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EFFECT OF DISSOLVED OXYGEN CONCENTRATION ON RED PIGMENT AND CITRININ PRODUCTION BY *Monascus purpureus* ATCC 36928

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Abstract - The present study investigated the effects of agitation speed, N (200, 500, 600 or 700 rpm), and dissolved oxygen concentration, C (120, >70, 70, 60, 10 or ≤ 10%), on red pigment and citrinin production by *Monascus purpureus* ATCC 36928, cultivated in liquid medium by a batch process. The gas flow rate was the same for all runs with C controlled by means of the incoming gas composition control (air/N_2 or air/O_2). From the response surface plots it can be verified that the effect of C was greater than that of N on the production of both metabolites. The absorbance for red pigments varied from 1.6 U (C≤ 10%; N=200 rpm) up to 3.3 U (C=60%; N=600 rpm), an increase of 106%, while citrinin concentration increased 257%, from 14.2 to 50.7 mg.L^{-1}. The most appropriate conditions were C=60% and N=600rpm, under which the highest red pigment absorbance (3.3U) and half of the highest citrinin concentration were obtained.
Keywords: Agitation speed; Citrinin; Dissolved oxygen; *Monascus*; Red pigments.

INTRODUCTION

The filamentous fungus *Monascus* sp. produces several secondary metabolites, including at least six pigments: two yellow-colored (ankaflavin and monascin), two orange-colored (rubropunctatin and monascorubrin) and two red-colored (rubropunctamine and monascorubramine) (Wong and Koehler, 1983). The other secondary metabolites comprise an anticholesterolemic molecule, antibiotics and antitumorals (Juslová et al., 1996).

Red pigments have been successfully employed as total or partial substitutes of nitrate and nitrite salts in coloration, flavoring and preservation of red meat, on account of their bacteriostatic properties and mostly because the inorganic colorant used, nitrosamine, is a carcinogenic substance (Bakošová et al., 2001).

The majority of *Monascus* sp. secrete a mycotoxin, citrinin, which rules out the use of the red pigments. Citrinin synthesis depend on the *Monascus* strain, carbon source, nutritional factors (yeast extract and rice are enhancers) and environmental factors (oxygen and temperature) (Xu et al., 2006; Wang et al., 2003; Hajjaj et al., 2000a).

Some strategies to reduce the levels of citrinin in *Monascus* fermentations include genetic modification, degradation of the mycotoxin, the use of nonproducing strains and the manipulation of submerged culture conditions (Hajjaj et al., 1999; Hajjaj et al., 2000b; Lakrod et al., 2000).

The biosynthesis of pigments and citrinin begins with the condensation of one acetyl-CoA molecule and three malonyl-CoA molecules, followed by a series of reactions producing the orange-colored

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molecule in the cytosol. Oxidation of orange-colored molecules yields the yellow-colored pigments and complexation with L-glutamate renders the red pigments (Lin and Demain, 1991; Hajjaj et al., 1999). Although pigments and citrinin are derived from the same tetraketide, the independent level of production of each suggests that the enzymes involved in their synthesis have independent regulatory mechanisms of their genes (Pisareva et al., 2005); therefore, a given reduction in citrinin synthesis does not correlate with an increase in red pigments.

Production of secondary metabolites by filamentous microorganisms is affect by respiration rate and hyphal morphology (Pamboukian et al., 1998), which makes oxygen transfer rate and N variables relevant to the process. Oxygen transfer rate can be enhanced by N or gas flow rate, by pressurizing the reactor or by sparging air enriched with oxygen. Nevertheless, except air enrichment with oxygen, all others affect shear stress, which, in turn, causes morphological changes (Cui et al., 1998; Amanullah et al., 1999). Therefore, ideally, the effect of respiration rate influence should be studied keeping N and gas flow rate constant.

A few authors have studied the independent effects of dissolved oxygen concentration, N or gas flow rate on secondary metabolite production by filamentous microorganismS (Cui et al., 1998; Yegneswaran et al., 1991).

The present study investigated the independent effects of N and dissolved oxygen concentration, C, on red pigment and citrinin production by *Monascus purpureus* ATCC36928. In order to maintain constant the shear stress from the incoming gas flow and to maintain a given C value, the gas flow rate was the same for all runs and composition was varied by means of the control of an air/N_2 or air/O_2 stream. Gas composition was varied after a growth phase with no oxygen limitation so as not to impair cell growth and above all, to be able to analyze the effect of C and N on only red pigment and citrinin production.

MATERIALS AND METHODS

Microorganism and Storage

Monascus purpureus ATCC 36928 was obtained from Centro de Culturas Tropicais (CCT) at Fundação Tropical de Pesquisas André Tosello (Campinas, SP, Brazil) as *Monascus purpureus* CCT 3802. Suspended hyphae, grown in complex liquid medium, were added to glycerol 20% (v/v), (1:1) and kept at -80ºC.

Culture Media

The complex medium was composed of (g . L^{-1}): glucose, 10; meat extract, 3; peptone, 5.

The semi-synthetic medium was composed of (g . L^{-1}): $MgSO_4.7H_2O$ 4.8; KH_2PO_4 1.5; K_2HPO_4 1.5; $ZnSO_4.7H_2O$ 0.01; monosodium glutamate 7.6; NaCl 0.4; $FeSO_4$ 0.01; yeast extract 1.0; glucose 10.0. (Pereira and Kilikian, 2001).

The media were prepared with distilled water in two fractions: one with glucose and the other with the remaining nutrients. The pH was adjusted to 5.5 prior to sterilization at 121º C for 20 minutes.

Inoculum

A volume of 80 mL of semisynthetic medium in 500 mL Erlenmeyer flasks was inoculated with 24 mL of a defrosted cell suspension and incubated in a rotary shaker at 30ºC, 300 rpm for about 35h, when cells were at the end of the exponential phase.

Culture Conditions

Seven batch cultures were carried out in Bioflo III reactors (New Brunswick Scientific, Edison, NJ) with 4 L of working volume at 500 rpm (N), 30ºC, pH 5.0 ±0.3 and a total gas flow rate of 4 L . min^{-1}. During the first 25 to 30 h of cultivation, when cells were growing and red pigment production had not exceeded 0.1 U of absorbance, C was not controlled and varied between 70 and 100% of saturation concentration relative to air. Then, N and dissolved oxygen concentration, C, were changed as indicated in Table 1 and maintained throughout the end of the culture.

Analytical Methods

a) Cell Concentration (X)

Cell dry weight was measured by means of filtration through a 1.2 μm membrane. The filtrate was further analyzed for glucose and citrinin concentration and red pigment absorbance.

b) Citrinin Concentration (Cc)

The filtrate was added to a C-18 μBondapak column (10 μ, 3.9 mm, 300 mm, Waters) in a gradient eluent flow of water and methanol (0.5 mL/min, 25ºC). The UV absorbance was measured at λ of 330 nm. A calibration curve, 0.1 to 100 mg.L^{-1}, was obtained with pure citrinin (product C1017, Sigma Chemical Co.).

c) Red Pigment (P)

The highest absorbance (U) was measured for λ between 485 and 500 nm with a scanning spectrophotometer (Beckman, DU 530 UV/Vis).

Dissolved Oxygen Concentration (C)

Dissolved oxygen concentration (C) was monitored with an InPro6100 oxygen sensor (Mettler-Toledo GmbH, Switzerland) and expressed as a percentage of saturation relative to air (Figure 1). A blend of oxygen and air at a fixed total flow rate of 4L . min^{-1}, was sprayed into the bioreactor at a constant agitation rate in order to keep shear stress constant. Two mass flow controllers (5850E, Brooks Instruments) were used to adjust the flow rate of each gas, according to an algorithm written in LabVIEW 6i (National Instruments) to control the C value by adjusting the oxygen molar fraction in the inlet gas. Alternatively, nitrogen, instead of oxygen, was used to control low C levels.

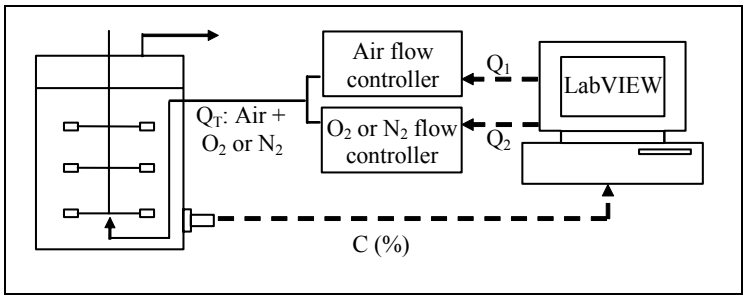


Figure 1: Schematic diagram of dissolved oxygen concentration (C) data acquisition and control system, applied in the batch cultures: Q_1: air flow rate; Q_2: O_2 or N_2 flow rate; Q_T: air plus oxygen or nitrogen flow rate (constant).

RESULTS AND DISCUSSION

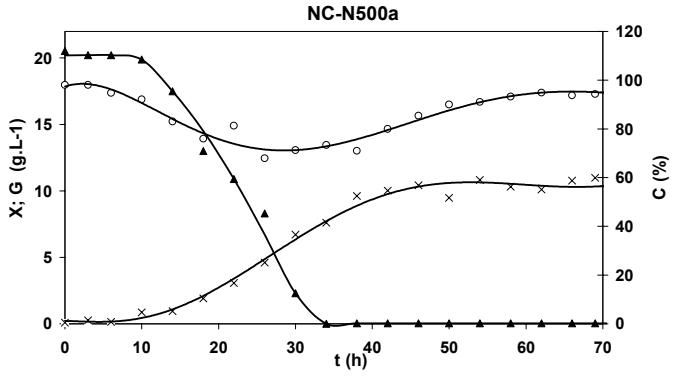
In Table 1 the seven runs performed are identified and the results of the highest cell concentration (X_{max}) and red pigment absorbance (P) are shown. The reproducibility of the cultures was analyzed for three standard batch runs (NC-N500 a, b and c, Figures 2 and 3) conducted simultaneously with some of the seven runs.

It is possible to conclude that there was reproducibility because the highest cell concentration of 10g . L^{-1} was reached at around 40h, glucose depletion at around 35h, the lowest dissolved oxygen concentration of 70% at around 25 h of cultivation and the hoghest absorbance for red pigments of 2.8U at around 50 h in all of the three runs. In addition, red pigment production began in the middle of the cell growth phase and continue after cell growth had ceased, in a pattern typical of secondary metabolite synthesis, which was verified in all runs, as depicted in Figure 3.

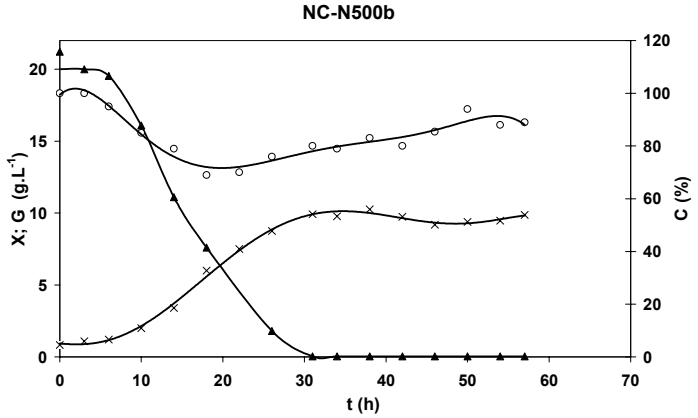
Table 1: Identification, characteristics and results of the runs.

Identification	N (rpm)	C (%)	X_{max} (g.L^{-1})	P (U)
NC - N200	200	5 < C < 10	9.8	1.8
C70 - N200	200	70	10.0	2.2
NC - N500	500	70 < C < 100	10.1	2.8
C120 - N500	500	120	10.0	1.6
C60 - N600	600	60	10.0	3.3
C10 - N700	700	10	10.0	2.3
NC - N700	700	70 < C < 100	9.8	3.1

NC: Runs without dissolved oxygen concentration control
NC-N500: Standard runs



(a)



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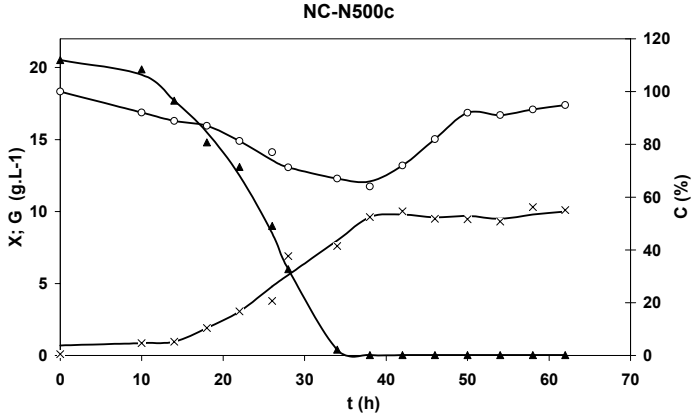

(c)

Figure 2: Dissolved oxygen concentration (C, ○), cell concentration (X, x) and glucose concentration (G, ▲) in standard batch runs [NC-N500a (A), NC-N500b (B) and NC-N500c (C)] of *Monascus purpureus* ATCC 36928.

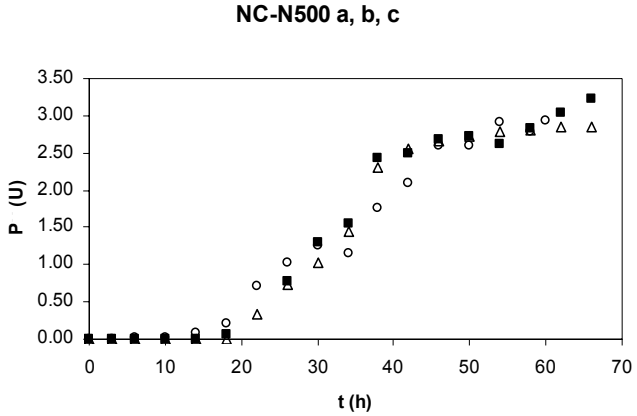

Figure 3: Absorbance of red pigments (P) in standard batch runs [NC-N500a (A), NC-N500b (B) and NC-N500c (C)] of *Monascus purpureus* ATCC 36928.

In order to analyze the effect of N (agitation speed) and C (dissolved oxygen concentration) on red pigment (P) and citrinin (C_c) production, results of the seven batch runs performed were arranged in response surface plots (Figure 4). For runs without C control, C corresponds to the average value of dissolved oxygen concentration throughout the production phase with the standard deviation being less than 12%.

As can be observed in Figure 4, the effect of dissolved oxygen concentration, C, was greater than that of agitation speed on red pigment production. The highest values of absorbance were achieved for high C values of around 60%, which is in accordance with oxygen dependence of the metabolic pathway for polyketides. However, for oxygen concentrations higher than 60%, red pigment production was reduced up to 51% for C as high as 120%. This is possibly due to the oxidation of the orange colored pigment yielding the yellow colored pigments instead of the red ones (Lin and Demain, 1991). Morphological measurements as hyphal length and degree of branching, were made, but no reliable correlation with red pigment production was found (data not shown). In Figure 5 it can be observed that an increase in C (10 to 60% and 80%) results in a five fold in citrinin production, from 17.5 up to 93.4 $mg.L^{-1}$, whileproduction of red pigments almostdoubled. As well, C_C values almost did not change for C values higher than 80%, including 120%. Agitation speed had no effect on C_C production.

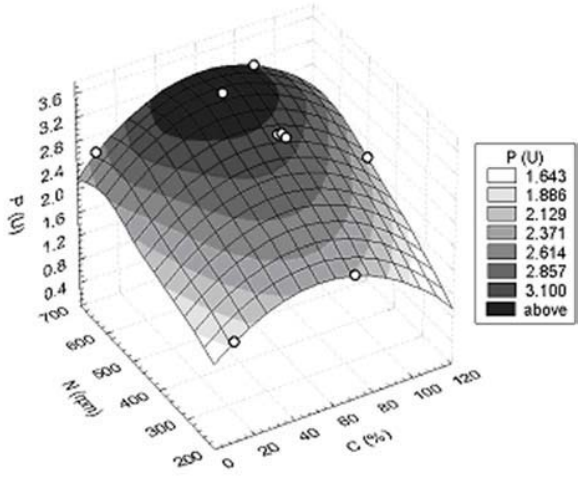

Figure 4: Response surface plots for the effect of dissolved oxygen concentration (C) and agitation speed (N) on red pigment production (P) by *Monascus purpureus* ATCC 36928 in batch cultures (cultivation time=60 h). C corresponds to the average value of dissolved oxygen concentration throughout the production phase, with a standard deviation of less than 12%.

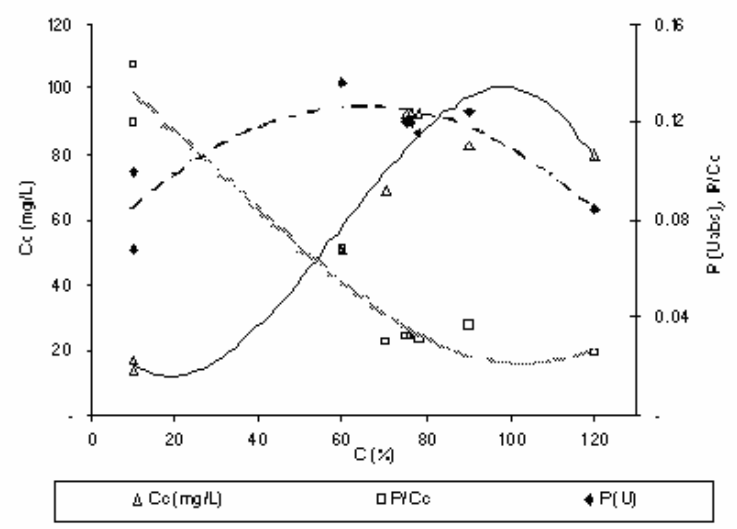

Figure 5: Citrinin concentration (Cc), red pigment absorbance (P) and the ratio of red pigments to citrinin (P/Cc) as a function of dissolved oxygen concentration (C) for *Monascus purpureus* ATCC 36928 batch cultures.

Citrinin production was also strain and culture medium-dependent. Twenty-nine strains of *Monascus sp.*, belonging to four species (*M. ruber*, *M. purpureus*, *M. anka* and *M. rubiginosus*), were cultivated in our laboratory in the same semisynthetic liquid medium employed in the runs presented in this paper, with the production of citrinin by *Monascus purpureus* ATCC 36928 being the highest (47.7mg/L) and the lowest, for nine strains was bellow 0.1mg . L^{-1}. Considering that the lowest citrinin concentration in the present runs was about 20mg . L^{-1}, certainly the strain used for production must be carefully identified from the strains with a lower citrinin production than that of *Monascus purpureus* ATCC 36928. Choosing the strain which had the highest citrinin production for the present runs enable analysis of the effect of C on citrinin production.

Although an ideal situation, high P and low C_c values simultaneously, is not possible, the best conditions for red pigment production, C=60% and N=600 rpm, correspond to those for half of the highest citrinin concentration. The relationship between red pigment, citrinin production and oxygen concentration must be examined for other *Monascus* strains.

CONCLUSIONS

The effect of dissolved oxygen, C, on red pigment and citrinin production was greater than that of agitation speed, N. Red pigment absorbance increased 106%, from 1.6 U at C≤10% and N=200rpm up to 3.3 U at C=60% and N=600 rpm. Under the same conditions of aeration and agitation, citrinin production increased 257%, from 14.2 to 50.7 mg.L^{-1}, showing that C had a greater effect on citrinin than on red pigment production.

Despite this behavior, the highest red pigment absorbance, 3.3U, corresponds to a citrinin concentration that is 50% lower than the maximum production, which is achieved at C=60% and N=600rpm, indicating the relevance of an appropriate concentration of oxygen.

Furher studies on the effects of dissolved oxygen concentration and shear stress on red pigment and citrinin production for low citrinin and high red pigment *Monascus* strains must be done.

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NOMENCLATURE

C	dissolved oxygen concentration	%
Cc	citrinin concentration	mg . L^{-1}
G	glucose concentration	g . L^{-1}
Q_1	air flow rate	NLPM

Q_2	oxygen or nitrogen flow rate	NLPM
Q_T	air plus oxygen or nitrogen flow rate	NLPM
N	agitation speed	rpm
NC	runs without dissolved oxygen concentration control	(-)
NLPM	normal liter per minute	(-)
P	absorbance of red pigments	U
X	cell concentration	$g \cdot L^{-1}$
X_{max}	maximum cell concentration	$g \cdot L^{-1}$

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