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Submerged Culture Conditions for the Production of Alternative Natural Colorants by a New Isolated *Penicillium purpurogenum* DPUA 1275[S]

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This work aims at investigating the production of yellow, orange, and red natural colorants in a submerged culture of *Penicillium purpurogenum* DPUA 1275. For this purpose, different experimental conditions evaluating the effect of incubation time, type and size of inoculum, and different carbon and nitrogen sources were performed. Furthermore, the growth kinetics were obtained in the conditions of 10^8 spores/ml and 5 mycelia agar discs during 360 h. These experiments showed that 5 mycelia agar discs and 336 h promoted the highest yellow (3.08 UA_{400nm}), orange (1.44 UA_{470nm}), and red (2.27 UA_{490nm}) colorants production. Moreover, sucrose and yeast extract were the most suitable carbon and nitrogen sources for natural colorants production. Thus, the present study shows a new source of natural colorants, which can be used as an alternative to others available in the market after toxicological studies.

Key words: Natural colorants, filamentous fungi, submerged culture, production

Synthetic and natural colorants are used extensively in the food, cosmetic, and pharmaceutical industries [16]. However, natural colorants have recently gained popularity over synthetic coloring agents, which can be potential carcinogens [19]. In this way, there is a growing demand for eco-friendly/non-toxic colorants, specifically for health sensitive applications, such as the coloration of food and dyeing of children's fabrics and leather garments [42].

Natural colorants can be obtained from sources like plants [3, 6, 45], insects [26, 41], and microorganisms [5, 28, 43, 46]. In the industrial-scale production of natural colorants, the microorganisms have the advantage of versatility and productivity over higher forms of life. Microbial colorants have advantages over artificial and inorganic colors: they produce metabolites from fermentation and their genes are easy to manipulate [43].

The interest in colorants production by fungi is increasing owing to the capacity of these microorganisms to produce colorants with different chemical structures and with a color range, which may add new or additional hues to the color palette of the existing colorants arising from contemporary sources [24]. Fungi colorants are produced as secondary metabolites of known or unknown function. Polyketide colorants from commercially available *Monascus* have been used as food colorants for hundreds of years in the Orient. However, *Monascus* species have been also reported to co-produce the mycotoxin citrinin and other potential toxic metabolites [25]. Therefore, it is of interest to search for alternative colorant-producing organisms [16].

Penicillium strains have been recently reported as a potential producer of colorants to be used in the food industry because they do not produce citrinin. The colorants produced by these microorganisms are homologs of *Monascus* colorants with similar chromophores [29]. Mendez *et al*. [29] mention that *P. purpurogenum* can produce colorants in both solid and liquid media. Furthermore, in studies carried out by Teixeira *et al*. [39], *P. purpurogenum* DPUA 1275 showed potential to produce natural colorants with significant antimicrobial activities and a total absence of toxicity against *Artemia salina*.

Studies to optimize the production and the microorganism characteristics during the process are required. Regarding filamentous fungi, a variety of metabolites such as antibiotics, enzymes, and pigments are produced industrially using submerged culture [13]. This process has the advantages of the facility to control parameters like aeration,

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agitation, and temperature. However, the production of colorants by fungi filamentous in submerged culture can be affected by several environmental factors [44]. Moreover, the carbon and nitrogen sources are two important factors affecting the cell's growth and the product's development of microorganisms [44]. It is known that the fungus culture in liquid broth requires carbohydrates (such as sucrose), nitrogen (corn extract, yeast autolysate or extract), zinc, and magnesium sulfate [9].

P. purpurogenum DPUA 1275 was studied to produce yellow, orange, and red extracellular colorants during culture on an orbital shaker. Initially, we evaluated the following parameters: inoculum size, inoculation methodology, and the influence of time in the production. The second part was a study of the effects of different nitrogen and carbon sources on the production of colorants.

Materials and Methods

Chemicals

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

Microorganism Reactivation and Authentication

Penicillium purpurogenum DPUA 1275 was provided by the Culture Collection of Federal University of Amazon, AM, Brazil. The method described by Pitt [33] was used to isolate this strain directly from soil samples. The stock culture preserved in distilled water was reactivated in Czapek Yeast Extract Agar (CYA) medium at 25°C, for 7 days. From the reactivated culture, a spore was transferred to Water-Agar 1.8% (w/v) at 25°C for 7 days, to produce a monosporic culture.

The authentication of species was carried out by transferring a spore from the monosporic culture to a Sabouraud Agar (SAB) medium. *P. purpurogenum* authentication was performed throughout the morphologic characteristics, using the identification key proposed by Rapper and Fennel [35], Pitt [33], and Samson *et al*. [37]. The macroscopic colony was observed in Water-Agar, CYA, and Yeast extract sucrose agar (YES) media. After the authentication, a spore from the monosporic culture was transferred to tubes containing CYA medium and kept at 25°C for 7 days. After this period, the culture was maintained at 4°C.

Media Composition

CYA medium was used as the inoculum and production medium in the initial experiments. The CYA medium had the following composition (g/l in deionized water): K_2HPO_4 (1), yeast extract (5), sucrose (30), agar (15), and 10 ml/l of concentrated Czapeck. Concentrated Czapeck, which is a salts solution, had the following composition (g/100 ml of deionized water): $NaNO_3$ (30), KCl (5), $MgSO_4.7H_2O$ (5), $FeSO_4.7H_2O$ (0.1) [33].

For the study of the best carbon and nitrogen sources, CYA medium was considered the standard one. The carbon sources evaluated were sucrose, glucose, fructose, maltose, soluble starch, and control (without addition). In this condition, the nitrogen source was yeast extract. The nitrogen sources evaluated were yeast extract, malt extract, ammonium sulfate, peptone, tryptone, and control (without addition). Sucrose was the carbon source.

To calculate the carbon:nitrogen (C:N) ratio, the amount of carbon and nitrogen in each nitrogen source was determined by elemental analysis at Central Analytical of Chemistry Institute from São Paulo University (São Paulo, Brazil). The C:N ratio of 15 was chosen because it was the ratio found in the CYA medium. In this way, the amount of each nitrogen source was calculated considering the required C:N ratio, as well as its composition on carbon and nitrogen. As in the control condition to evaluate the nitrogen source, for any additional nutrient, except sucrose, that was added, there was more carbon available in the middle than nitrogen, which results in a C:N ratio of 28.

All media were autoclaved at 121°C for 15 min.

Submerged Culture Conditions

For the first set of experiments, two methodologies of inoculation (spores suspension and mycelia agar discs) for the influence of incubation time and the inoculum size in the production of extracellular colorants by *P. purpurogenum* were evaluated. For the spore suspension methodology, the microorganism was grown on CYA slant for 7 days, at 25°C. After the incubation, the spores were scraped off with a spatula and the spore concentration was adjusted to 10^6, 10^7, and 10^8 spores/ml with a Neubauer camera [22] and transferred to Erlenmeyer flasks.

For the mycelia agar discs methodology from a stock culture grown on a CYA medium plate during 7 days at 25°C, 5, 10, or 15 mycelia agar discs were punched out with a sterilized self-designed cutter (8 mm diameter) and transferred to Erlenmeyer flasks. These experiments were carried out in 125 ml Erlenmeyer flasks with 25 ml of liquid CYA medium, pH 6.5, in a rotary shaker incubator at 30°C, 150 rpm. Samples were withdrawn after 120, 216, and 288 h, in order to determine the production of colorants, the pH, and the biomass.

Growth kinetics was determined by both inoculation methodologies. However, the inoculum size consisted of 5 mycelia agar discs or 10^8 spores/ml. The experiments were carried out at the same conditions described above during 360 h. Samples were withdrawn every 24 h.

Secondly, experiments evaluating the best carbon and nitrogen sources were performed with 5 mycelia agar discs of *P. purpurogenum* in 125 ml Erlenmeyer flasks containing 25 ml of liquid medium, under the following specific conditions: 150 rpm, 30°C, 336 h.

Analytical Methods

The fermented broth was filtrated (Whatman No. 1 filter paper; Whatman, Kent, 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*. [8] and the pH was measured using a pH meter.

The concentration of extracellular colorants was estimated by measuring the absorbance of filtrates. The wavelength of each colorant was scanned at 350–600 nm. The supernatant was read at 400, 470, and 490 nm (a wavelength that represents the maximum absorption for yellow, orange, and red colorants, respectively), using a spectrophotometer and considering the dilution factor of each

sample. The results were expressed in terms of Units of Absorbance (UA). The maximum absorption obtained for each colorant is in agreement with Johns and Stuart [17].

Statistical Analyses

All the analyses and experiments were performed in triplicate, and the results expressed as mean values.

RESULTS AND DISCUSSION

Monosporic Culture and Authentication of *P. purpurogenum* DPUA 1275

The monosporic culture aims at working with a fungal strain, since this technique provides not only a pure colony from a spore, but also a reduction in the morphophysiological, biochemical, and genetic microorganism variation when it is submitted to successive cultures [12]. As the traditional and commonly used method for identification of *Penicillium* species is still the visualization of morphological characteristic of the colonies (texture, color, and colony diameter in a specific medium) and reproductive structures (types and sizes of conidia and conidiophore) [33, 38], this method was used.

P. purpurogenum DPUA 1275 showed phenotypic characteristics similar to the ones described by Pitt [33]. From the macroscopic colony obtained in Water-Agar, CYA, and YES, not only the color was observed, but also the texture of the mycelium in each one of these media (Supplementary Fig. S1). In CYA medium, the diffusion of a reddish color was observed, indicating the production of colorants by the microorganism. In this medium, the colony diameter was measured (29 mm) and its value was the one expected as reported in the literature [7]. In the Water-Agar medium, the color obtained in both surface and back of the colony was greenish, without colorants production. This result was expected, since the Water-Agar medium does not have the essential components to stimulate the production of these metabolites by the microorganism.

At the end of the culture on slides and staining of structures, the following structures were observed: conidiophore or stipe, metula and sterigmata arranged in a brush shape, and conidial chain. With these data, it was possible to make the microorganism authentication. Thus, the monosporic culture provided to obtain a pure culture, which can suffer less morphological and physiological changes.

Effects of Incubation Time, and Type and Size of Inoculum on Colorants Production

In the literature, a range of methods to inoculate filamentous fungi can be found. However, the most used are the spores suspension and mycelia agar discs. In this way, both methods were used to evaluate the extracellular colorants production by *P. purpurogenum* DPUA 1275 in submerged culture at different incubation times (120, 216, and 288 h) (Table 1).

Considering the experiments performed with the spore suspension, the highest production of yellow, orange, and red colorants was achieved with 10^8 spores/ml but at

Table 1. Colorants production at different inoculation methods (spore suspension and agar mycelia discs) and incubation time by *P. purpurogenum* DPUA 1275.

Inoculation method		Time (h)	Yellow colorants (UA_{400nm})	Orange colorants (UA_{470nm})	Red colorants (UA_{490nm})
Spore suspension (spores/ml)	10^6	120	0.88 ± 0.01	0.08 ± 0.02	0.02 ± 0.01
		216	0.67 ± 0.02	0.29 ± 0.03	0.17 ± 0.01
		288	0.66 ± 0.07	0.22 ± 0.04	0.22 ± 0.03
	10^7	120	0.90 ± 0.06	0.08 ± 0.01	0.04 ± 0.01
		216	0.50 ± 0.01	0.21 ± 0.06	0.17 ± 0.03
		288	0.67 ± 0.01	0.28 ± 0.01	0.21 ± 0.01
	10^8	120	1.15 ± 0.02	0.06 ± 0.01	0.09 ± 0.01
		216	0.71 ± 0.03	0.29 ± 0.02	0.22 ± 0.01
		288	0.70 ± 0.03	0.42 ± 0.08	0.34 ± 0.01
Agar mycelial discs	5	120	0.79 ± 0.07	0.27 ± 0.07	0.17 ± 0.06
		216	1.79 ± 0.02	0.88 ± 007	1.13 ± 0.04
		288	1.38 ± 0.08	1.61 ± 0.02	0.99 ± 0.02
	10	120	0.55 ± 0.05	0.18 ± 0.02	0.65 ± 0.04
		216	1.55 ± 0.03	0.69 ± 0.04	0.50 ± 0.05
		288	1.48 ± 0.03	1.50 ± 0.04	0.86 ± 0.02
	15	120	0.89 ± 0.04	0.41 ± 0.02	0.75 ± 0.06
		216	0.91 ± 0.02	0.73 ± 0.03	0.94 ± 0.02
		288	1.42 ± 0.04	1.24 ± 0.07	0.78 ± 0.07

The error represents a 95% confidence limit for the measurements.

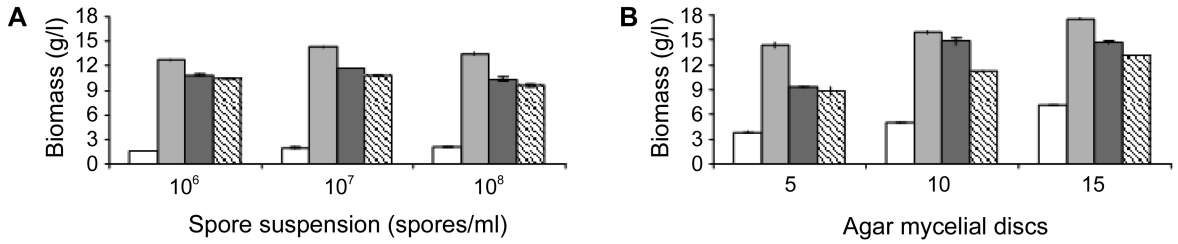

Fig. 1. Biomass at different incubation times [0 (white bar), 120 (light gray bar), 216 (dark gray bar), and 288 (merged bar) h] during submerged culture of *P. purpurogenum* DPUA 1275 with agar mycelial discs (**A**) and spore suspension (**B**) as the inoculation method. The error bars represent a 95% confidence limit for the measurements.

different incubation times. For the yellow colorants, 120 h of incubation promoted an absorbance of 1.15 UA_{400nm}, while for the orange and red colorants, a higher production was achieved at 288 h of incubation.

Regarding the experiments carried out with agar mycelia discs, the highest yellow colorants production occurred at 5 agar mycelia discs after 216 h of incubation. On the other hand, for the same incubation time but with 10 and 15 agar mycelia discs and after 216 h, the yellow colorants production decreased 3% and 20%, respectively, as compared with 5 agar mycelia discs. The same conditions obtained for the yellow colorants promoted the highest red colorants production. However, the orange colorant was produced in a higher amount with the same number of mycelia agar discs, but after 288 h of incubation.

During a culture process, several metabolites can be produced as a result of the metabolic pathways. The results above indicate that yellow colorants are the first metabolites to be produced during the submerged culture of *P. purpurogenum.* Moreover, orange and red colorants can be produced in more complex metabolic pathways. Furthermore, during the metabolic process, the yellow colorants can have their chemical structure modified, which can generate orange and red colorants, as occurs with the colorants produced by *Monascus*. In addition to that, the loss of yellow colorants during the process may happen owing to their photochemical degradation, as happens with pigments derived from contemporany sources that are sensitive to heat, light, and oxygen [23]. According to Pastrana *et al.* [31], the pigments produced by *Monascus* spp. are degraded in a few days in aqueous solutions.

Figs. 1 and 2 show the biomass and pH behavior from both methodologies of inoculation.

Biomass concentration changed in the course of the study in both methodologies. Regarding the spore suspension (Fig. 1B), the highest biomass concentration was obtained after 120 h of incubation, regardless of the initial spore concentration. At this time, the biomass concentration was 14.31 g/l per 10^7 spores/ml, a value 7-fold higher than the initial one (1.97 g/l).

When agar mycelia discs (Fig. 1A) were used, 120 h of incubation promoted the highest biomass concentration as well. However, in this case, a higher number of agar mycelia discs promoted a higher biomass concentration, which was 14.38, 15.92, and 17.54 g/l for 5, 10, and 15 agar mycelia discs, respectively. Thus, for 216 and 288 h of incubation, the cell concentrations were lower than the one obtained after 120 h in all conditions evaluated.

For both inoculation methods, the initial pH of the culture medium was 6.50 and its behavior through the submerged culture in all conditions studies was similar, except when 15 agar mycelia discs were used. In the latter condition, in which the pH increased through the bioprocess after 120 h of culture, its value was 7.70, which remained constant in the other evaluated times. The highest agar mycelia discs generated the highest biomass concentration

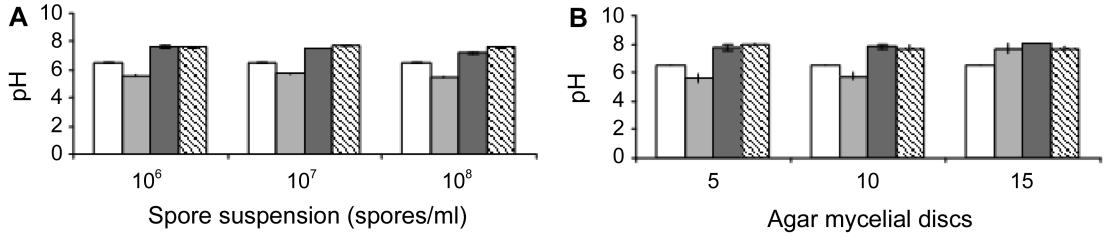

Fig. 2. pH at different incubation times [0 (white bar), 120 (light gray bar), 216 (dark gray bar), and 288 (merged bar) hours] during submerged culture of *P. purpurogenum* DPUA 1275 with agar mycelial discs (**A**) and spore suspension (**B**) as the inoculation method. The error bars represent a 95% confidence limit for the measurements.

(17.54 g/l), which may have promoted a difficulty for the microorganism to be metabolically developed, as occurred in the other evaluated conditions.

For the additional 120 h of incubation experiments, the pH value decreased, ranging from 5.51 to 5.75. Thereafter, the pH increased to values of 7.50 and 8.00 after 216 and 288 h of bioprocess. The changes observed to pH at different incubation times can be related with substances produced during the bioprocess due to the microorganism metabolism. In the beginning, substances with acid characteristics can be produced, and they are consumed during the microorganism metabolism to generate the target molecules. A similar behavior was observed by Teng and Feldheim [40] in fermentation with *Monascus purpureus* during the production of red colorants.

From the results above, regardless of the colorants evaluated, the production was more favorable with the use of agar mycelia discs as inoculation method. This fact can be explained by the physiology of the microorganism in both conditions. To extend the discussion about this hypothesis, experiments regarding the growth curve were performed.

Growth Curve

The growth curve of *P. purpurogenum* DPUA 1275 was carried out through spore suspension (10^8 spores/ml) and agar mycelia discs (5) as inoculation method. Fig. 3 shows the growth curve and sucrose consumption.

From Fig. 3, it can be observed that when the microorganism is inoculated with spore suspension (filled symbol), a "lag" phase occurs in the first 24 h of incubation. After the microorganism's growth, the exponential phase was reached, in which the cells duplicate, followed by the stationary and death phases. Initially, the biomass concentration was 2.41 g/l, and at the end of the study, its concentration was 10.07 g/l. The highest biomass concentration (14.17 g/l) was attained after 144 h of incubation.

A different behavior occurred with the spore suspension; the inoculation with agar mycelia discs (empty symbols) promoted a grown curve without the "lag" phase and quickly reached the exponential phase. This situation happened as a result of the presence of spores, enzymes, and other metabolites produced during the inoculum period.

As for the spore suspension, a solution prepared by scraping, the microorganism arrives in the culture medium in other physiologic state (as compared with agar mycelia discs), and it has to readapt to this medium, justifying the lag phase observed in Fig. 2.

Furthermore, the difference observed among the results obtained with both inoculation methods can be a result of a 20% slower biomass in the spore suspension compared with the initial biomass in the methodology of agar mycelia discs. In this way, the growth curve with agar mycelia discs begins with an exponential phase, followed by the stationary and death phases. Initially, the biomass concentration was 3.07 g/l, and then it reached 15.48 g/l after 72 h. After 360 h, it decreased to 7.81 g/l.

The sucrose was consumed in the same way with both methodologies. After 120 h of fermentation, around 97% of this carbon source had been consumed, reaching the exhaustion of this source. Fig. 4 shows the pH changes throughout the 360 h of incubation.

The initial pH was 6.5 in all conditions, and as occurred before, this parameter decreased in the beginning of the bioprocess. The pH value kept at around 4.5 from 48 until 120 h of incubation for all the studied conditions. After this time, the pH value increased, reaching pH values close to 7.50, up to the end of the submerged culture. As the orange and red colorants production occurred more often in the end of the bioprocess, these metabolites could have been formed and/or excreted more under alkaline conditions in both inoculation methods evaluated.

Saha *et al*. [36] studied the production of phenazine pigments from *Pseudomonas aeruginosa*. The highest biomass concentration was achieved after 6 h of incubation (60 g/l) and the production of pigments increased while the biomass concentration was decreasing.

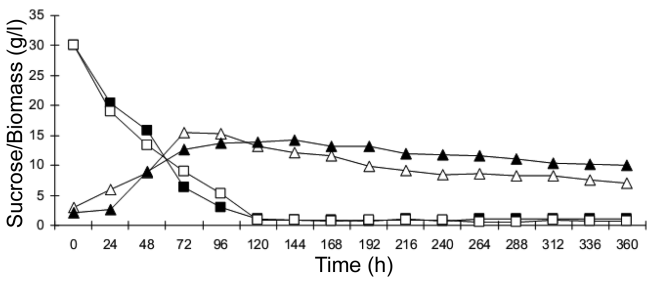

Fig. 3. Growth curve (square), sucrose concentration (triangle), and pH behavior (circle) during submerged culture of *P. purpurogenum* DPUA 1275 at 30°C for 360 h using as inoculation method a spore suspension (△) and agar mycelial discs (■).

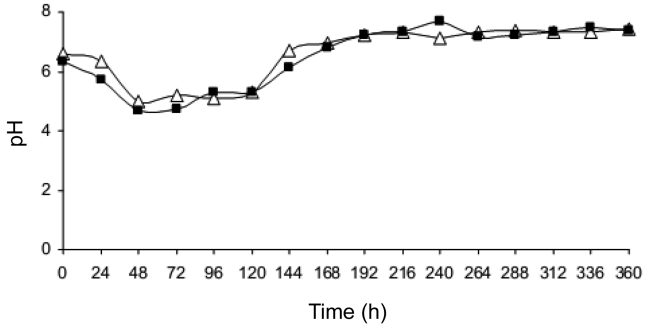

Fig. 4. pH changes during submerged culture of *P. purpurogenum* DPUA 1275 at 30°C for 360 h using as inoculation method a spore suspension (△) and agar mycelial discs (■).

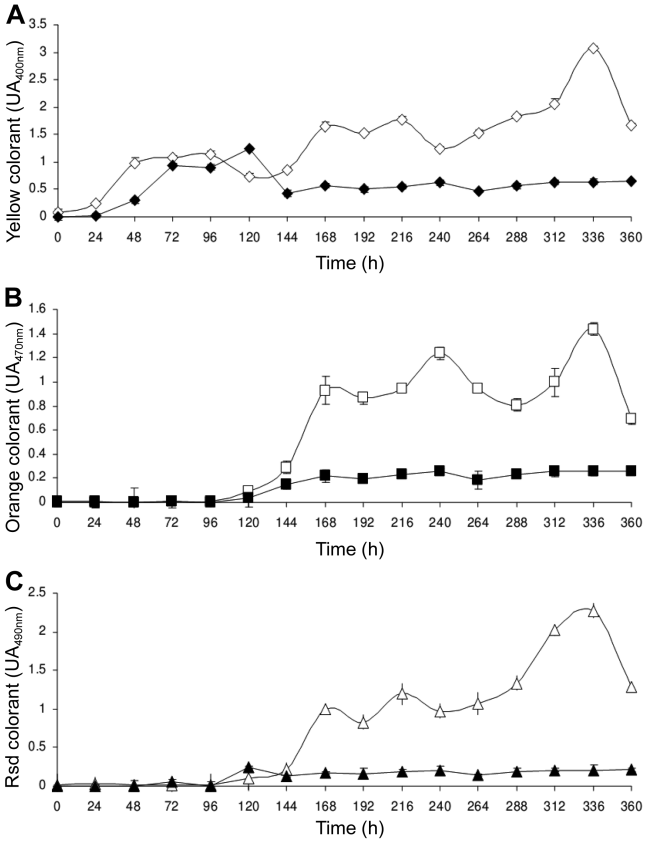

Fig. 5. Yellow (◇ - **A**), orange (□ - **B**), and red (△ - **C**) colorants production by *P. purpurogenum* DPUA 1275 using as inoculatin method a spore suspension (filled symbol) and agar mycelial discs (empty symbol) in submerged fermentation at 30°C for 360 h.

Fig. 5 shows the red colorants production throughout the 360 h of incubation, and also shows that larger amounts of yellow colorant were produced, as compared with the amount of the orange and red ones produced during the submerged culture. Moreover, the production was more pronounced when agar mycelia discs were used. The highest yellow colorants production occurred with agar mycelium disc and 336 h of fermentation, 3.08 UA_{400nm}. This value was 146% higher than the one obtained in the best conditions and with a spores suspension (120 h).

The orange and red colorants production increased after 168 h of incubation, which means that these metabolites have characteristics to be secondary ones, because 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. The maximum value of orange and red colorants was 1.44 UA_{470nm} and 2.27 UA_{490nm}, respectively, after 336 h and with 5 agar mycelia discs. These results show that both orange and red colorants can be produced in the decline and/or death phase and meet the statement in the literature, which says that colorants in general are secondary metabolites [33, 34]. As supporting information, (Supplementary Fig. S2) shows the colorants produced with 5 agar mycelia discs, after 336 h.

Study of Different Carbon and Nitrogen Sources

The choice of suitable nutrients to produce the product of interest is related with the metabolic activity developed by the microorganism. In this way, the knowledge about the nutrients required by the microorganism is very important. Thus, it is necessary to supplement the culture medium or control the components that can allow an efficient and fast conversion of carbon source on a product with the highest possible productivity. Furthermore, organisms require nitrogen for the synthesis of compounds such as proteins, vitamins, and nucleic acids. Nitrates are mainly used as the nitrogen source in culture media [7]. In this work, the C:N ratio, independently of carbon or nitrogen sources, was kept as 15, since the objective was to evaluate the source and not the C:N ratio in the colorants production.

Table 2 shows the carbon sources evaluated and the results of the colorants production, biomass concentration, and pH.

Fungi are heterotrophic and require an exogenous carbon source. Some fungi use complex compounds containing carbon, but others are more selective in their requirements.

Table 2. Biomass concentration, final pH, and yellow, orange, and red colorants production at different carbon sources in submerged culture of *P. purpurogenum* DPUA 1275.

Carbon souces	Biomass (g/l)	Final pH	Colorants		
			Yellow (UA_{400nm})	Orange (UA_{470nm})	Red (UA_{490nm})
Control	2.29 ± 0.26	7.57 ± 0.06	0.69 ± 0.24	0.16 ± 0.03	0.02 ± 0.00
Glucose	11.94 ± 0.15	7.48 ± 0.00	0.95 ± 0.17	0.50 ± 0.07	0.69 ± 0.02
Fructose	11.98 ± 0.09	7.59 ± 0.08	0.97 ± 0.08	0.28 ± 0.06	0.50 ± 0.01
Sucrose	9.70 ± 0.08	7.41 ± 0.09	1.65 ± 0.06	0.83 ± 0.05	0.93 ± 0.05
Maltose	10.07 ± 0.04	7.65 ± 0.05	0.88 ± 0.15	0.22 ± 0.04	0.52 ± 0.04
Starch	9.27 ± 0.25	7.31 ± 0.09	1.77 ± 0.07	0.84 ± 0.03	1.01 ± 0.06

The error represents a 95% confidence limit for the measurements.

Table 3. Biomass concentration, final pH and yellow, orange and red colorants production at different nitrogen sources in submerged culture of *P. purpurogenum* DPUA 1275.

Nitrogen souces	Biomass (g/l)	Final pH	Colorants		
			Yellow (UA_{400nm})	Orange (UA_{470nm})	Red (UA_{490nm})
Control	8.02 ± 0.60	4.15 ± 0.12	0.36 ± 0.07	0.14 ± 0.05	0.15 ± 0.05
Malt extract	14.61 ± 0.36	6.70 ± 0.04	1.02 ± 0.23	0.53 ± 0.08	0.48 ± 0.06
Ammonium sulfate	11.51 ± 0.86	3.10 ± 0.06	0.13 ± 0.09	0.04 ± 0.00	0.07 ± 0.01
Peptone	9.03 ± 0.42	7.48 ± 0.09	1.13 ± 0.09	0.54 ± 0.04	0.44 ± 0.03
Yeast extract	12.12 ± 0.26	7.64 ± 0.15	2.01 ± 0.10	0.94 ± 0.14	0.71 ± 0.09
Tryptone	13.71 ± 0.47	7.62 ± 0.03	0.46 ± 0.16	0.19 ± 0.01	0.18 ± 0.09

The error represents a 95% confidence limit for the measurements.

In this way, many compounds can be used as a carbon source to fungi [12]. Monosaccharides (glucose and fructose), disaccharides (sucrose and maltose), and starch were analyzed. From all the carbon sources evaluated, regardless the colorants, the best results were obtained with sucrose and starch.

It was expected that the addition of glucose and/or fructose in the fermentation medium could have promoted a higher production of colorants. However, it did not happen. A possible catabolic repression obtained with the monosaccharides added directly in the medium may be responsible for the results obtained [7].

Similar results were obtained with maltose, a disaccharide composed of glucose. The difference obtained between sucrose and maltose may have been from the easy hydrolysis of sucrose, which has no free anomeric carbon atoms, leading to an easy metabolization of the carbohydrate, if compared against maltose [20].

Some works report that the direct addition of glucose in the fermentation broth can affect the production of enzymes. This kind of relation occurs not only in bacteria, but also in fungi like *A. níger* [21], *P. expansum* [32], and *P. purpurogenum* in the production of β-glucosidase [7].

In the present study, it can be observed that *P. purpurogenum* has the capability to adapt and assimilate a varied range of carbon sources. However, these carbon sources were not the ones that promote the production of colorants: the carbon sources that promoted the highest growth were glucose and fructose. The control condition produced 80% less biomass than the one obtained with fructose. The preference for fructose and glucose, which are isomers compounds, compared with the other sources can be related to the fact that these sugars are easily metabolized during cell respiration [14]. The final pH in all conditions was close to 7.0 (Table 2).

Cho *et al*. [5] studied the influence of 11 carbon sources in the production of red colorants by *Paecilomyces sinclairii*. The results showed that glucose, fructose, mannose, sucrose, and maltose were favorable for the microorganism's growth. Sucrose and starch were the most efficient sources in the production of red colorants. In a later work [40], the same authors studied the influence of different carbon sources and the aeration rate, which showed that sucrose was the best source for mycelia growth whereas starch was the best one for the production of red pigments.

Gunasekaran and Poorniammal [15] studied the influence of 11 carbon sources in the production of red colorants by *Penicillium* sp. Starch promoted the highest pigment production, followed by maltose and glucose.

From the results above and others found in the literature, the carbon source consumption seems to be strain-dependent, since in some reports from the literature, both were observed: the growth and the production of colorants in medium containing sucrose as the main carbon source. As the best results were obtained with sucrose and starch, the first one was chosen to be used in the subsequent experiments, since it is a source less expensive than starch. Table 3 shows the results of varying the nitrogen source.

The nitrogen source that showed the highest colorants production was yeast extract, followed by malt extract. Yeast extract may have promoted the best result because it is an excellent source of amino acids and vitamins [1], which are available in a way that the microorganism can perform a metabolization more efficiently as compared with the other sources studied.

Ammonium sulfate, as a main nitrogen source, strongly inhibited the production of colorants. The direct presence of this subtract in the culture medium may have caused the production of toxic substances and increased the production of acid compounds, which resulted in a medium with an acidic pH (3.10). Regarding the microorganism growth, a favorable biomass (11.51 g/l) was obtained with this nitrogen source, probably because the microorganism assimilated it more easily than the other ammonia sources [34]. However, the production of metabolites depends not only on the assimilation of the source, but also on the metabolism related to a favorable metabolic pathway.

The initial pH in all media was 6.5 and its value ranged, according to the nitrogen source evaluated. As mentioned

before, the ammonium sulfate promoted the lowest pH value (3.10). The absence of a main nitrogen source (control) promoted a pH of 4.15, which means that the microorganism did not have conditions to synthetize the same metabolites when there was a main nitrogen source in the medium, excluding the experiment with ammonium sulfate. As for the other nitrogen sources, the pH varied from 6.0 to 7.0. Although yeast extract promoted the lowest biomass concentration, this nitrogen source promoted the highest absorbance to all colorants, as can be observed in Table 3.

It is interesting to note that without the addition of a main nitrogen source, the cell growth was similar to the medium with yeast extract. In this way, the microorganism grew up in a medium with a low amount of nitrogen, but the production of colorants was hindered, since some metabolic pathway did not occur owing to the absence of nitrogen compounds. As mentioned by Babitha *et al*. [2], the nitrogen source is an important factor for the growth and the production of pigments.

Cho *et al*. [5] studied the influence of different nitrogen sources in the growth and the production of pigments by *Paecilomyces sinclairii*. Among the nitrogen sources evaluated, soy peptone and malt extract strongly inhibited the synthesis of the pigments. Yeast extract promoted intermediate values and the highest yield in terms of pigment production occurred with meat peptone.

Gunasekaran and Poorniammal [15] evaluated different nitrogen sources in the pigments production by *Penicillium* sp. Among the sources studied, peptone and yeast extract+peptone, tryptone, and monosodium glutamate had a positive effect on the production of pigment, whereas soy peptone, meat extract, and potassium nitrate inhibited the synthesis of the pigments. These results are in accordance with the other ones reported in this work, in which the best production occurred with yeast extract, followed by peptone and tryptone.

Despite some other reports, which state that peptone is more favorable than other nitrogen sources in the production of colorants for a wide range of fungi [4, 5, 11, 18], in this work, the best nitrogen source was yeast extract.

In conclusion, the present work describes the conditions for the production of colorants by *P. purpurogenum* DPUA 1275, a new producer of natural colorants that may represent a safer alternative for the use of synthetic colorants in submerged cultures. The conditions established in this microorganism for yellow, orange, and red colorants are the following: 5 agar mycelia discs, 336 h of fermentation, and sucrose and yeast extract as carbon and nitrogen source, respectively. In this way, our results indicate an economically advantageous way for the production of yellow, orange, and red colorants for the pharmaceutical and food industries, after toxicologic tests.

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