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# Red pigment production by *Penicillium purpurogenum* GH2 is influenced by pH and temperature*

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**Abstract:** The combined effects of pH and temperature on red pigment production and fungal morphology were evaluated in a submerged culture of *Penicillium purpurogenum* GH2, using Czapek-Dox media with D-xylose as a carbon source. An experimental design with a factorial fix was used: three pH values (5, 7, and 9) and two temperature levels (24 and 34 °C) were evaluated. The highest production of red pigment (2.46 g/L) was reached with a pH value of 5 and a temperature of 24 °C. Biomass and red pigment production were not directly associated. This study demonstrates that *P. purpurogenum* GH2 produces a pigment of potential interest to the food industry. It also shows the feasibility of producing and obtaining natural water-soluble pigments for potential use in food industries. A strong combined effect ($p$<0.05) of pH and temperature was associated with maximal red pigment production (2.46 g/L).

**Key words:** *Penicillium purpurogenum*, Food colorants, Combined effects, Water-soluble pigment
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## 1 Introduction

Pigments are compounds with characteristics of importance to many industries. In the food industry they are used as additives, color intensifiers, antioxidants, etc. Pigments come in a wide variety of colors and some are water-soluble. For these reasons, many of these compounds have been produced, isolated, and characterized (Durán *et al*., 2002). The scrutiny and negative perceptions of synthetic food pigments by the modern consumer have given rise to a strong interest in natural coloring alternatives (Dufossé, 2006). Many companies have decided to utilize natural pigments mainly from plant and animal sources. However, these additives have numerous drawbacks such as instability and low water solubility, and are often not available throughout the year (Gunasekaran and Poorniammal, 2008). Microbial pigments are of industrial interest because they are often more stable and soluble than those from plant or animal sources (Gunasekaran and Poorniammal, 2008). Microorganisms can grow rapidly, which can lead to high productivity, and can produce a product throughout the year (Jiang *et al*., 2005). Special attention has been focused on the strains belonging to the *Monascus* genus of filamentous fungi. Some authors refer to these fungi as potent producers of natural pigments (Blanc *et al.*, 1994; Tseng *et al.*, 2000; Carvalho *et al.*, 2003). However, there are other microorganisms which have the ability to produce pigments in high quantities, such as those belonging to the genus *Paecilomyces* (Cho *et al*., 2002), producing red, yellow, and violet pigments in quantities of up to

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4.73 g/L. Microorganisms belonging to the genera *Aspergillus* and *Penicillium* have also been studied as potential producers of natural pigments (Engstrom *et al.*, 1982; Suhr *et al.*, 2002; Dufossé, 2006; Méndez-Zavala *et al.*, 2007; Hernández-Rivera *et al.*, 2008).

The production of *Monascus*-like pigments from *Penicillium* strains has recently been reported. These pigments have a potential use in the food industry because they are not associated with citrinin production. They are homologues of pigments of *Monascus* which have similar chromophore polyketides (Mapari *et al*., 2008a) and of fungal strains of the species *Epicoccum nigrum* that produce yellow pigments (Mapari *et al.*, 2008b).

We previously reported the isolation and characterization of three *Penicillium* sp. strains capable of producing red pigments (Espinoza-Hernández *et al.*, 2004). These xerophilic strains were isolated from the Mexican semi-desert, and they produced pigments at 24 °C and at an initial pH of 10 in potato dextrose. The fungi were also able to produce pigments in submerged fermentation using malt extract as a nutrient source. However, the effect of culture parameters on pigment production was unknown.

Among the most important variables affecting biotechnological processes, pH and temperature are environmental conditions with a strong effect on the biosynthesis of metabolites such as pigments. Thus, it is very important to control them in industrial bioprocesses. Metabolically, the effects of pH and temperature are associated with changes in the activities of proteins, so the culture conditions can control some activities such as cellular growth, production of primary and secondary metabolites, fermentation, and the oxidation processes of the cell. In this paper we present a study of the effects of temperature and pH on the production of red pigment by *Penicillium purpurogenum* GH2 in submerged culture.

## 2 Materials and methods

### 2.1 Microorganism and culture media

*P. purpurogenum* GH2 from the DIA-UAdeC collection was used in this study. The strain was conserved in a spore solution at −20 °C, and was inoculated into a 250-ml Erlenmeyer flask containing 30 ml of malt extract agar prepared using deionized water (Milli-Q) and incubated at 30 °C for 5–7 d.

### 2.2 Culture conditions

A concentration of $2\times10^7$ spores/ml was inoculated into 250-ml Erlenmeyer flasks with 40 ml of Czapek-Dox modified broth (Hernández-Rivera *et al*., 2008) containing the following: 15 g/L D-xylose, 3 g/L $NaNO_3$, 1 g/L $K_2HPO_4$, 0.5 g/L $MgSO_4{\cdot}7H_2O$, 0.5 g/L KCl, and 0.1 g/L $FeSO_4{\cdot}7H_2O$, in deionized water. The culture media were sterilized by filtering through 0.45-μm sterile membranes (cellulose, Millipore) and were incubated in the dark at 24 or 34 °C with an agitation of 200 r/min for 240 h. The media were adjusted to three different pH levels (5, 7, and 9) with 0.1 mol/L HCl or NaOH, prior to filtration.

### 2.3 Analytical methods

Pigment production was spectrophotometrically monitored every 48 h in aseptic conditions. A sample of 3 ml of culture medium was carefully taken. Some samples were previously centrifuged at 10000 r/min for 5 min to remove suspended cells and all samples were filtered using 0.45-μm sterile membranes. Pigment production was measured at 500 nm (Méndez-Zavala *et al.*, 2007), and pH and reduction/oxidation potential (p-Redox) were determined potentiometrically. Biomass was determined gravimetrically (dry weight) after the incubation at 60 °C. A portion of mycelium was used for microscopic observations. The yield of product per unit biomass ($Y_{P/X}$) was calculated as the ratio of the amount of pigment produced ($P_t-P_0$) in a certain time ($t$) to the biomass generated in the same time ($X_t-X_0$).

### 2.4 Statistical analysis

The results were evaluated in triplicate using a divided parcel design with a factorial fix 2×3, and the analysis was performed using the Statistical Program Infostat (Córdoba, Argentina).

## 3 Results and discussion

### 3.1 Combined effect of pH and temperature on red pigment production

*P. purpurogenum* GH2 produced red pigment under different treatments of initial pH and temperature

in submerged culture. Red pigment production was observed at 150 h of culture and showed the highest level at 240 h of culture (final sampling time). The highest level of production was obtained with a treatment of pH 5 and 24 °C (T1; Table 1), followed by the treatment of pH 9 and 34 °C (T6). For the rest of the treatments, pigment production was very low in relation to treatment T1 (about one tenth or less). Fig. 1 shows the effects of pH and temperature on the fungal growth and pigment production.

The morphological and metabolic differences found among *Monascus* and *Penicillium* strains demonstrated different patterns in pigment production in relation to temperature and pH. However, it has been shown that *Penicillium* strains produce pigments with chemical structures similar to those produced by *Monascus* (Mapari *et al.*, 2006; 2008a).

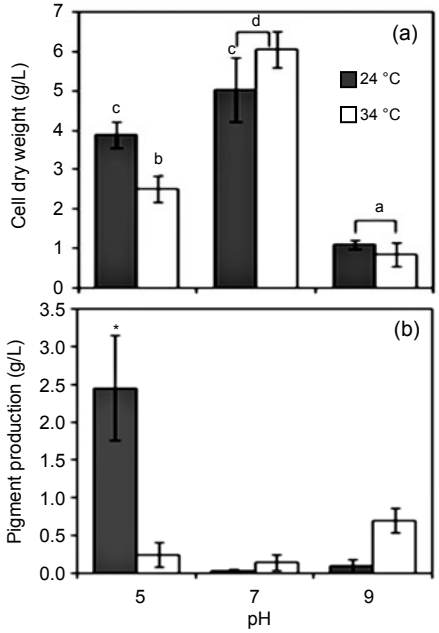


**Fig. 1 Effects of pH and temperature on growth (a) and pigment production (b) of *P. purpurogenum* GH2**

Different letters represent significant differences according to the Tukey test ($p<0.05$). * means significant difference at $p<0.05$

Pigment production in extracts obtained from *P. purpurogenum* GH2 following different treatments is shown in Table 2. The reddest culture medium was achieved with treatment T1 as a result of the highest pigment production, which was 2.46 g/L at 240 h. The color present in the extract was influenced by the initial pH of the culture medium. Gunasekaran and Poorniammal (2008) also found that the initial pH has a strong effect on red pigment production by *Penicillium* sp., but in that case the highest product accumulation was reached at an initial pH of 9.0. Chen and Johns (1993) in a study of a *Monascus purpureus* culture demonstrated the presence of yellow pigments (ankaflavin) at a low pH (4.0) and red pigments (monascorubramine) at higher pH (6.0), contrary to the results obtained in our research. Other authors (Lin and Demain, 1991; Orozco and Kilikian, 2008) found a positive effect on cellular growth due to the pH of the culture medium, favoring the production of red pigments intracellularly and extracellularly (secondary metabolites) at a pH of 5.5, as shown in our study. These results suggest that pH can affect the activities of enzymes involved in the biosynthesis of pigments, but the effect depends on the particular microorganism utilized.

A temperature of 24 °C in combination with acidic pH favors pigment production; this temperature might be involved in the regulation of enzymatic processes inside the fungal cell. Su (1983) found that temperature did not have a significant effect on the growth of *Monascus* (between 25 and 35 °C). However, the effect was significant for pigment production with temperatures between 28 and 30 °C

**Table 1 Kinetic parameters of pigment production at different values of pH and temperature**

| Treatment | Temperature (°C) | pH | Pigment (g/L)* | Biomass (g/L)* | Final pH* | $Y_{P/X}$* |
|---|---|---|---|---|---|---|
| T1 | 24 | 5 | 2.460±0.696$^{b}$ | 3.890±0.335$^{c}$ | 6.076±1.029$^{a}$ | 0.6320 |
| T2 | 24 | 7 | 0.024±0.023$^{a}$ | 5.045±0.808$^{c,d}$ | 5.733±0.344$^{a}$ | 0.0046 |
| T3 | 24 | 9 | 0.090±0.093$^{a}$ | 1.110±0.114$^{a}$ | 7.547±0.138$^{a}$ | 0.0802 |
| T4 | 34 | 5 | 0.246±0.164$^{a}$ | 2.500±0.318$^{b}$ | 8.236±0.727$^{a}$ | 0.0980 |
| T5 | 34 | 7 | 0.146±0.105$^{a}$ | 6.045±0.461$^{d}$ | 6.723±1.788$^{a}$ | 0.0240 |
| T6 | 34 | 9 | 0.700±0.156$^{a}$ | 0.833±0.303$^{a}$ | 7.216±0.408$^{a}$ | 0.8401 |

* Data are obtained at the maximum time of pigment production (240 h) and are expressed as the mean±SD of three samples. Values with different letters in the same column are significantly different according to the Tukey test ($p<0.05$)

**Table 2 Aqueous extracts of the pigments produced by *P. purpurogenum* GH2 with different treatments**

| *T* (°C) | pH | Intensity | Color |
|---|---|---|---|
| 24 | 5 | ++++ |  |
| | 7 | +++ | |
| | 9 | ++ | |
| 34 | 5 | ++ | |
| | 7 | + | |
| | 9 | +++ | |

producing higher levels. In other studies with *Monascus* cultures, red pigment production was highest at 30 °C, and decreased at temperatures higher than 40 °C accompanied by an increase in the production of yellow pigments (Babitha *et al.*, 2007). Hernández-Rivera *et al*. (2008) found that the best results for the production of yellow pigments were obtained at initial pH values from 3.0 to 3.5, while the best results for the production of red pigments were reached at pH levels between 7.0 and 7.5. Similar to the results of our research, Ahn *et al.* (2006) found that 10 times more pigment could be obtained at 25 °C than at 30 °C. These results suggest that the combined effect of pH and temperature on the red pigment production observed may be due to a stress from environmental conditions that favor pigment biosynthesis but not the production of biomass.

### 3.2 Combined effect of pH and temperature on the growth and morphology of *P. purpurogenum*

The biomass detected through the gravimetric method, showed a maximal growth of this microorganism with treatment T5 (34 °C and pH 7), followed by treatment T2 (Fig. 1), a result consistent with the mesophilic nature of this microorganism (isolated from a semi-arid zone).

Nevertheless, treatments at pH 9, at both temperatures (T3 and T6), showed the least growth in relation to the composition of the culture medium. Cellular growth was inhibited as a result of pH but not of temperature. In treatments at pH 5, at both temperatures (T1 and T4), biomass production was good, but not maximal, showing no relationship between biomass concentration and pigment production (Fig. 1). This treatment was found to be the best for the production of pigments, but not of the biomass. Mapari *et al*. (2008b) evaluated growth, morphology, and yellow pigment production in *Epicoccum nigrum* and demonstrated that a direct relation does not exist between maximal growth and pigment production. Rather, production is related to genetic and environmental factors. In addition, they observed that the growth-type pellet favored the production of yellow pigments, information that coincides with our research. Data reported by Cho *et al*. (2002) do not agree with our results. They tested a *Paecilomyces sinclairii* strain in submerged culture and found that at 25 °C and pH 6, fungal growth and pigment production were favored to the same extent. This fungus is very closely related morphologically and taxonomically to *P. purpurogenum*.

The maximal biomass concentration was obtained with treatments at pH 7. However, for these treatments, pigment production was minor, meaning that the conditions for the production of biomass are not suited for obtaining pigments. Ahn *et al*. (2006) observed high viscosity in the culture medium at 30 °C due to fungal growth, causing poor oxygenation of the culture and a decrease in pigment production. Some other microorganisms (algae), however, overproduce the pigment lutein as a function of growth at high temperatures (Boskou, 2008).

Miyake *et al.* (2006) suggested that the signalization of cyclic adenosine monophosphate (cAMP) suppresses the production of secondary metabolites in *Monascus* (lovastatin and red pigments), regulating the pigment biosynthesis for the repression of the carbon source (glucose). This can be strongly dependent on the route of the cAMP signal, which might explain the variations in the concentration of biomass expressed in the results. This implies a stronger effect caused by pH than by temperature, explaining the mesophilic nature of the microorganism isolated from a zone with a great variation in temperature and a high concentration of salt and alkaline soils. Nevertheless, pigment production is not associated with this behavior. In pigment production, temperature and pH together activate mechanisms probably related to genetic and metabolic controls or a defense mechanism.

The production of biomass at pH 7 and 34 °C could be related to oxidative metabolism caused by high production of adenosine triphosphate (ATP) that would accelerate the process of oxidation, leading to the formation of biomass but not the formation of secondary metabolites (Vázquez-Duhalt, 2002).

The experimental data obtained demonstrated a higher yield of pigments from the treatment at pH 9 and 34 °C (T6). However, these conditions can halt the metabolic activities of the microorganism (minor formation of biomass), and at a certain time the microorganism reaches its maximal rate of growth. It then begins to decline as a result of metabolic stress produced under these conditions, causing a failure of the culture to reach the maximal production of biomass. This leads to a lack of an association between microbial growth and the yields of pigment produced. Rather, maximal biomass will occur at a certain point in the production process.

The combined effect of pH and temperature on fungal morphology was evaluated microscopically. In the photomicrographs (Fig. 2), the mycelial growth following the different treatments can be observed. At pH 7 and 34 °C, an abundant mass of long hyphae with very few aerial hyphae and reproductive forms was obtained, associated with the optimal conditions for growth but not with the metabolic stress which was clearly associated with pigment production (Fig. 2e). In treatments at pH 5 and 24 °C, and pH 9 and 34 °C, pigmentation was observed in the hyphae (Figs. 2a and 2f). In the treatment at pH 9, slow fungal growth was observed. However, at this pH, the high quantity of aerial hyphae observed indicated that the organism was in a state of environmental stress caused by pH and that it produced these structures to produce spores as a defense mechanism, allowing it to conserve the species. The main effect of temperature in *Monascus* is specifically in the type of reproduction.

An abundant cellular concentration was not present in the growth-type pellets (data not shown), which can favor suitable oxygenation of the culture media and the best metabolism of the xylose as a carbon source. This would explain the best growth found under these conditions. Similar information has been reported by Hajjaj *et al.* (1999). Nevertheless, more thorough research is needed to evaluate the conditions of the culture medium that fungi favor for each type of growth and what their effects are on pigment production.

These changes in the morphology can be explained by the influence of the pH on microbial growth. It is possible that the concentration of intracellular hydrogen ions together with the electrical potential of the membrane determines the force motorboat of protons that directs the reactions of the membrane. The microbial morphology therefore can be affected by this change at the membrane level, because the change in the pH of the culture medium can affect the composition and the chemical nature of its components (Quintero-Ramírez, 1981). Shin *et al.* (1998) demonstrated that coculturing *Monascus* with *Saccharomyces cerevisiae* and filtering the yeast culture increases the production of pigments by up to 30 or 40 times compared to monocultures of these fungi and changes *Monascus* morphology. These behaviors suggest a mechanism of defense in mould, producing molecules such as the pigments that help to regulate damage principally at the membrane level. This type of behavior has been documented in *Monascus* cultures (Shin *et al*., 1998; Suh and Shin,

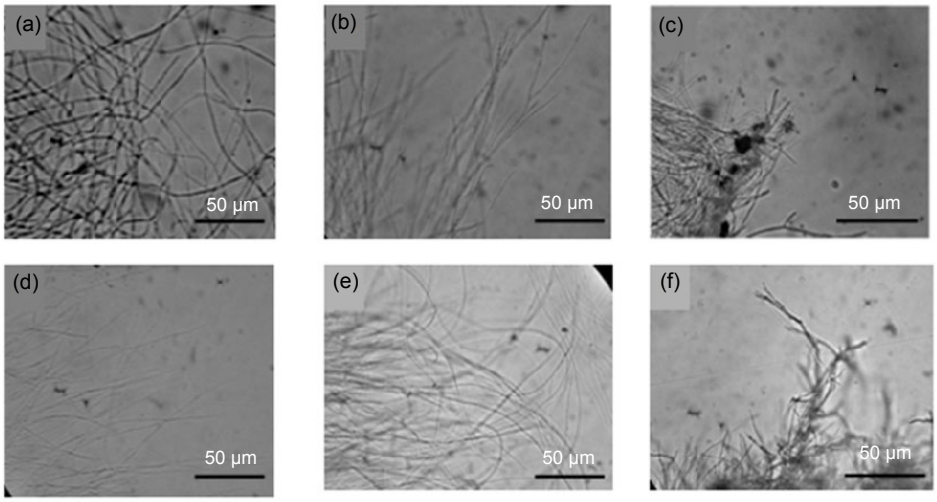


**Fig. 2 Combined effects of pH and temperature on the morphology of *P. purpurogenum* GH2 at different treatments**
(a) 24 °C/pH 5; (b) 24 °C/pH 7; (c) 24 °C/pH 9; (d) 34 °C/pH 5; (e) 34 °C/pH 7; (f) 34 °C/pH 9

2000) grown in presence of hydrolytic enzymes that induce morphologic changes in the cell wall, leading to an overproduction of pigments and a stimulation of reproduction for cellular proliferation as a mechanism of defense against the activities of these enzymes.

This information might aid in the understanding of the processes involved in the production of pigments, caused by chemical or structural changes in the membranes of the microorganism.

### 3.3 Kinetic evaluation of red pigment production

The kinetics of pigments formed by the *P. purpurogenum* strain was monitored every 48 h in triplicate (Fig. 3), where the highest level of production was obtained with T1 followed by T6. The maximal red pigment production was 2.46 g/L for treatment T1 at 240 h of culture and the maximal biomass was 6.045 g/L for treatment T5. These values demonstrate the feasibility of producing and obtaining natural, water-soluble pigments with a possible use for the food industry.

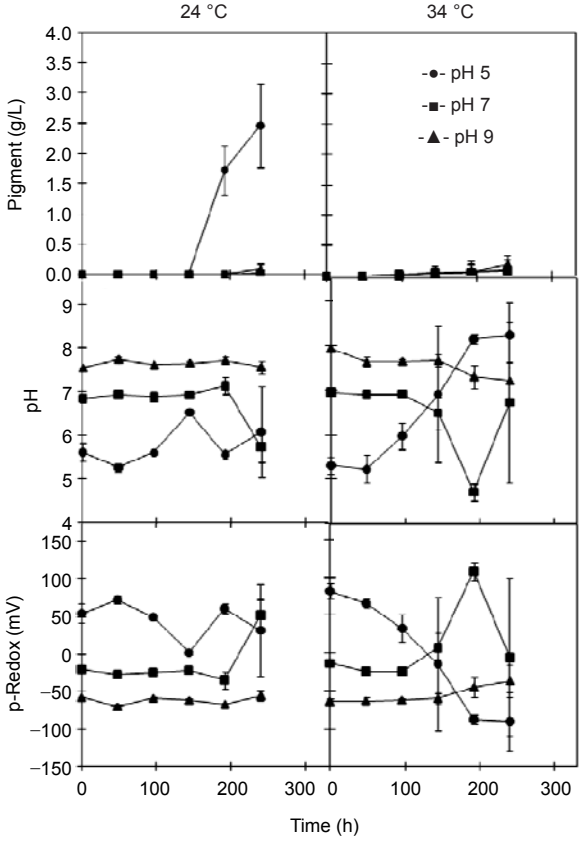


**Fig. 3 Kinetics of water-soluble red pigments with different treatments**

Table 1 shows the kinetic data obtained for all treatments, including values of pigments and biomass produced, and the pH values obtained at the end of the culture time.

The high concentration and productivity of pigments might be regulated by the effect of the pH and temperature of the culture medium on the regulation of molecules such as ATP that have an important function in the regulation of metabolic pathways, coupled reactions, and functional yields at the level of the membrane and cell wall (Zhou *et al.*, 2009).

This study demonstrates that changes in the oxidation/reduction process of the molecules in the cell due to the concentration of hydrogen ions can regulate the redox fluxes and the oxidative state of major energy molecules such as ATP in the culture medium (Vázquez-Duhalt, 2002). The change in pH can modify these states causing different metabolic flows and diverse mechanisms of osmotic, metabolic, and oxidative regulation, leading to diverse metabolisms and, therefore, diverse products.

The best yields of pigments were obtained with T6 ($Y_{P/X}$=0.8401). However, these conditions caused an inhibition of the metabolic activities of the microorganism (minor formation of biomass). The amount of time for the microorganism to reach its maximal growth rate is prolonged under these conditions, and, therefore, microbial growth is not associated with the yields of pigment produced. Moreover, under these conditions of pH, the microorganism has a low activity. This might have been observed in the kinetic modification of pH and p-Redox values at 24 and 34 °C (T4 and T6), where no considerable variation with respect to time existed, indicating a low metabolic activity. In the other treatments, the pH and p-Redox values changed with respect to time, as a result of the metabolic activities of the microorganism, such as different product formation (primary and secondary metabolites), respiration process, and substrate oxidation.

## 4 Conclusions

This study shows that the red pigment production and cellular growth of *P. purpurogenum* in a submerged culture can be controlled by the temperature and pH of the medium. It was possible to produce

a water-soluble red pigment from *P. purpurogenum* GH2 in a submerged culture using a minimal medium. Among the evaluated conditions, maximal red pigment production (2.46 g/L) was obtained with the treatment of pH 5 and 24 °C. In contrast, the maximal biomass concentration (6.045 g/L) was obtained at pH 7 and 34 °C. These results demonstrate that a direct relationship between biomass concentration and pigment production does not exist. This study shows the feasibility of producing and obtaining natural water-soluble pigments, and the potential for using a fungus as a potential high yielding industrial source of red pigment for possible use in the food industry. However, more detailed experiments could improve the production of pigment.

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