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Optimization of pigment extraction from quinoa flour fermented by *Monascus purpureus* supplemented with sodium chloride †

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Abstract: Depending on the substrate and fermentation conditions in different investigations, *Monascus purpureus* has shown to produce different pigments with importance for the food industry, therefore it is necessary to determine an optimal extraction method for this matrix. A yield (%) of 26.15 ± 0.26 was obtained at ethanol graduation conditions of 49.0°, extraction temperature of 60 °C and ethanol:sample ratio of 35.9. In addition, a linear equation (R^2=0.964) was modelled to estimate extract concentration from absorbances measured at 400, 470 and 500 nm.

Keywords: Extracts; sodium chloride; yield; density; Box-Benhken 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].

The production of natural pigments by fermentation has advantages due to lower cost in production and better management of parameters [1]. Also, having a ratio of sodium chloride, as a stress factor, could increase the production of beneficial secondary metabolites [7]. Taking into consideration the factors in pigment production, it is necessary to explore on the suitable parameters for the extraction of these pigments for future

research.

In this regard, the objectives of this study were to optimize the hydroethanol extraction of pigments from quinoa flour fermented by *M. purpureus* 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 $x10^6$ spores/ml was collected, counted and adjusted as inoculum for solid state fermentation.

2.2. Inoculation of M. purpureus in quinoa grains

White quinoa was used as substrate where 30 g of quinoa grains, NaCl 0.05 % (w/w) with 25 ml of distilled water was added per flask. Triplicate flasks were sterilized in an autoclave (PRESOCLAVE III 80, J.P. SELECTA, s.a., Spain) at 121°C for 15 minutes. 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, Poland) at 30°C for up to 8 days, then the fermented substrate was dried at 65°C to constant weight. It was milled to obtain the pigmented quinoa flour.

2.3. Hydroethanol extraction of pigments and spectrophotometric analyses

Pigment extraction was made from fermented quinoa flour, where 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 temperature 50, 55 and 60°C. Mixing was performed in round base tubes, then centrifuged at 10000 rpm at 25°C for 20 min. The supernatant was used for UV-Vis spectrophotometer (C-7100, PEAK INSTRUMENTS INC., USA) measurements at 400, 470 and 500 nm for yellow, orange and red pigments respectively, at a dilution of 1:6 (v:v).

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 ~ 2 days.

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. The three independent variables were based on ethanol graduation, 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).

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.

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 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

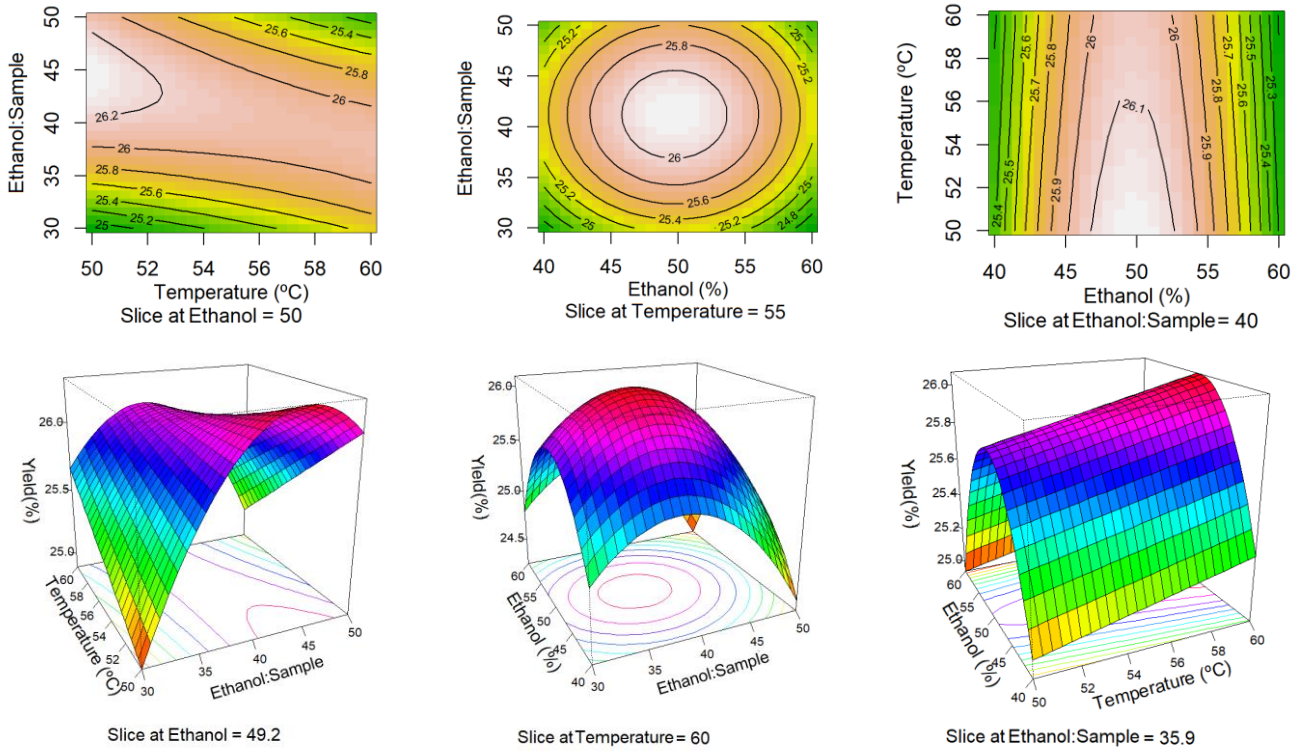
The results of the final model of response surface analysis are shown in Table 2. Such a second order polynomial model presented an adjusted regression coefficient (R^2=0. 7289), which indicated that 72.89% of the variability could be jointly explained by the independent variables. In addition to the linear terms for ethanol graduation, extraction temperature and ethanol: sample ratio, the quadratic terms for ethanol graduation, the ratio ethanol: sample and the interaction temperature×ethanol:sample were highly significant predictors ($p<.0001$) 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×Etanol:Sample may raise issues related to ethanol evaporation, since it implies that at the same ethanol:sample ratio, higher temperatures produce lower extraction yields.

Figure 1(a) illustrates a higher extraction yield in the contour plot when working with a low temperature and ethanol:sample ratio between 40 and 50, having as central point the ethanol grade at 50 %. The same is suggested in the response surface plot, at the optimal central point for ethanol grade of 49.2. In Figure 1(b), in the contour plot 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 1(c), it is observed that at lower temperature and with ethanol grade extraction between 45 and 55 (%), better yields are obtained at temperatures below 56 ºC. 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.

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.

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	<.0001
Temperature (°C)	0.3437	0.07828	<.0001
Etanol (%)	0.8219	0.1058	<.0001
Etanol: Sample (v/v)	1.044	0.1357	<.0001
Etanol^2	-0.008265	0.001056	<.0001
Etanol:Sample ratio^2	-0.006747	0.001056	<.0001
Temperature×Etanol:Sample	-0.008864	0.001927	<.0001
Goodness of fit			
Multiple R-squared	0.769		
Adjusted R-squared	0.729		
Residuals	0.111		



(a) (b) (c)

Figure 1. (a) Contour plot cut at the center point Ethanol= 50 and response surface at the maximum optimal value Ethanol= 49.2 as a function of Temperature and Ethanol: Sample. (b) contour plot cut at the center point Temperature= 55 and response surface at the maximum optimal value Temperature = 60 as a function of Ethanol and Ethanol: Sample. (c) contour plot cut at the center point Ethanol: Sample=40 and response surface at the maximum optimal value Ethanol: Sample = 35.9 as a function of Ethanol and Temperature.

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 ex-

traction. The use of the equation, however, requires that the extract solution be always diluted 1:6 in ethanol at the same graduation (%) 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	<.0001
(Abs 400 + Abs 470 +Abs 500)	0.0088	0.00019	<.0001
Goodness of fit			
Multiple R-squared	0.964		
Adjusted R-squared	0.964		
Residuals	0.00075		

4. Conclusions

This study has optimized the conditions for the hydroethanolic extraction of pigments from quinoa flour fermented by *M. purpureus* when supplemented with sodium chloride. Contrarily to what is commonly used in hydroethanolic extractions, a low ethanol graduation of 49% was found to maximise the yield. This implies that extraction of pigments from fermented quinoa flour can be economically feasible. This study also derived a very useful equation for future rapid estimations of extract concentrations.

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