Research Paper

Improvement of submerged culture conditions to produce colorants by *Penicillium purpurogenum*

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Abstract

Safety issues related to the employment of synthetic colorants in different industrial segments have increased the interest in the production of colorants from natural sources, such as microorganisms. Improved cultivation technologies have allowed the use of microorganisms as an alternative source of natural colorants. The objective of this work was to evaluate the influence of some factors on natural colorants production by a recently isolated from Amazon Forest, *Penicillium purpurogenum* DPUA 1275 employing statistical tools. To this purpose the following variables: orbital stirring speed, pH, temperature, sucrose and yeast extract concentrations and incubation time were studied through two fractional factorial, one full factorial and a central composite factorial designs. The regression analysis pointed out that sucrose and yeast extract concentrations were the variables that influenced more in colorants production. Under the best conditions (yeast extract concentration around 10 g/L and sucrose concentration of 50 g/L) an increase of 10, 33 and 23% respectively to yellow, orange and red colorants absorbance was achieved. These results show that *P. purpurogenum* is an alternative colorants producer and the production of these biocompounds can be improved employing statistical tool.

Key words: natural colorants; filamentous fungi; optimization; submerged culture.

Introduction

In the industries of food, cosmetic and pharmaceutical both natural and synthetic colorants are used extensively (Mapari *et al.*, 2005; Hailei *et al.*, 2011). But, some characteristics of synthetic colorants such as detrimental effects on environment (Shahid *et al.*, 2013), the development of mental illness, allergies and diverse type of cancers associate with the use of artificial colorants (Ruiz-Ruiz *et al.*, 2013) reduced the number of synthetic colorants approved by federal regulatory agencies (Santos-Ebinuma *et al.*, 2013a). Furthermore, there is a growing demand for ecofriendly/non-toxic colorants, specifically for health sensitive applications, such as the coloration of food and dyeing of children's fabrics and leather garments (Santos-Ebinuma *et al.*, 2013b). Natural colorants have been extracted from natural sources, such as plant and insect tissues (Unagul *et al.*, 2005; Boo *et al.*, 2012; Deveoglu *et al.*, 2012), but also these compounds can be produced by microorganisms (Cho *et al.*, 2002; Jiang *et al.*, 2005; Yang *et al.*, 2007; Méndez *et al.*, 2011). The production by the latter is very interesting to industries because microorganisms can grow rapidly and achieve high productivity with a product available throughout the year (Jiang *et al.*, 2005;

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Méndez *et al.*, 2011). Furthermore, microbial colorants are often more stable and soluble than those produced by plants or animal (Gunasekaran and Poorniammal, 2008).

Many ascomycetous fungi naturally synthesize and secrete natural colorants with improved functionalities (Dubois *et al.*, 1956; Mapari *et al.*, 2009). The diversity of fungal colorants is not only found in their chemical structures, but also in the color range of these compounds (Mapari et al., 2009). As most of the studies found in the literature about fungal colorants were performed with *Monascus* species and there is a large number of other fungi to be explored, it is of interest to search for alternative colorant-producing organisms (Hailei *et al.*, 2011).

It has recently been reported in the literature (Mapari et al., 2008; Hailei et al., 2011; Méndez et al., 2011) that *Penicillium* strains are potential producers of natural colorants, which have chromophore similar to *Monascus* colorants (Mapari *et al.*, 2008). Among *Penicillium* species, *P. purpurogenum* can produce colorants not only in solid medium but also in liquid media (Méndez *et al.*, 2011). In studies performed by our research group (Teixeira *et al.*, 2012), *P. purpurogenum* DPUA 1275 showed potential to produce natural colorants with significant antimicrobial activities and total absence of toxicity against *Artemia salina*.

According to Meinicke *et al.* (2012), different factors can influence the production of secondary metabolites. So, the optimization of operating conditions such as pH, temperature and orbital stirring speed and nutritional factors for maximum colorants production is an essential step (Mukherjee and Singh, 2011). Comparing statistical methods and classic ones for the optimization of processes, the first represent a safe and reliable alternative because they are based on the study of only one independent variable at a time, while all of the other factors are maintained at a fixed level (Gonçalves *et al.*, 2012). Furthermore, classical approaches are intrinsically more complicated to pinpoint interactions among processing parameters (Queiroga *et al.*, 2012).

Response surface methodology (RSM) is frequently used to optimize a process using statistic tool. The response surface methodology (RSM) model is usually constructed for the defined medium factors by quadratic polynomial equation to depict the interaction effect among the variables and is then used to optimize the bioprocess (Zafar *et al.*, 2012). The aim of this set of statistics techniques is to execute the experimental planning by building empirical models and evaluating the effect of independent variables on the desired variable response (Box and Wilson, 1951; Gonçalves *et al.*, 2012). It can reduce the number of experimental runs and provide sufficient information for a statistically acceptable result (Guo *et al.*, 2012). In this way, response surface analysis and orthogonal experimental design are commonly used approaches which are useful in

fermentative process optimization (Gösungur *et al.*, 2011; Yu *et al.*, 2012).

In this study, the influence of six independent variables (sucrose and yeast extraction concentrations, pH, temperature, orbital stirring speed and incubation time) on the production of yellow, orange and red colorants by submerged culture of *P. purpurogenum* DPUA 1275 was evaluated employing statistical designs aiming to increase the production of natural colorants by *P. purpurogenum*.

Materials and Methods

Chemicals

Sucrose and Yeast extract were purchased respectively from Synth (São Paulo, Brazil) and Acumedia (Lansing, Michigan, USA). All the other reagents were of analytical grade and were used as received.

Microorganism

Penicillium purpurogenum DPUA 1275 was provided by the Culture Collection by Federal University of Amazon (DPUA), AM, Brazil. The stock culture was maintained on Czapeck Yeast Extract Agar (CYA) tubes. Tubes and plates were inoculated at 30 °C for 7 days and subsequently stored at 4 °C.

Culture medium and inoculum preparation

CYA medium was used as the growth medium. This medium had the following composition (g/L in deionized water): K_2HPO_4 (1.0), yeast extract (5.0), sucrose (30.0), Agar (15.0) and 10 mL/L of concentrated Czapeck. Concentrated Czapeck, which is a salt solution, had the following composition (g/100 mL of deionized water): $NaNO_3$ (30.0), KCl (5.0), $MgSO_4.7H_2O$ (5.0), $FeSO_4.7H_2O$ (0.1) (Pitt, 1985). The composition of the production medium was similar to the one used for the inoculum, except for the sucrose and yeast extract concentrations, which was varied according to the selected experimental design.

For production experiments, 125 mL-Erlenmeyer flasks (Vidrolabor - ISO 1773) containing 25 mL of required medium were inoculated with 5 mycelial agar discs punched out with a sterilized self-designed cutter (8 mm diameter) from a stock culture grown at CYA medium in Petri plates during 7 days at 30 °C. The Erlenmeyer flasks were closed using cotton plug. The pH values and other experimental conditions varied according to the factorial design. All experiments were performed in orbital shaker. At the end of submerged culture, which varied according factorial design, samples were collected and assayed for pH and yellow, orange and red colorants production.

Analytical methods

The fermented broth was filtrated (Whatman N° 1 filter paper, Whatman, England), and the resulting supernatant was filtered through a Millipore filter (0.45 μm). The

concentration of fungal biomass was determined by dry weight. The filtrate was used to measure the sucrose consumption, pH and colorants production. Sucrose concentration was determined according to Dubois *et al.* (1956) and the pH was measured using pHmeter.

The concentration of extracellular colorants was estimated by measuring the absorbance of filtrates. Wavelength of each colorant was scanned at 350-600 nm. The supernatant was read at 400, 470 and 490 nm (a wavelength which represents the absorption maxima for yellow, orange and red colorants, respectively), using the spectrophotometer model UV-1650PC (Shimadzu, Kyoto, Japan) and taking the dilution factor of each sample into consideration. The results were expressed in Units of Absorbance (UA). The absorption maxima got to each colorant is in agreement with Johns and Stuart (1991).

Statistical design

Initially, a selection of variables that influence the production of yellow, orange and red colorants by *P. purpurogenum* was carried out using three different factorial design, as follows: first a 2^{6-2} fractional factorial statistical design (20 experiments), second a 2^{4-1} fractional factorial statistical design (12 experiments) and third a 2^3 full factorial design (12 experiments). After each experiment, some variables were kept constant. In this way, after the first factorial design, the temperature and fermentation time were kept at 30 °C and 336 h, respectively. In the second and third factorial design, the pH chosen was 4.5 and the orbital stirring speed, 150 rpm. The variables range and levels of the components under study to each factorial design are given in Table 1.

In the second stage, the influence of the independent variables, specifically sucrose and yeast extract concentrations, on the responses yellow, orange and red colorants production was investigated using a 2^2 central composite designs. A set of 12 experiments which contained a factorial or fractional factorial matrix with center points and star points to allow estimation of the curvature (Mokhtarani *et al.*, 2008; Viana Marques *et al.*, 2011) was performed. The range and levels of the components under study are given in Table 2.

For statistical elaboration, the actual values of each independent variable (X_i) were coded, to give coded levels, according to the equation:

Table 1 - Variables and factor levels used in the 2^{6-2}, 2^{4-1} and 2^3 design for studying yellow, orange and red colorants production by *P. purpurogenum* DPUA 1275.

Factors	Levels		
	Lower (+1)	Center (0)	Higher (-1)
2^{6-2} design			
Orbital stirring speed (rpm)	120	150	180
pH	4.5	6.0	7.5
Temperature (°C)	25	30	35
Sucrose (g/L)	10	30	50
Yeast extract (g/L)	3	5	7
Incubation time (h)	144	240	336
2^{4-1} design			
Orbital stirring speed (rpm)	120	150	180
pH	4.0	4.5	5.0
Sucrose (g/L)	50	60	70
Yeast extract (g/L)	6.0	7.0	8.0
2^3 design			
Orbital stirring speed (rpm)	150	180	250
Sucrose (g/L)	45	50	55
Yeast extract (g/L)	7.0	8.0	9.0

$$x_i = \frac{X_i - X_o}{\Delta X_i} \tag{1}$$

where x_i represents the corresponding coded values, X_o, the actual values at the central point, and ΔX_i, the step change value.

To identify the best conditions for yellow, orange and red colorants production, a quadratic model expressed by the Eq. (2) was used:

$$\hat{y}_i = b_0 + \sum b_i x_i + \sum b_i x_i^2 + \sum b_{ij} x_i x_j \tag{2}$$

where $\hat{y}_i$ are the predicted values for each response, b_o and b_i the intercept and linear and quadratic coefficients, respectively, and b_{ij} the interaction ones.

The "Statistica" Version 7.0 (Statsoft, Tulsa, OK, USA) software was used for regression and graphical analysis of data. The statistical significance of the regression

Table 2 - Factor levels used in the 2^2 central composite design for studying yellow, orange and red colorants production by *P. purpurogenum* DPUA 1275.

Factors	Levels				
	Axial (- 1.41)	Lower (-1)	Center (0)	Higher (1)	Axial (+ 1.41)
2^2 design					
Sucrose (g/L)	48	50	55	60	62
Yeast Extract (g/L)	7.6	8.0	9.0	10.0	10.4

coefficients was determined by the Fischer's test for analysis of variance (ANOVA) at a significance level $(p) \leq 0.05$, and the extent of variance explained by each model was given by the determination coefficient R^2. To minimize the error of ANOVA, the tests corresponding to the central point were repeated four times. The experimental and predicted values were compared in order to determine the validity of the models.

Results and Discussion

Experimental designs to produce natural colorants

It is known that metabolite production by microorganisms is largely influenced by the medium compounds, such as carbon and nitrogen sources, physicochemical factors, such as pH, temperature, inoculum size, stirring intensity and incubation time (Oh *et al.*, 2000; Celik and Calik, 2004; Mapari *et al.*, 2005). In previous work (Santos-Ebinuma *et al.*, 2013b) different carbon (glucose, fructose, sucrose, maltose and starch) and nitrogen (malt extract, ammonium sulfate, peptone, yeast extract and tryptone) sources was evaluated in order to produce natural colorants by submerged culture of *P. purpurogenum* and sucrose and yeast extract were the more suitable sources. In this way, the effect of different independent variables, specifically orbital stirring speed, pH, temperature, incubation time and sucrose and yeast extract concentrations on the yellow, orange and red natural colorants production by *P. purpurogenum* DPUA 1275 were evaluated by using statistical tools. The experimental design methodology is very important because it allows reducing the number of experiments as well as identifying both the independent variables with statistical significance for the process and their significant interactions (Santos *et al.*, 2011).

First fractional factorial design to produce natural colorants

Initially, a 2^{6-2} fractional factorial design, which is a resolution IV screening design, was performed. Its analysis is very interesting for an initial investigation, since it decreases the number of experiments, in comparison to the full factorial design with six variables. This statistical design is not completely saturated, meaning it does not mix the main effects with the first order (interaction), which secures the calculation of the variable effects in the responses studied, without losing the statistic quality of the information. This strategy is well suitable to an initial results screening, once it enables the evaluation of the main effects and, from such, deciding the strategy for the following design to obtain an optimized condition (Rodrigues and Iemma, 2005). It is natural, therefore, to assume that third-order terms are much less important than first-order ones, and, at least at a first approach, maybe neglected (Cavalcanti *et al.*, 2006; Lima *et al.*, 2009). In this way, the third-order interactions in the present work are negligible. The main results

of natural colorants production by submerged culture of *P. purpurogenum* according to the 2^{6-2} fractional factorial design are shown in Table 3.

It can be observed that among the 20 runs performed, the yellow colorants production varied from 0.110 to 2.803 UA_{400nm}, while the orange and red ones varied from 0.016 to 1.875 UA_{470nm} and 0.018 to 1.660 UA_{490nm}, respectively. However, while the highest yellow and red colorants production was obtained in run 10, it occurred in run 9 for the orange production. These runs have four variables in common: pH (4.5), temperature (25 °C), sucrose concentration (50 g/L) and incubation time (336 h).

The Pareto chart to all responses is shown in Figure 1. This chart represents the estimated effects of the variables, and their interactions on the responses variables in decreasing the order of magnitude. The length of each bar is proportional to the standardized effect. The vertical line can be used to judge which effects are statistically significant. Bars extending beyond this line correspond to the statistically significant effects at a confidence level of 95% (Zea *et al.*, 2004).

It can be seen from Figure 1A that all independent variables had a significant effect under the response yellow colorants production. However, for the orange colorants (Figure 1B), the temperature did not show a significant effect and, for the red ones, not only the temperature, but also the orbital stirring speed did not have a significant effect.

In this way, the main independent variables that showed a significant effect to all responses were incubation time, pH and yeast extract and sucrose concentrations. Incubation time was the main significant effect, with a positive one, which means that an increase of this parameter can improve the results. As it is known that natural colorants are, generally, secondary metabolites and a modification in the nutritional components concentration in the culture medium can promote changes not only in microorganism growth, but also in their metabolism, different incubation times were studied. However, the highest colorants production was achieved with 336 h of incubation time. According to previous results obtained by our research group (Santos-Ebinuma *et al*., 2013b) an, an incubation time higher than 336 h promoted a decrease on colorants production. In this way, this variable was not included in the next factorial designs and it was kept at a highest level (336 h).

Regarding pH, the analysis indicated that low levels of this parameter can promote the highest results for colorant production. On the other hand, the variables yeast extract and sucrose concentrations showed a positive effect, and higher concentrations of both sources would improve the results. As the most part of the articles found in the literature about colorants production by *Penicillium* strain is using CYA medium (30 g/L of sucrose and 5 g/L of yeast extract) and this is the first attempt to optimize their production using *P. purpurogenum,* there is not a concentration of both nutrients source to follow. It is known from

Table 3 - Matrix of the 2^{6-2} fractional factorial design with the real variables and results of the selected responses (yellow, orange and red colorants production).

Run	Orbital stirring speed (rpm)	pH	Temperature (°C)	Sucrose (g/L)	Yeast extract (g/L)	Incubation time (h)	Yellow Colorants (UA_{400nm})	Orange Colorants (UA_{470nm})	Red Colorants (UA_{490nm})
1	120	4.5	25	10	3	144	0.11	0.02	0.02
2	180	4.5	25	10	7	144	0.60	0.24	0.16
3	120	7.5	25	10	7	336	0.75	0.30	0.19
4	180	7.5	25	10	3	336	0.59	0.22	0.14
5	120	4.5	37	10	7	336	2.61	1.73	1.00
6	180	4.5	37	10	3	336	0.85	0.45	0.34
7	120	7.5	37	10	3	144	0.66	0.28	0.22
8	180	7.5	37	10	7	144	0.72	0.38	0.28
9	120	4.5	25	50	3	336	2.62	1.88	1.17
10	180	4.5	25	50	7	336	2.80	1.33	1.66
11	120	7.5	25	50	7	144	0.71	0.04	0.02
12	180	7.5	25	50	3	144	0.30	0.10	0.07
13	120	4.5	37	50	7	144	0.91	0.29	0.18
14	180	4.5	37	50	3	144	0.37	0.14	0.09
15	120	7.5	37	50	3	336	1.45	0.40	0.25
16	180	7.5	37	50	7	336	2.24	1.07	0.98
17 (C)	150	6.0	30	30	5	240	1.25	0.66	0.45
18 (C)	150	6.0	30	30	5	240	1.12	0.78	0.56
19 (C)	150	6.0	30	30	5	240	1.24	0.80	0.58
20 (C)	150	6.0	30	30	5	240	1.11	0.67	0.59

C - Central points.

the literature that it is required not only to supplement the culture medium but also to control the components in order to achieve an efficient and fast conversion of carbon source in the desired product with the highest possible productivity. Furthermore, once the nutritional needs are supplied, most of the fungi grow under pH values from 4.0 to 7.0 (Carlile and Watkinson, 1996).

The orbital stirring speed [1] showed a significant effect when interacting with pH [1*5], sucrose [1*2] and yeast extract [1*4]. The interaction between pH and incubation time [2*6] also showed a significant effect under all responses. The interaction 1*5 was significant in the highest level [+]. As independent variable, the yeast extract concentration was significant in the highest level. Thus, to keep the interactions with this nitrogen source in the highest level, the orbital stirring speed has to be kept in the same level. For interaction 1*4, the same argument used for interaction 1*5 can be applied, and the orbital stirring speed level has to be used in the highest level. For interaction 1*2, the factorial design showed that working with interactions in the highest level is more favorable. As for variable pH, the factorial design showed that the lowest level was better

to obtain the highest level among the interactions, and the orbital stirring speed has to be used in the highest level.

As the temperature was significant only for yellow colorants, in the next factorial design this variable will be kept at 30 °C, which was the central point used in this factorial design. It is known that many fungi usually require long periods to grow by submerged culture, and are exposed to contamination risk; this temperature is considered favorable to keep the physiological properties of the microorganisms (Gunasekaran and Poorniammal, 2008).

To all answers evaluated the error pure was lower than 0.006 and the determination coefficient for the yellow, orange and red colorants was 96.51, 91.64 and 97.34. According to Silva *et al.* (2009) values of $R^2 > 90\%$ are very good in the experimental design of bioprocesses.

Even though the orbital stirring speed as main variable did not have a significant effect on all responses, its interaction with many independent variables was significant. This fact is probably related to microorganism metabolism. As the microorganism used in this work is a filamentous fungus, it can grown in the form of a *pellet*, increasing the medium viscosity and promoting problems in mass trans-

fer, which results in lower productivity. It is known from the literature that for each culture, the optimum conditions of stirring intensity will partially depend on hyphae resistance to mechanic force and to its physiologic study, as well (Papagianni, 2004). Thus, finding the best orbital stirring speed is essential for improving the production and this

Figure 1 - Pareto chart for the effects of variables orbital stirring speed (1), pH (2), temperature (3), sucrose (4), yeast extract (5) and incubation time (6) on yellow (A), orange (B) and red (C) natural colorants production according to a 2^{6-2} fractional factorial design.

variable was included in the next factorial design in the same range previously studied.

Figure 2 shows the results about biomass, sucrose consumption and pH in each run carried out according to a 2^{6-2} fractional factorial design.

It was expected that the runs 1, 2, 7 and 8, which ended up with 240 h of incubation time, showed higher biomass concentration as compared with the other runs because this time is located in the exponential phase of cell growth (Santos-Ebinuma *et al.*, 2013b); however, these results were not achieved. This behavior probably occurred due the low sucrose concentration (10 g/L) in these runs. Comparing these results with the ones obtained in the runs with initial sucrose concentration of 50 g/L at the same incubation time the hypothesis cited before can be confirmed since the biomass concentration obtained in this condition was higher than the others. According to Santos-Ebinuma *et al.* (2013b), the orange and red colorants production increases after 168 h of incubation, which means that these metabolites have characteristics to be secondary ones, since their formation started to be more significant in the moment that the main carbon source had been almost completely consumed and the microorganism was in the decline phase.

Regarding sucrose consumption, the runs 3, 4, 5 and 6 promoted the maximum consumption of sucrose leading the lowest final sucrose concentration. In these runs, the initial sucrose concentration was 10 g/L, which can be the responsible for the microorganism sucrose consumption until exhaustion. In the other runs, the sucrose consumption occurred as expected.

Based on the analyzed results, four variables - yeast extract and sucrose concentrations, pH and orbital stirring speed - were selected to be evaluated by a 2^{4-1} fractional factorial design. The other variables were fixed at 336-h incubation period and 30 °C of temperature.

Second Fractional Factorial design to produce natural colorants

The fractional factorial design 2^{4-1} is an useful method to evaluate the impact of the variables under the responses and to define the conditions of the following facto-
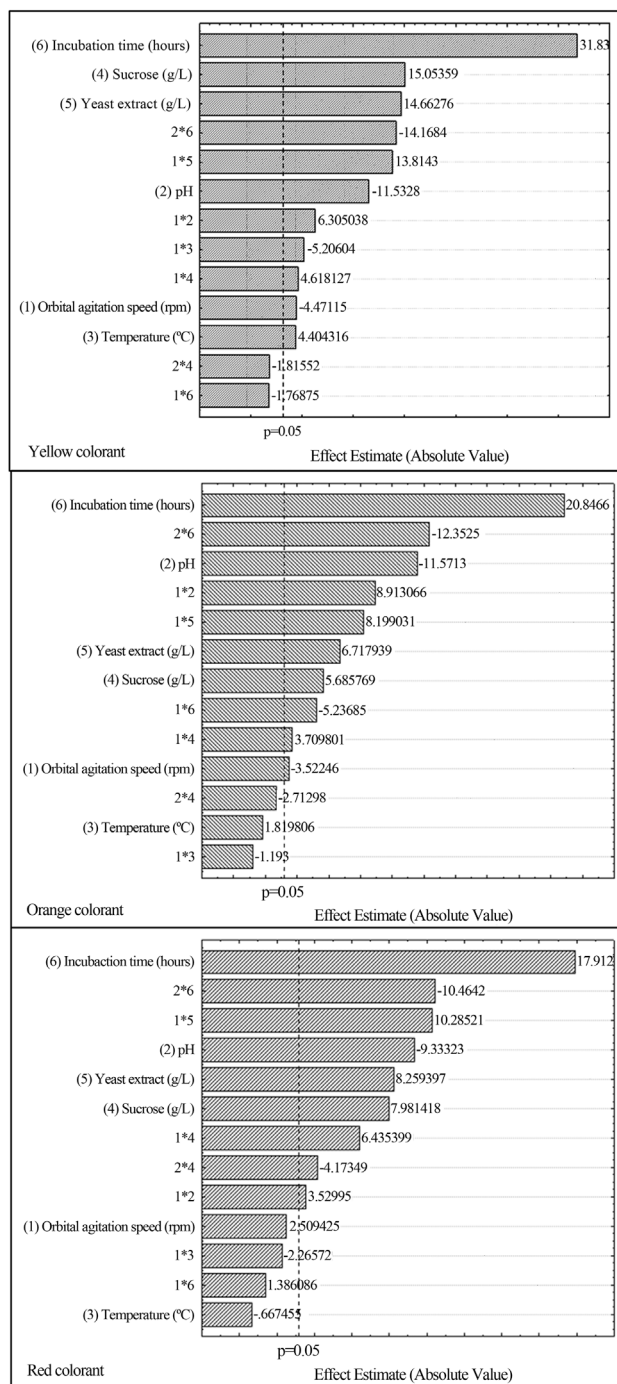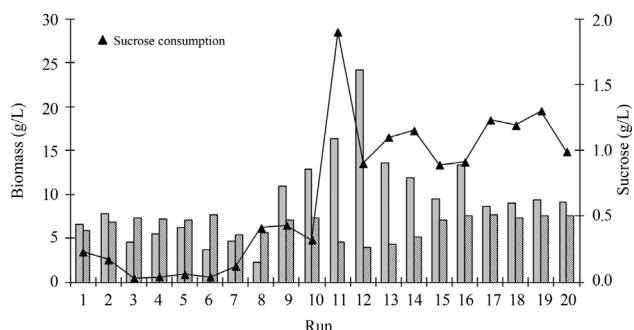
Figure 2 - Biomass (gray bars), sucrose consumption and pH (striped bars) in the runs performed according to a 2^{6-2} fractional factorial design.

Table 4 - Analysis of variance applied to the regression models used for yellow colorants production as function of pH, orbital stirring speed and sucrose and yeast extract concentrations, according to 2^{4-1} fractional factorial design.

Source	p value Yellow Colorants	p value Orange Colorants	p value Red Colorants
(1) pH	0.071514	0.634352	0.093393
(2) Orbital stirring speed (rpm)	0.005608*	0.093774	0.002189*
(3) Yeast extract (g/L)	0.000104*	0.007302*	0.000024*
(4) Sucrose (g/L)	0.000004*	0.000978*	0.000361*
1*2	0.001015*	0.002324*	0.008177*
1*3	0.006540*	0.089051	0.000196*
1*4	0.000346*	0.134367	0.201925

*significant level $p < 0.05$; $R^2 = 97\%$. $R^2 = 92\%$ (orange colorants). $R^2 = 93\%$ (red colorants).

rial design. Table 4 shows the p-value to responses yellow, orange and red colorants.

The statistical analysis of the results showed that, in the range studied, the yellow, orange and red colorants production was significantly affected by the yeast extract and sucrose concentrations. Moreover, the interaction between pH and orbital stirring speed [1*2] also showed a significant effect on all responses. The orbital stirring speed and the interactions between pH and yeast extract also had a significant effect on yellow and red colorants. In addition, the interaction between pH and sucrose was significant to the yellow colorants production.

The fit of the model was checked by the coefficient of determination R^2, which was calculated as 0.97, 0.92 and 0.93 to yellow, orange and red colorants, respectively. It indicates that 97, 92 and 93% of the variability could be explained by the model for the respective response. The analysis of variance (ANOVA) for the yellow, orange and red colorants are presented in the Supporting Information (Table A.1, A.2 and A.3, respectively).

The effect of the interaction between the variables pH and orbital stirring speed was negative. In this way, a variable should be in the lowest level while the other is in its higher level to promote the best results. So, when the pH was in its lower level (4.0) and the orbital stirring speed was in its higher level (180 rpm), the highest effect was observed.

The highest production was obtained in the highest yeast extract concentration, regardless of pH and orbital stirring speed. In this study, it was observed that the pH, despite being significant in the interactions, can be considered inert, which means that it did not have any effect in the responses variables. In this way, this variable was fixed at 4.5 in the next designs, which represents the central point in this study.

The yeast extract and sucrose concentrations were essentially significant to the responses, at a positive level, which means that an increase in their concentrations can improve the results. High yeast extract concentration promoted an increase in the extracellular colorants production,

probably due to its reaction with groups containing amine component in the medium to form water soluble complexes (Pastrana *et al.*, 1995).The biomass concentration, sucrose consumption and pH for all runs every 48 h are presented in the Supporting Information (see Figure A.1).

Not only sucrose concentration, but also yeast extract was kept to be evaluated in a future factorial design. As the orbital stirring speed was significant to two responses (yellow and red colorants production), this independent variable was also studied in a new factorial design.

Full Factorial design to produce natural colorants

After a selection of the variables that significantly influenced the natural colorants production by *P. purpurogenum* DPUA using fractional design, the independent variables orbital stirring speed, yeast extract and sucrose concentrations were evaluated for their effects under the yellow, orange and red natural colorants through a 2^3 full factorial design. Figure 3 shows the Pareto chart under the response variable yellow colorants.

Regarding the independent variables, sucrose and yeast extract concentrations had a significant effect on all responses. Considering the interactions, the ones among the orbital stirring speed and both sucrose and yeast extract concentrations showed significant effect. All significant effects showed a positive one, except the interaction between orbital stirring speed and yeast extract. As the highest yeast extract concentration promoted a high production of colorants, the orbital stirring speed has to be used in the lowest level and in the next factorial design this variable will be fixed in the central point (150 rpm), which can promote a good homogenization of the medium to avoid problems in the mass transfer during the fermentation process.

Analyzing all results obtained for the studied responses, a higher influence of yeast extract and sucrose concentrations under the natural colorants production, both in the highest level, can be observed. This result shows that the studied concentrations have not yet exert an effect of catabolic repression in microorganism metabolism. In this way, both variables were analyzed using a central compos-
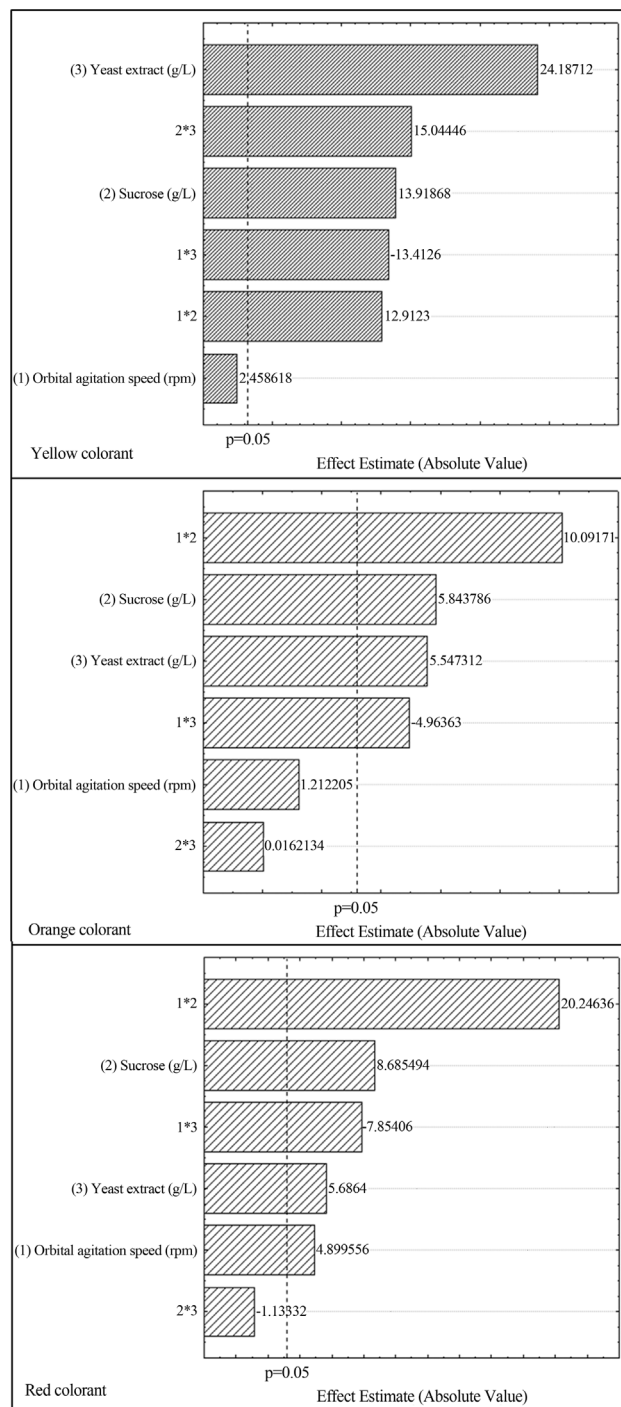
Figure 3 - Pareto chart for the effects of variables orbital stirring speed (1), pH (2), temperature (3), sucrose (4), yeast extract (5) and incubation time (6) on yellow (A), orange (B) and red (C) natural colorants production according to a 2^3 full factorial design.

ite design. The biomass concentration, sucrose consumption and pH for all runs every 48 h are presented in the Supporting Information (see Figure A.2).

Central composite factorial design to produce natural colorants

From the factorial designs showed above, sucrose and yeast extract concentrations were the most significant variables in the production of natural colorants by *P. purpurogenum* DPUA 1275. These variables were, therefore, analyzed by a central composite factorial design with 4 replications in the central point, with the purpose of optimizing the production of these metabolites. The results from these runs are showed at Table 5.

From Table 5, it can be observed that the yellow colorants production varied from 1.79 to 3.14 UA_{400nm}, the orange colorants varied from 1.20 to 2.50 UA_{470nm} and the red colorants, from 1.10 to 2.04 UA_{490nm}. For the yellow colorants, the highest result was obtained in run 8, in which the yeast extract and sucrose concentrations were 10.4 and 55 g/L, respectively. For the orange and red colorants, the highest production occurred in run 2 (50 g/L of sucrose and 10 g/L of yeast extract concentrations). The results obtained in this factorial design are higher than the ones obtained in the factorial designs above, which shows the conduction of a factorial design to an increase in the production of natural colorants. The central composite factorial design for the yellow, orange and red colorants did not show lack of fit (see Table A.4, Table A.5 and Table A.6 from Supporting Information).

Figure 4 shows the response surface indicating the simultaneous effects of the independent variables under the response variables yellow, orange and red colorants. It can be observed that, to all responses studies, there is a possibility to improve the colorants production by varying the conditions of the fermentative process. The surface response indicates that the best results would be possible if higher concentrations of yeast extract were used. Table 6 shows the probability values (p) for the yellow, orange and red colorants. From the *p* value, it is possible to determine which variables were significant to the process. Since in this work a confidence level of 95% was considered, the independent variables with p values lower than 0.05 are considered significant.

Through the results of the statistical analysis for the responses yellow, orange and red colorants, it was observed that the yeast extract in the linear and quadratic terms had a significant effect. Furthermore, the interaction between the linear terms of the variables also showed significant effects to the responses orange and red colorants. The biomass concentration, sucrose consumption and pH for all runs every 48 h are presented in the Supporting Information (see Figure A.3).

On the basis of the results of the complete regressions, the following models were determined to sucrose (x_1) and yeast extract (x_2) under the production of yellow (y_1), orange (y_2) and red (y_3) colorants, as described by the following equations:

$$y_1 = 2.10 - 0.05x_1 - 0.04x_1^2 + 0.31x_2 + 0.32x_2^2 - 0.28x_1x_2 \tag{3}$$
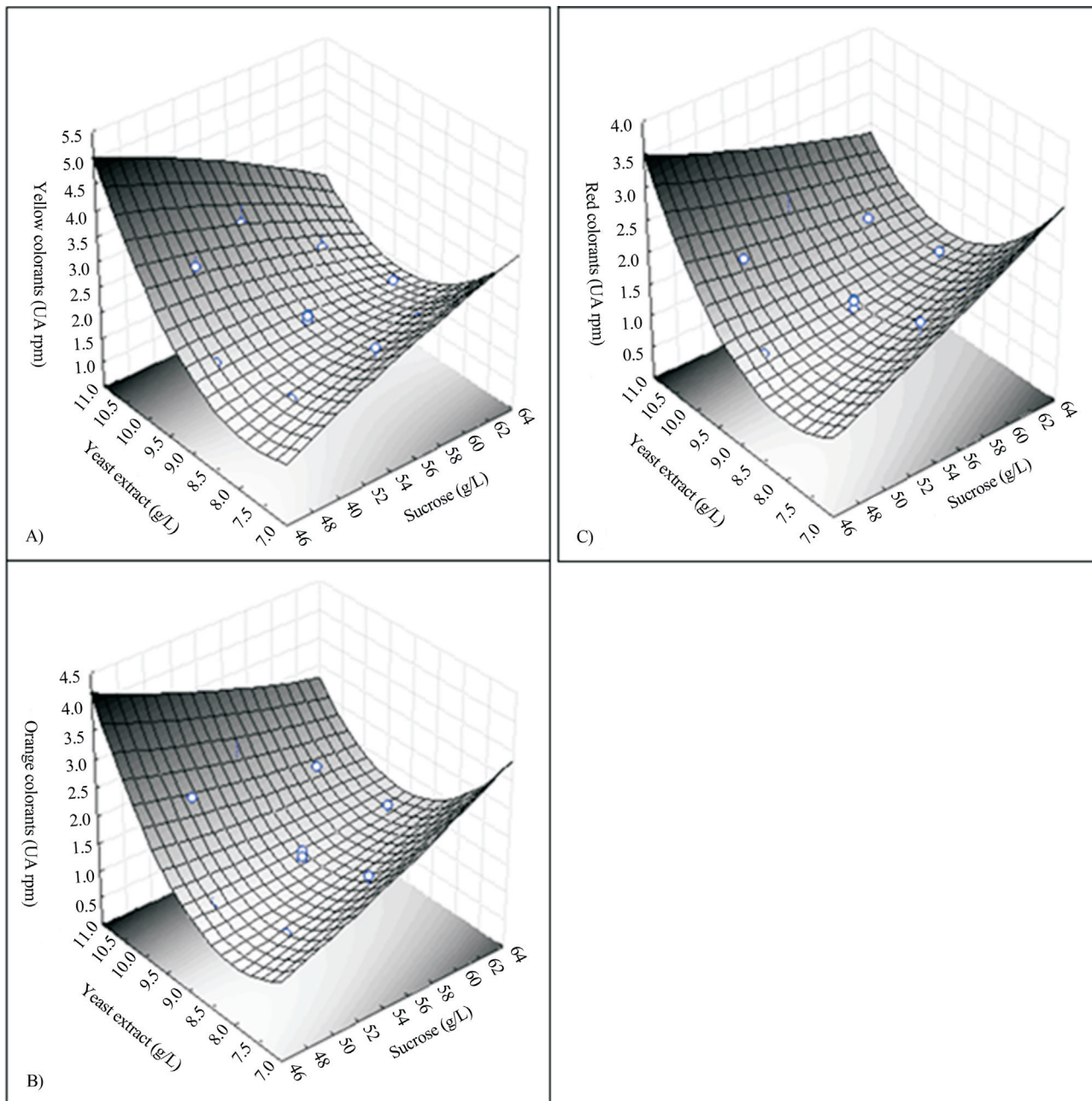
Figure 4 - Response surface showing the simultaneous effects of sucrose and yeast extract concentrations on the yellow (A), orange (B) and red (C) colorants by submerged culture of *P. purpurogenum* DPUA 1275.

$$y_2 = 1.44 + 0.07x_1 + 0.03x_1^2 + 0.27x_2 + 0.35x_2^2 - 0.23x_1x_2 \qquad (4)$$

$$y_3 = 1.33 + 0.09x_1 + 0.02x_1^2 + 0.20x_2 + 0.28x_2^2 - 0.20x_1x_2 \qquad (5)$$

So, according to convenience different conditions can be profitably exploited to maximize the production of yellow, orange and red colorants. However, the run 2 (50 g/L of sucrose and 10 g/L of yeast extract) can be selected as the best ones for the colorants production, being able to ensure , at the same time, satisfactory results of yellow, orange and

red colorants production. A comparison between the best results presented in the in the first experimental design (Table 3) and the ones achieved in the run 2 shows that an increase of 10, 33 and 23% was achieved to yellow, orange and red colorants, respectively.

According the results obtained through all designs, it is very important the control of the sucrose and yeast extract concentrations during the colorants process by *P. purpurogenum,* an alternative colorant producer. Moreover, from the economic/industrial point of view, the use of a cheap carbon source such as sucrose is interesting. Fur-

Table 5 - Matrix of 2^2 central composite factorial design to responses yellow (UA$_{400nm}$), orange (UA$_{470nm}$) and red colorants (UA$_{490nm}$).

Run	Sucrose (g/L)	Yeast Extract (g/L)	Yellow Colorants (UA$_{400nm}$)	Orange Colorants (UA$_{470nm}$)	Red Colorants (UA$_{490nm}$)
1	50	8	1.79	1.20	1.01
2	50	10	3.10	2.50	2.04
3	60	8	2.16	1.67	1.57
4	60	10	2.34	2.04	1.79
5	48	9	2.07	1.27	1.26
6	62	9	2.05	1.68	1.56
7	55	7.6	2.42	1.92	1.80
8	55	10.4	3.14	2.25	2.03
9 (C)	55	9	2.20	1.35	1.17
10 (C)	55	9	1.97	1.57	1.44
11 (C)	55	9	2.07	1.38	1.30
12 (C)	55	9	2.15	1.47	1.42

C - Central points.

Table 6 - p-values to the independent variables sucrose and yeast extract in the production of yellow, orange and red colorants by submerged culture of *P. purpurogenum* DPUA 1275.

Source	p value Yellow Colorants	p value Orange Colorants	p value Red Colorants
(1) Sucrose (g/L) (L)	0.243554	0.133879	0.129119
Sucrose (g/L) (Q)	0.374487	0.486239	0.698317
(2) Yeast extract (g/L) (L)	0.002839*	0.005012*	0.021401*
Yeast extract (g/L) (Q)	0.003586*	0.003417*	0.011214*
1L*2L	0.010056	0.019799*	0.047279*

*significant level $p < 0.05$; L = Linear terms; Q = Quadratic terms.

ther investigation to improve the colorants production in a bench-scale fermenter is a future effort aiming the scale-up for an industrial application.

Conclusions

The results obtained from the factorial designs, which analyzed the independent variables incubation period, orbital stirring speed, pH, temperature, sucrose and yeast extract concentrations under the production of yellow, orange and red colorants, suggested that sucrose and yeast extract concentrations are the most significant variables to the process of obtaining natural colorants by submerged culture of *P. purpurogenum* DPUA 1275, and the ideal concentrations of these subtract are values close to 50 and 10 g/L, respectively. Furthermore, the new strain showed potential to be used as a new source of natural colorants.

Acknowledgments

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