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Proceeding Paper

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

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Abstract: In recent years, different substrates have been used in the production of pigments from *M. purpureus*; thus, pigment extraction would benefit from the appraisal of an optimal method. In this study, we employed quinoa flour fermented by the fungus, which was supplemented with sodium chloride to improve pigment production. The optimization of the hydroethanolic extraction of the pigments was found to reach a maximum at an ethanol grade of 49.0%, an extraction temperature of 60 °C, and an ethanol:sample ratio of 35.9 with a yield (%) of 26.15 ± 0.26. In addition, a linear equation (R^2 = 0.964) was modelled to estimate the extract concentration from absorbances measured at 400, 470, and 500 nm.

Keywords: extracts; sodium chloride; yield; density; Box–Behnken design; response surface

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1. Introduction

The most important sensory attribute when buying a food is color, which influences the other sensations (aroma, flavor, texture) giving a suggestion to the buyer of a product with good general attributes [1]. Synthetic dyes are the most widely employed coloring agents, but in recent years, the interest in and production of natural pigments have increased, because chemical dyes are related to be potentially dangerous and may have carcinogenic effects on human health, in addition to being pollutants to the environment in their production [2,3].

A good pigment producer is the fungus *Monascus purpureus* belonging to the family *Monascaceae* that produces different secondary metabolites with important polyketide structures [4]. These include red pigments (rubropunctamine and monascurubramin), orange pigments (rubropunctatin and monascorubrin), and yellow pigments (monascine and ankaflavin) among others [2]. *Monascus* is a saprophytic fungus used for more than a thousand years in Asian countries, in the production of fermented foods, red tofu, red wines, Kaoliang, etc., in addition to attributing to them antimutagenic, anticancer, antimicrobial properties, and possible anti-obesity activities [5,6]. In addition, the fungus produces monacolin K, a secondary metabolite beneficial to human health that regulates cholesterol biosynthesis [7].

The production of natural pigments by fermentation has advantages due to lower costs in production and the better management of parameters [1]. In addition, a minimal

proportion of sodium chloride during fermentation stimulates the production of secondary metabolites such as pigments [8]. Taking into consideration the factors in pigment production, it is necessary to explore suitable parameters for the extraction of these pigments for future research; the response surface methodology is an efficient statistical optimization tool that uses fewer trials, and one of the most widely used methodologies is the Box–Behnken design for optimization experiments, which provides the relationship between the independent variables and the response [9].

In this regard, the objectives of this study were to optimize the hydroethanol extraction of pigments from quinoa flour fermented by *M. purpureus* supplemented with sodium chloride, and to build a linear equation by spectrophotometry to estimate the concentration of the hydroethanol extracts.

2. Materials and Methods

2.1. Fungal Strain

The filamentous fungus *Monascus purpureus* CECT 2955 was acquired from the Spanish Type Culture Collection (CECT). It was resuspended and seeded in PDA (Potato Dextrose Agar) in a Petri dish at 30 °C for 7 days; then, it was seeded in QFA (Quinoa Flour Agar) with pH adjusted to 6 and cultured at 30 °C for 7 days. The amount of 1.0×10^6 spores/mL was collected, counted, and adjusted as the inoculum for solid-state fermentation.

2.2. Inoculation of M. purpureus in Quinoa Grains

White quinoa was used as a substrate wherein 30 g of quinoa grains, NaCl 0.05% (*w*/*w*), and 25 mL of distilled water were added per flask. Triplicate flasks were sterilized in an autoclave (Presoclave III 80, J.P. Selecta, S.A., Barcelona, Spain) at 121 °C for 15 min. Solid-state fermentation was carried out by inoculating 1 mL of the *M. purpureus* spore suspension into the sterile substrate. Flasks were placed in an incubator (ILW, Pol Eko, Śląski, Poland) at 30 °C for up to 8 days, then the fermented substrate was dried at 65 °C to a constant weight. It was milled to obtain the pigmented quinoa flour.

2.3. Hydroethanol Extraction of Pigments and Spectrophotometric Analyses

Pigment extraction was carried out from fermented quinoa flour, wherein 1 g of sample was mixed with ethanol at 40, 50, and 60% (*v*/*v*) at an ethanol:sample ratio of 30:1, 40:1, and 50:1 mL/g with agitation (400 rpm) for 3 h at a temperature of 50, 55, and 60 °C. Mixing was performed in round base tubes, then samples were centrifuged at 10,000 rpm at 25 °C for 20 min. A sample of 1 mL of the supernatant was taken, diluted in ethanol at a ratio of 1:6 (*v*:*v*), and the sample was measured with a UV–Vis spectrophotometer (C-7100, Peak Instruments Inc., Houston, TX, USA) at 400, 470, and 500 nm for yellow, orange, and red pigments, respectively. The result is expressed in absorption units (AU).

2.4. Obtaining Yields

For each treatment, the yield was obtained as the quotient of the dry weight of the ethanolic extract of pigments (in grams) and the dry weight of quinoa flour pigmented by *M. purpureus* (in grams), expressed as a percentage. Previously, the hydroethanol extract was dried in hot air at 65 °C for 48 h.

2.5. Response Surface Methodology

In this study, the experiments were conducted using the Box–Behnken design (BBD) with three levels to fit the response surfaces [9]. The three independent variables were based on ethanol grade, extraction temperature, and ethanol:sample ratio, with 14 experimental runs and three replicates. The conditions of the 14 runs are shown in Table 1. The experimental data were fitted to the quadratic model using a second-order polynomial model. Statistical analysis was conducted in the R software (version 4.1.0, R Foundation for Statistical Computing, Vienna, Austria).

Table 1. Mean yield, density, and absorbances of hydroethanolic extracts produced in a BBD for three factors: ethanol (%), temperature (°C), and ethanol: sample ratio (mL:g) for pigment extraction from quinoa flour fermented by *Monascus purpureus* supplemented with 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	25.0 ± 0.10	0.260 ± 0.0081	0.151 ± 0.0046	0.194 ± 0.0061	0.894 ± 0.0056
2	50	55	40	26.2 ± 0.26	0.270 ± 0.0068	0.155 ± 0.0044	0.198 ± 0.0055	0.922 ± 0.0209
3	40	50	40	25.4 ± 0.00	0.241 ± 0.0032	0.136 ± 0.0031	0.169 ± 0.0036	0.909 ± 0.0214
4	40	55	50	24.8 ± 0.47	0.199 ± 0.0078	0.112 ± 0.0052	0.140 ± 0.0058	0.935 ± 0.0101
5	40	60	40	25.6 ± 0.14	0.246 ± 0.0070	0.141 ± 0.0050	0.177 ± 0.0065	0.936 ± 0.0074
6	50	55	40	26.2 ± 0.26	0.270 ± 0.0068	0.155 ± 0.0044	0.198 ± 0.0056	0.922 ± 0.0209
7	60	55	50	25.1 ± 0.28	0.221 ± 0.0085	0.127 ± 0.0055	0.163 ± 0.0070	0.901 ± 0.0044
8	50	50	50	26.1 ± 0.40	0.203 ± 0.0069	0.114 ± 0.0044	0.144 ± 0.0064	0.924 ± 0.0057
9	60	55	30	24.4 ± 0.53	0.362 ± 0.0200	0.212 ± 0.0130	0.275 ± 0.0167	0.900 ± 0.0112
10	40	55	30	24.2 ± 0.04	0.309 ± 0.0095	0.177 ± 0.0059	0.222 ± 0.0079	0.935 ± 0.0156
11	50	60	50	24.8 ± 0.25	0.216 ± 0.0100	0.122 ± 0.0060	0.156 ± 0.0076	0.913 ± 0.0033
12	50	50	30	25.1 ± 0.16	0.325 ± 0.0053	0.186 ± 0.0030	0.237 ± 0.0031	0.927 ± 0.0031
13	60	60	40	25.1 ± 0.22	0.273 ± 0.0067	0.161 ± 0.0044	0.209 ± 0.0059	0.893 ± 0.0134
14	50	60	30	25.7 ± 0.16	0.341 ± 0.0029	0.199 ± 0.0015	0.256 ± 0.0023	0.916 ± 0.0086

3. Results and Discussion

Table 1 compiles the results of the 14 experimental runs for varying ethanol, temperature, and ethanol: sample ratio. The three extraction conditions appeared to affect the yield, density, and absorbances—and therefore amount of pigment extracted.

The results of the final model of response surface analysis are shown in Table 2. Such a second-order polynomial model presents an adjusted regression coefficient (R^2 = 0.7289), which indicated that 72.89% of the variability could be jointly explained by the independent variables. In addition to the linear terms for ethanol grade, extraction temperature, and ethanol: sample ratio, the quadratic terms for ethanol grade, the ratio of ethanol: sample, and the interaction temperature × ethanol:sample were highly significant predictors ($p < 0.0001$) of the yield of pigment extraction from fermented quinoa flour. Other terms were not significant and therefore removed from the model. The negative interaction term for temperature × ethanol:sample may raise issues related to ethanol evaporation, since it implies that at the same ethanol:sample ratio, higher temperatures produce lower extraction yields.

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

	Mean	Std. Error	*p*_Value
Intercept	−24.68	5.425	<0.0001
Temperature (°C)	0.3437	0.07828	<0.0001
Ethanol (%)	0.8219	0.1058	<0.0001
Ethanol:sample ratio (*v*/*v*)	1.044	0.1357	<0.0001
Ethanol^2	−0.008265	0.001056	<0.0001
Ethanol:sample ratio^2	−0.006747	0.001056	<0.0001
Temperature × ethanol:sample	−0.008864	0.001927	<0.0001
Goodness of fit			
Multiple R-squared	0.769		
Adjusted R-squared	0.729		
Residuals	0.111		

Figure 1a illustrates a higher extraction yield in the contour plot when working with a low temperature and ethanol:sample ratio between 40 and 50, taking as a central point an ethanol grade of 50%. The same is suggested in the response surface plot, at the optimal central point for an ethanol grade of 49.2. In the contour plot of Figure 1b, a higher extraction yield is observed in the central zone of the ethanol grade and ethanol:sample ratio with a temperature center point of 55 °C. In the surface plot derived at an optimized

temperature of 60 °C, the highest yield corresponds to the maximum point of the surface. In the contour plot of Figure 1c, it is observed that at a lower temperature and with an ethanol grade extraction between 45 and 55 (%), better yields are obtained at temperatures below 56 °C. The respective surface plot was derived based on an optimal ethanol: sample ratio of 35.9, which is out of the domain area of the experiment.

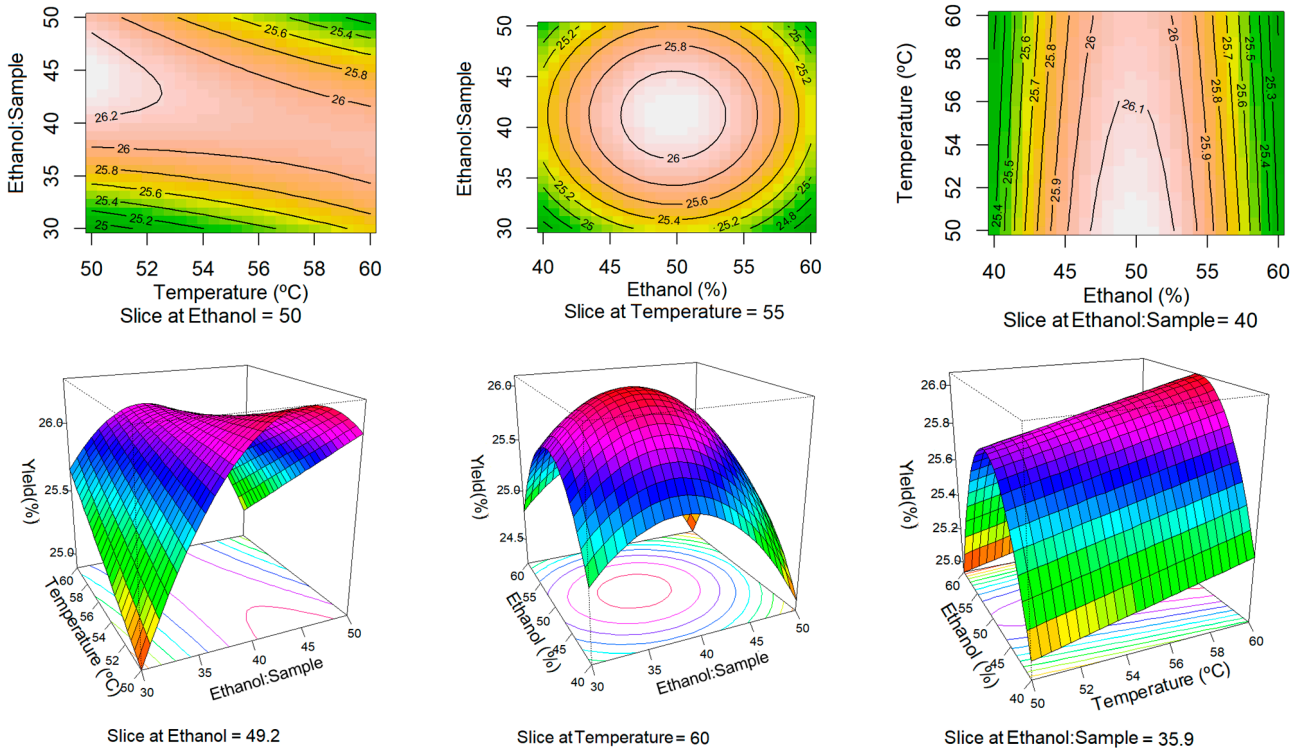

Figure 1. (**a**) Contour plot and response surface as a function of temperature and ethanol:sample. (**b**) Contour plot and response surface as a function of ethanol and ethanol:sample. (**c**) Contour plot and response surface as a function of ethanol and temperature.

Thus, the optimal conditions for extraction of pigments were determined at ethanol 49.2°, extraction temperature of 60 °C and ethanol: sample ratio of 35.9. At these conditions, a maximum yield (%) of 26.15 ± 0.26 can be achieved.

In addition, a linear equation was built to predict the concentration of extract in solution from the added values of absorbances measured at 400, 470, and 500 nm at a dilution of 1:6. The coefficient of determination evidenced a strong association (R^2 = 0.964). The estimates of the linear equation are shown in Table 3; and it is intended that this equation is used for a rapid estimation of the concentration of extracts in solution, right after extraction. The use of the equation, however, requires that the extract solution is always diluted 1:6 in ethanol at the same grade (%) used in the extraction process.

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

	Mean	Std. Error	*p*_Value
Intercept	0.0016	0.00019	<0.0001
(Abs 400 + Abs 470 +Abs 500)	0.0088	0.00019	<0.0001
Goodness of fit			
Multiple R-squared	0.964		
Adjusted R-squared	0.964		
Residuals	0.00075		

4. Conclusions

This research has optimized the conditions for the hydroethanolic extraction of pigments from quinoa flour fermented by *M. purpureus* supplemented with sodium chloride. A maximum extraction yield of 26.15 ± 0.26% was obtained using a low percentage of ethanol in the extraction of 49%, which is favorable when scaling up because it is less costly and non-polluting compared with other solvents. This study has also derived a linear regression equation that may be useful for the future estimation of hydroethanol extracts.

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