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Proceeding Paper

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

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Abstract: *Monascus purpureus* has been tested with different food matrices in solid state fermentation and has had a different behavior in the production of its pigments. Therefore; in a new matrix it is important to optimize the extraction of these pigments obtaining 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 ± 0.18. In addition, the best equation to predict the concentration of hydroethanol extract was linear and was obtained by summing 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 comprise 31% of the market, 40% are synthetic and 29% semi-synthetic. Due to their potential undesirable mutagenicity and carcinogenicity the number of synthetic dyes allowed will continue to decrease over time, which is why nowadays new sources of production such as plants, animals and microorganism are sought, among them presenting greater advantages 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 culture [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); Rubropunctamine and Monascorubramine (red pigments) [4].

Fish hydrolysate is considered a rich source of amino acids, since the hydrolysis process decreases the size of 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, essential for amino acid biosynthesis through the ammonia pathway and other pathways that are linked for 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 pigments, that is why including sodium chloride in the fermentation would generate salt stress, and the fungus would produce a greater amount of pigments, this concentration of salts must be low because 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 chlorite, in order to maximize the yield, using a response surface design; and to construct a linear equation by spectrophotometry to predict the concentration of 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 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, vortexed for 5 min, spores were counted in Neubauer chamber and adjusted to 1.0×10^6 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 pH adjusted to 6, and then sterilized at 121°C for 15 min. 1 mL of inoculum (1.0×10^6 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. It was milled in a mill (CS-1000, Shang-Jun, 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 30:1, 40:1 and 50:1 mL/g, agitated at 400 rpm for 180 min at 50, 55 and 60 °C. After the time 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. 1 mL of sample was taken and diluted with ethanol, where the ratio was 1:6 (v:v), as blank for quantification ethanol was used.

2.5. Yield

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

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

where, MS1 represents the dry weight of the ethanolic extract of 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, taking into account 14 treatments in the design with three repetitions, to generate a second order polynomial quadratic model on the response surface. 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 response surface was used, having as variables the ethanol:sample ratio, ethanol graduation and extraction temperature, with a total of 14 experimental trials in triplicate, where the extraction variables affect the yield, density and absorbance in pigment extraction (Table 1).

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

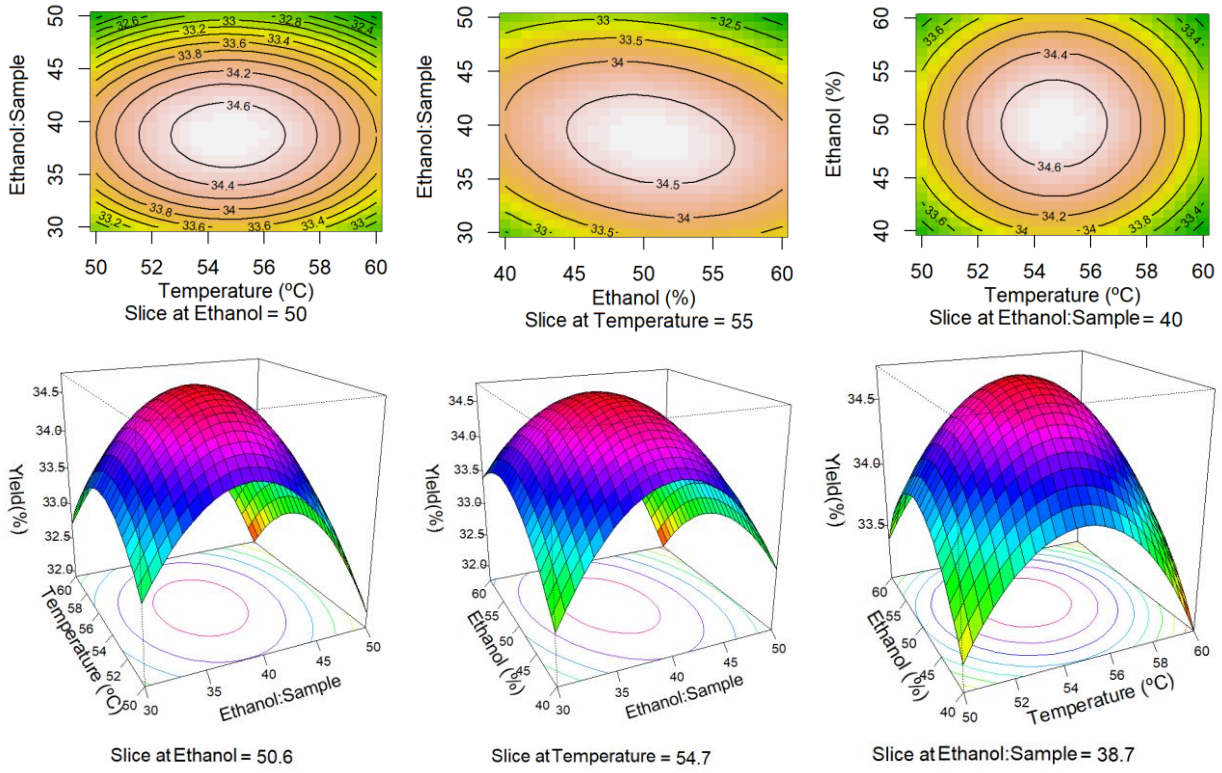
Run Order	Ethanol (%)	Temperature (°C)	Ethanol: Sample (ml/g)	Yield (%)	Abs 400 nm	Abs 470 nm	Abs 500 nm	Density (g/mL)
1	60	50	40	33.9 ± 0.14	0.274 ± 0.0137	0.179 ± 0.0085	0.231 ± 0.0111	0.909 ± 0.0142
2	50	55	40	34.7 ± 0.18	0.275 ± 0.0075	0.174 ± 0.0047	0.222 ± 0.0054	0.919 ± 0.0128
3	40	50	40	33.2 ± 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 variability could be explained by the independent variables as a whole. Also, the linear terms for extraction temperature, ethanol strength, ethanol: sample and ethanol:sample ratio, the quadratic terms for 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.

The negative interaction term for Ethanol × Ethanol:Sample may raise issues related to the polarity of ethanol gradient, as it implies that at the same ethanol:sample ratio, higher ethanol grade yields 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 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 of the ethanol: sample ratio. It was also possible to obtain the highest extraction yield in % which was 34.72 ± 0.18 with the optimum points of ethanol degree of 50.6°, extraction temperature of 54.7 °C and ethanol: sample ratio of 38.7 taking into account the data of the second order polynomial equation.



(**a**) (**b**) (**c**)

Figure 1. (**a**) Contour plot cut at the center point Ethanol = 50 and response surface at the optimum maximum value Ethanol = 50.6 as a function of Temperature and Ethanol:Sample. (**b**) Contour plot cut at the center point Temperature = 55 and response surface at the optimum maximum value Temperature = 54. 7 as a function of Ethanol and Ethanol:Sample. (**c**) Contour plot cut at the center point EA = 40 and response surface at the optimum maximum value Ethanol:Sample = 38.7 as a function of Ethanol and Temperature.

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^2	−0.006871	0.001678	0.000246
Temperature^2	−0.03359	0.00671	<0.0001

Ethanol:Sample ratio^2	−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		

In addition, the concentration of extract in solution can be predicted with a linear equation of the sum of the absorbances at 400, 470 and 500 nm at 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 quick information in the calculations after extraction, as long as 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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