Cadavez <sup>1,2</sup> and Ursula Gonzales-Barron <sup>1,2,\*</sup>

<sup>1</sup> Centro de Investigação de Montanha (CIMO), Instituto Politécnico de Bragança, Campus de Santa Apolónia, 5300-253 Bragança, Portugal

<sup>2</sup> Laboratório para a Sustentabilidade e Tecnologia em Regiões de Montanha, Instituto Politécnico de Bragança, Campus de Santa Apolónia, 5300-253 Bragança, Portugal

<sup>3</sup> Laboratorio de Microbiología de Alimentos, Universidad Nacional Agraria La Molina (UNALM), Av. La Molina s/n La Molina, Lima 15024, Peru

\* Correspondence: [ubarron@ipb.pt](mailto:ubarron@ipb.pt); Tel.: +35-12-7330-3325

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**Abstract:** *Monascus purpureus* has been tested with different food matrices in solid state fermentation and has shown different behaviors in the production of its pigments. Therefore, in a new matrix it proved important to optimize the extraction of these pigments and obtain optimal conditions (ethanol graduation of 50.6°, extraction temperature of 54.7 °C and ethanol: sample ratio of 38.7); the extraction yield (%) was  $34.72 \pm 0.18$ . In addition, the best equation to predict the concentration of the hydroethanol extract was linear and was obtained by summing the absorbances, measured at 400, 470 and 500 nm at a dilution of 1:6 ( $R^2 = 0.974$ ).

**Keywords:** fish hydrolysate; fermentation; pigment; yield; concentration; response surface design



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## 1. Introduction

Pigments or colorants are the most attractive additives that consumers can perceive when buying their food; even if the color is changed the consumer could perceive another flavor. In 2017, natural pigments comprised 31% of the market, 40% were synthetic and 29% semi-synthetic. Due to their potentially undesirable mutagenicity and carcinogenicity the number of synthetic dyes allowed will continue to decrease over time, which is why currently new sources of production such as plants, animals and microorganisms are sought; among them presenting greater advantages are the pigments that come from microorganisms, since optimal conditions can be given for their production [1,2].

*Monascus purpureus* is a fungus used thousands of years ago as a food additive in Asian cultures [3]. This fungus, in the process of solid state fermentation, produces several secondary metabolites of interest such as pigments, which are a mixture of different metabolites classified as polyketides, where the best known are: Monascine and Ankaflavine (yellow pigments); Rubropunctatin and Monascorubrin (orange pigments); and Rubropunctamine and Monascorubramine (red pigments) [4].

Fish hydrolysate is considered a rich source of amino acids, since the hydrolysis process decreases the size of the peptides making these amino acids more available [5]. In addition, this substrate is a source of nitrogen, important for the growth and metabolism of *Monascus* and essential for amino acid biosynthesis through the ammonia pathway and other pathways that are linked to pigment production [6].

On the other hand, it has been shown that environmental factors affect the response of the fungus to produce a greater or lesser amount of pigment, which is why including sodium chloride in the fermentation would generate salt stress, and the fungus would produce a greater amount of pigment, although this concentration of salts must be low otherwise it could be detrimental to the microorganism [7].

Therefore, the objectives of this study were to optimize the hydroethanol extraction conditions for the pigments of the fermented product supplemented with fish hydrolysate and sodium chloride, in order to maximize the yield, using a response surface design, and to construct a linear equation by spectrophotometry to predict the concentration of the hydroethanol extracts.

## 2. Materials and Methods

### 2.1. Microbial Strain and Culture Conditions

The filamentous fungus *Monascus purpureus* CECT 2955, from the Spanish Type Culture Collection (CECT), was used in this study. The strain was resuspended and seeded in PDA (Potato Dextrose Agar) in a Petri dish at 30 °C for 7 days, then seeded in QFH (Quinoa Flour Agar) adjusted to pH 6, and incubated at 30 °C for 7 days. The spore suspension was made by adding 10 mL of 0.01% Tween-80; hyphae and spores were removed, then filtered and vortexed for 5 min, and the spores were counted in a Neubauer chamber and adjusted to  $1.0 \times 10^6$  spores/mL.

### 2.2. Solid-State Fermentation of Quinoa

Solid-state fermentation was carried out in 250 cc flasks containing quinoa grains (30 g), NaCl 0.05% (*w/w*), fish hydrolysate 1% (*w/w*) and distilled water (25 mL) with the pH adjusted to 6, and then sterilized at 121 °C for 15 min. Subsequently, 1 mL of inoculum ( $1.0 \times 10^6$  spores/mL) was added to the flasks previously cooled to room temperature, and each flask was capped with sterile absorbent cotton and then kept in an incubator (ILW, Pol Eko, Poland) at 30 °C for up to 8 days. After the time had elapsed, the substrate was dried at 65 °C for ~24 h and milled in a mill (CS-1000, Shang-Jun, Beijing, China) to obtain the pigmented quinoa flour.

### 2.3. Pigment Extraction

In centrifuge tubes (round base) 1 g of fermented quinoa flour supplemented with fish hydrolysate and sodium chloride was mixed with ethanol at 40, 50 and 60% (*v/v*), maintaining an ethanol: sample ratio of 30:1, 40:1 and 50:1 mL/g, respectively, and then agitated at 400 rpm for 180 min at 50, 55 and 60 °C, respectively. After the time had elapsed, it was centrifuged at 10,000 rpm for 20 min, and the supernatant was recovered for later analysis.

### 2.4. Spectrophotometric Analyses of Ethanol Extracts

The ethanol extracts of the pigments were measured with a UV-Vis spectrophotometer (C-7100, Peak Instruments Inc., Houston, TX, USA) at 400 (yellow), 470 (orange) and 500 (red) nm. A 1 mL of sample was taken and diluted with ethanol, where the ratio was 1:6 (*v:v*); as a blank for quantification, ethanol was used.

### 2.5. Yield

The ethanol extract was taken to an oven (J.P. SELECTA, Barcelona, Spain) and dried at 65 °C to a constant weight for approximately 48 h, then the extraction yield was calculated from Equation (1), being 1 g dry weight of sample:

$$\text{Yield (\%, w/w)} = (\text{MS1/MS2}) \times 100 \quad (1)$$

where, MS1 represents the dry weight of the ethanolic extract of the pigments (g), MS2 is the dry weight of quinoa flour pigmented by *M. purpureus* (g).

### 2.6. Experimental Design Using a Box–Behnken Design with RSM

The design used in the experiment was the Box–Behnken design, having three independent variables, ethanol graduation, extraction temperature and ethanol: sample ratio, and applying 14 treatments in the design with three repetitions, to generate a second-order polynomial quadratic model on the response surface. The statistical analysis was conducted in the R software (version 4.1.0, R Foundation for Statistical Computing, Vienna, Austria).

### 3. Results and Discussions

To obtain a higher yield in pigment extraction, the Box–Behnken design with a response surface was used, having as the variables, the ethanol: sample ratio, ethanol graduation and the extraction temperature, with a total of 14 experimental trials in triplicate, where the extraction variables affected the yield, density and absorbance in the pigment extraction (Table 1).

**Table 1.** The mean yield, density and absorbances of hydroethanolic extracts produced in a Box–Behnken design for three factors: Ethanol (%), Temperature (°C) and Ethanol: Sample ratio (mL:g) for the pigment extraction from quinoa flour fermented by *Monascus purpureus* supplemented with fish hydrolysate and sodium chloride.

Run Order	Ethanol (%)	Temperature (°C)	Ethanol: Sample (mL/g)	Yield (%)	Abs 400 nm	Abs 470 nm	Abs 500 nm	Density (g/mL)
1	60	50	40	33.9 ± 0.14	0.274 ± 0.0137	0.179 ± 0.0085	0.231 ± 0.0111	0.909 ± 0.0142
2	50	55	40	34.7 ± 0.18	0.275 ± 0.0075	0.174 ± 0.0047	0.222 ± 0.0054	0.919 ± 0.0128
3	40	50	40	33.2 ± 0.15	0.244 ± 0.0026	0.153 ± 0.0025	0.189 ± 0.0031	0.938 ± 0.0068
4	40	55	50	33.2 ± 0.33	0.205 ± 0.0045	0.127 ± 0.0030	0.158 ± 0.0040	0.918 ± 0.0235
5	40	60	40	32.8 ± 0.09	0.258 ± 0.0081	0.164 ± 0.0059	0.206 ± 0.0072	0.934 ± 0.0123
6	50	55	40	34.7 ± 0.18	0.275 ± 0.0075	0.174 ± 0.0047	0.222 ± 0.0054	0.919 ± 0.0128
7	60	55	50	32.0 ± 0.31	0.229 ± 0.0006	0.147 ± 0.0006	0.191 ± 0.0012	0.895 ± 0.0074
8	50	50	50	31.4 ± 0.40	0.203 ± 0.0053	0.127 ± 0.0026	0.161 ± 0.0031	0.918 ± 0.0031
9	60	55	30	32.8 ± 0.13	0.357 ± 0.0174	0.234 ± 0.0122	0.305 ± 0.0163	0.897 ± 0.0173
10	40	55	30	32.2 ± 0.66	0.335 ± 0.0032	0.211 ± 0.0015	0.264 ± 0.0020	0.924 ± 0.0090
11	50	60	50	31.8 ± 0.16	0.211 ± 0.0112	0.134 ± 0.0081	0.173 ± 0.0097	0.917 ± 0.0066
12	50	50	30	33.1 ± 0.32	0.323 ± 0.0080	0.205 ± 0.0045	0.263 ± 0.0045	0.917 ± 0.0057
13	60	60	40	32.9 ± 0.33	0.281 ± 0.0053	0.187 ± 0.0052	0.243 ± 0.0053	0.888 ± 0.0194
14	50	60	30	33.3 ± 0.32	0.346 ± 0.0047	0.225 ± 0.0042	0.292 ± 0.0051	0.919 ± 0.0065

Table 2 shows the results of the final response surface analysis model, where the second-order polynomial model presented an adjusted regression coefficient ( $R^2 = 0.721$ ), i.e., 72.89% of the variability could be explained by the independent variables as a whole. In addition, the linear terms for the extraction temperature, ethanol strength, ethanol: sample and ethanol: sample ratio, and the quadratic terms for the ethanol strength, extraction temperature, ethanol: sample ratio and ethanol × ethanol: sample interaction were highly

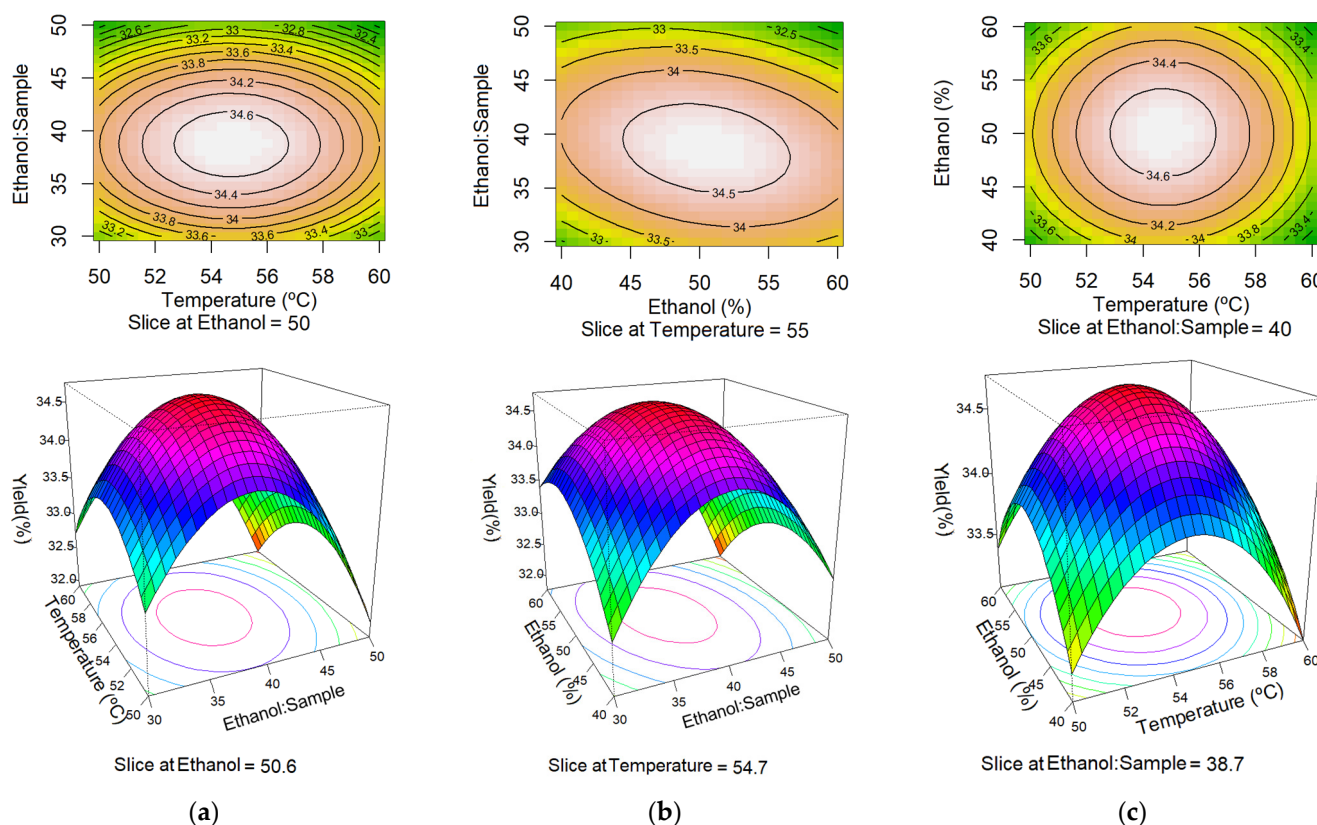
significant predictors ( $p < 0.0001$ ) of the pigment extraction yield of fermented quinoa flour. The other terms were not significant and were therefore removed from the model.

**Table 2.** Parameter estimates of the response surface model for estimating the yield (%) of extracts obtained from quinoa fermented by *Monascus purpureus* supplemented with fish hydrolysate and sodium chloride.

	Mean	Std. Error	p-Value
Intercept	−114.1	22.38	<0.0001
Temperature (°C)	3.676	0.7384	<0.0001
Ethanol (%)	0.8659	0.1785	<0.0001
Ethanol: Sample ( $v/v$ )	1.364	0.1541	<0.0001
Ethanol <sup>2</sup>	−0.006871	0.001678	0.000246
Temperature <sup>2</sup>	−0.03359	0.00671	<0.0001
Ethanol: Sample ratio <sup>2</sup>	−0.01473	0.001678	<0.0001
Ethanol × Ethanol: Sample	−0.004445	0.0015	0.005533
Goodness of fit			
Multiple R-squared	0.769		
Adjusted R-squared	0.721		
Residuals	0.270		

The negative interaction term for ethanol × ethanol: sample may raise issues related to the polarity of the ethanol gradient, as it implies that at the same ethanol: sample ratio, a higher ethanol grade results in lower extraction yields.

Figure 1a shows the contour plot with a central point of 50% ethanol, where it is observed that the highest extraction yield is found in the central zone of the temperature and the ethanol: sample ratio; the surface plot shows a maximum yield with a central point of 50.6% ethanol. Figure 1b shows the contour plot with a central temperature point of 55 °C, where it is observed that the highest extraction yield is found in the central zone of the ethanol:sample ratio and the ethanol grade; the surface plot shows a maximum yield with a central point of a 54.7 °C temperature. Figure 1c shows the contour plot with a central point of the ethanol:sample ratio at 40, where it is observed that the highest extraction yield is found in the central zone of the ethanol grade ratio and the temperature; the surface plot shows a maximum yield with a central point of 38.7 °C for the ethanol: sample ratio. It was also possible to obtain the highest extraction yield in %, which was  $34.72 \pm 0.18$  with the optimum points of: ethanol degree of 50.6°, an extraction temperature of 54.7 °C and an ethanol: sample ratio of 38.7, taking into account the data of the second-order polynomial equation.



**Figure 1.** (a) The contour plot cut at the center point of ethanol = 50 and the response surface at the optimum maximum value of ethanol = 50.6 as a function of temperature and ethanol: sample. (b) The contour plot cut at the center point at temperature = 55 and the response surface at the optimum maximum value at temperature = 54.7 as a function of ethanol and ethanol: sample. (c) The contour plot cut at the center point of ethanol: sample (EA) = 40 and the response surface at the optimum maximum value for ethanol: sample = 38.7 as a function of ethanol and temperature.

In addition, the concentration of the extract in solution can be predicted with a linear equation of the sum of the absorbances at 400, 470 and 500 nm at a 1:6 dilution; where a high coefficient of determination ( $R^2 = 0.974$ ) was obtained from the data in Table 3, the equation will allow for rapid concentration estimations in the calculations after extraction, providing that the extract is diluted 1:6 with ethanol at the same gradient (%).

**Table 3.** Parameter estimates of the linear regression model of concentration and absorbance of quinoa flour samples fermented by *Monascus purpureus* supplemented with fish hydrolysate and sodium chloride.

	Mean	Std. Error	p-Value
Intercept	0.0018	0.00021	<0.0001
(Abs 400 + Abs 470 + Abs 500)	0.0111	0.00020	<0.0001
Goodness of fit			
Multiple R-squared	0.975		
Adjusted R-squared	0.974		
Residuals	0.00083		

#### 4. Conclusions

This study helped to determine the optimal conditions for hydroethanol extraction of pigments from fermented quinoa flour by *M. purpureus* supplemented with fish hydrolysate and sodium chloride. In contrast to other investigations of hydroethanolic extractions, a low ethanol gradient of 50.6% at 50.6 °C was found to maximize yield. This implies that

pigment extraction from fermented quinoa flour may be economically feasible. A very useful equation was derived for future rapid estimates of extract concentrations.

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