Pigments and Citrinin Biosynthesis by Fungi Belonging to Genus *Monascus*

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Citrinin is a mycotoxin, which is produced by fungi belonging to the genus *Monascus*, known in biotechnology as producers of azaphilone pigments. The relation between biosynthesis of these secondary metabolites was investigated in different species of the genus *Monascus* in batch-culture at the following cultivation conditions: $T = 28$ °C, agitation 220 rpm, and a medium, which induce citrinin production, containing ethanol as a carbon source.

The screening was carried out with 16 fungal strains and the biosynthesis of citrinin and pigments was monitored quantitatively at the standard conditions mentioned above. Some kinetic parameters of the process have been determined. The values of the growth yield coefficient $Y_{X/C}$ were between 0.32 and 0.57. The amount of the extracellular red and orange pigments at the end of cultivation varied for the different strains between 0.09 and 1.33 OU/mg dry weight, and 0.15 and 0.96 OU/mg dry weight, respectively. The amount of the total pigments measured was between 0.16 and 3.6 OU/mg dry weight, and between 0.21 and 3.39 OU/mg dry weight. The determined ratio 500 nm/400 nm, characterizing the pigment production, ranged between 0.60 and 1.06. Twelve of the investigated strains produced citrinin and pigments, two of them produced only pigments. Two strains were not able to produce neither pigments nor citrinin.

Thus, the biosynthesis of citrinin appeared to be strain-specific and does not correlate with the pigments' biosynthesis by the fungal strains belonging to the genus *Monascus*.

Key words: Monascus, Citrinin, Pigments

Introduction

Fungi belonging to the genus *Monascus* are traditionally used in the production of natural colorants for the application in food industry (Wong and Koehler, 1981; Hajjaj *et al.*, 1999b; Blanc *et al.*, 1995; Rasheva *et al.*, 2003). These fungi are known to produce yellow (monascin and ankaflavin), orange (monascorubrin and rubropunktatin) and red pigments (monascorubramin and rubropunktamin). Their biosynthesis strongly depends on the nutrient media composition and physical parameters of the cultivation (Lin and Demain, 1991). A variety of processes have been developed for production of these metabolites based on solid phase and submerged fermentation (Rasheva *et al.*, 2003).

Recently, it has been shown that *Monascus purpureus* and *Monascus ruber* strains are able to produce, besides pigments, citrinin – a mycotoxin possessing nephrotoxic and antibiotic properties (Sankawa *et al.*, 1983). Such kind of molecule has been isolated also from cultures of *Penicillium* sp., *Aspergillus* sp., *Pythium* sp. and *Cercosporidium* sp. (Betina *et al.*, 1973; Ciegler *et al.*, 1977; Deruiter *et al.*, 1992). It was also proved that the *Monascus* albino strain does not produce pigments and citrinin, but preserves the production of other typical *Monascus* metabolites (Rasheva *et al.*, 2003). As it was shown that the pigment production by the above mentioned strains is compromised by the simultaneous production of citrinin, the question about the relationship between the biosynthesis of both metabolites is of special interest. For this reason a screening procedure favorising pigment and citrinin production (Pastrana *et al.*, 1996) has been performed in order to enlight the process of subordination of both events.

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Materials and Methods

Microorganisms and media

Sixteen strains belonging to the genus *Monascus* were used in this investigation. Five were newly isolated from different sources (cellulose, ketchup, red rice), two were selected mutants and nine were received from microbial collections (CBS, Utrecht, The Netherlands; DSMZ, Braunschweig, Germany). The biosynthesis of pigments and citrinin was carried out in batch-cultures at cultivation conditions as follows: The medium inducing citrinin production was composed of (g/l): monosodium glutamate (5), K_2HPO_4 (5), KH_2PO_4 (5), $MgSO_4 \cdot 7\ H_2O$ (0.5), $CaCl_2$ (0.5), $FeSO_4 \cdot 7\ H_2O$ (0.5), $ZnSO_4 \cdot 7\ H_2O$ (0.01), $MnSO_4 \cdot H_2O$ (0.03), ethanol (20) (Blanc *et al.*, 1995); the initial pH was adjusted to 6.5 with ammonium hydroxide; the cultures were cultivated for 9 d at 28 °C and agitation at 220 rpm.

Analytical control of the cultures

Growth of the cultures. The cultures' growth was estimated through determination of the biomass dry weight (Rasheva *et al.*, 1997).

Growth yield coefficient. $Y_{X/C}$, defined as biomass produced per carbon source utilized (g/g), was calculated according to Pirt (1975).

Pigment production. The pigment production ability of the strains was estimated spectrophotometrically at 400 nm (for the orange pigments) and 500 nm (for the red pigments). The orange and red pigments produced by *Monascus* fungi have an absorption peak at 400 nm and 500 nm, respectively (Chen and Johns, 1993). The total pigments (mycelium with culture broth), as well as the extracellular pigments' production (separated by filtration culture broth) were measured. The results are presented as relative optical units (OU/mg dry weight). An optical unit (OU) is defined as the absorbance (A) at 400 nm (for the orange pigments) and 500 nm (for the red pigments) multiplied by a dilution factor of 10 in those cases, in which $A > 1.0$.

Residual ethanol concentration. It was determined according to the method of Dawes *et al.* (1971).

Residual concentration of monosodium glutamate. It was estimated by titration. A sample of the culture broth (20 ml) was adjusted to pH 8.4 with 1 N NaOH and then 20 ml neutralized formaldehyde were added. This sample was titrated with 1 N NaOH to pH 8.4. The concentration of monosodium glutamate was calculated towards $V_{NaOH} \cdot N_{NaOH} \cdot M/V_{sample}$, where V_{NaOH} is the volume of NaOH used for titration, N_{NaOH} is the normality of NaOH, M is the molecular weight of monosodium glutamate and V_{sample} is the volume of the sample.

Phosphorus concentration. It was determined according to Herbert *et al.* (1971).

Assay for citrinin. The presence of citrinin in the cultivation broth was determinated after Rasheva *et al.* (2003). Commercially available pure citrinin obtained from Sigma Chemical Co. was used as a reference. The detection limit of the method is 2 ng/chromatogram zone (the sample application site). All data presented are mean values of at least three individual measurements.

Results and Discussion

Recently, data has been accumulated in respect to citrinin formation, indicating that this molecule arises as a result of postsynthetic modifications of the already synthesized polyketides within the cells of *Monascus* fungi (Hajjaj *et al.*, 1999a). During application of specific nutrient media and cultivation conditions, used by other authors for investigation of citrinin production (Blanc *et al.*, 1995; Pastrana *et al.*, 1996), a process of simultaneous biosynthesis of pigments and citrinin was investigated. In this study a set of 16 strains of *Monascus* fungi, belonging to the species *Monascus pilosus*, *Monascus purpureus* and *Monascus ruber*, was used in order to receive a better look of this event. The list of these strains and their characteristics are given in Table I. Some of them are type cultures, others are wild isolates having an anamorphous or teleomorphous status.

Growth of the cultures

The cultivation was performed as described in Materials and Methods and samples for analysis were collected after 9 d. The growth of different strains was evaluated through measurement of dry biomass and consumption of the elements C, N, P. The determined dry biomass ranged between 6.02 and 11.3 g/l for *Monascus ruber* 62478 and *Monascus purpureus* 288.34, respectively.

It is known that the type of carbon and nitrogen source used is very important for the biosynthesis of pigments and citrinin (Betina and Binovska, 1979). The consumption of the carbon source ethanol is shown in Fig. 1. All tested strains utilized it

Table I. Sexual status and production of secondary metabolites of the investigated strains.

Strains/sexual status	Source of the strain	Extracellular pigments [OU/mg dry weight]		Citrinin
		400 nm	500 nm	[μg/ml culturebroth]
M. pilosus 286.34 (A)	The Netherlands, CBS type culture	0.15	0.09	63*
M. pilosus C_1 (A)	Isolated (cellulose)	0.87	0.58	ND**
M. purpureus N8 (A) (T)	Isolated (ketchup)	0.40	0.28	59*
M. purpureus R_{13} (A)	Isolated (red rice)	0.92	0.82	ND**
M. purpureus 109.07 (A) (T)	The Netherlands, CBS type culture	0.44	0.37	65*
M. purpureus 285.34 (A)	The Netherlands, CBS type culture	0.17	0.10	61*
M. purpureus AKC (A)	Selected mutant	0.96	1.33	64*
M. purpureus 94–5 (A) (T)	Isolated (red rice)	0.45	0.36	66*
M. purpureus 1603 (A)	Type culture (Germany, DSMZ)	0.65	0.55	74*
M. ruber 62478 (A)	Type culture (Germany, DSMZ)	0.16	0.10	57*
M. purpureus M_{12} (A)	Selected mutant	–	–	ND**
M. purpureus 1604 (A)	Type culture (Germany, DSMZ)	0.34	0.32	62*
M. ruber 1561 (A) (T)	Type culture (Germany, DSMZ)	0.18	0.12	73*
M. ruber 291.34 (A)	The Netherlands, CBS type culture	0.16	0.11	68*
M. purpureus 288.34 (T)	The Netherlands, CBS type culture	0.19	0.10	67*
M. purpureus 94–1 (A) (T)	Isolated (red rice)	–	–	ND**

* All data presented are mean values of at least three individual measurements.
** No citrinin was detected in the investigated samples. If a recalculation in accordance with the method's resolution is made, the amount of citrinin in the samples, if available, is less than 0.005 μg/ml of culture broth.
T, teleomorphous sexual status.
A, anamorphous sexual status.

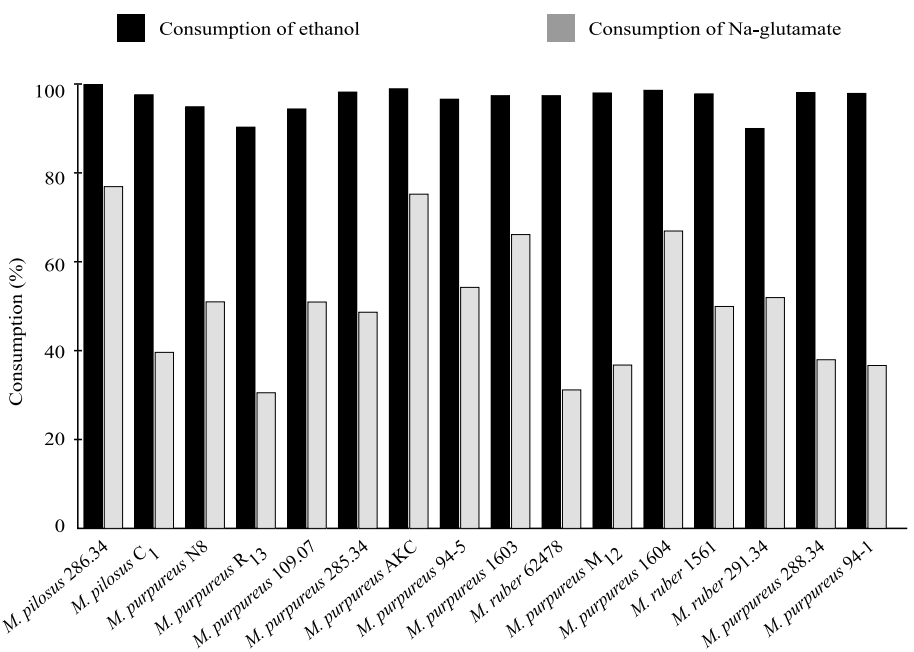

Fig. 1. Consumption of carbon and nitrogen after a 9-day period of cultivation.

quite well, between 90.1% and 100% during a 9-day cultivation period.

The utilization of the nitrogen source (monosodium glutamate) widely varied among the tested strains (between 30.56% and 77%). As the consumption of the nitrogen source influenced the pH value of the nutrient broth, its values were in the range 6.5–7.2. The strain with maximal consumption of monosodium glutamate indicated the largest change of pH, corresponding to the culture growth and transamination process.

The efficiency of growth and carbon substrate utilization by the tested strains was measured through calculation of the growth yield coefficients $Y_{X/C}$. As it is shown in Table II they ranged between 0.32 and 0.57. These values indicate that the composition of the nutrient medium used supported the biomass synthesis. An interesting fact is that there is no correlation between growth yield ($Y_{X/C}$) and amount of the pigments synthesized. The strains with equal values of $Y_{X/C}$ indicated very different amounts of total pigments. For instance, strain *M. pilosus* 286.34 with $Y_{X/C}$ = 0.55 had a total pigment production of 0.21 and 0.16 OU/mg dry weight, while *M. purpureus* R_{13} possessed the same value of $Y_{X/C}$ and a pigment production of 1.26 and 1.09 OU/mg dry weight measured at 400 nm and 500 nm, respectively. This observation indicates that the pigmentation is not connected with the growth efficiency and it is strongly strain-dependent. The best producers of pigments are strains *M. purpureus* AKC, R_{13}, 109.07, 285.34, 94–5 and *M. pilosus* C_1. They have also efficient growth and high $Y_{X/C}$ values (0.47–0.57). The calculated ratio of the total red and yellow pigments indicated that only *M. purpureus* AKC had a ratio higher then 1.0, which showed its ability to produce mainly red pigments. The other strains with relatively high values of the ratio 500 nm/400 nm were *M. purpureus* R_{13}, 1603 and 94–5. Only three of the strains – *M. purpureus* AKC, 1603 and 94–5 – are able to produce citrinin (Table I). Strain *M. purpureus* R_{13}, in spite of the high red pigment production, was not able to synthesize citrinin, which indicates that there is no correlation between red pigment synthesis and citrinin production.

It was found that the ability for excretion of pigments is also strain-dependent and it is not connected with the sexual status of the culture: teleomorphous (capable of sexual reproduction) or anamorphous (reproducing asexually). Strains possessing high pigmentation of the biomass indicated weak extracellular pigment production. This was observed for the strains *M. pilosus* C_1, *M. purpureus* 285.34 and *M. purpureus* AKC. As the composition of the nutrient media and the physical parameters of cultivation influenced the pigments biosynthesis, the high production of pigments was registered when monosodium glutamate, ammonia or peptone were used as a nitrogen source (Lin and Demain, 1991; Chen and Johns, 1993) and the

Table II. Dry weight, growth yield coefficient and pigments production of the tested strains.

Strain	Dry weight* [g/l]	$Y_{X/C}$	Pigment production: Total pigments [OU/mg dry weight] 400 nm	Total pigments [OU/mg dry weight] 500 nm	Ratio 500 nm/400 nm
M. pilosus 286.34	11.1	0.55	0.21	0.16	0.76
M. pilosus C_1	10.0	0.54	2.46	1.79	0.73
M. purpureus N8	9.5	0.52	0.78	0.52	0.67
M. purpureus R_{13}	9.5	0.55	1.26	1.09	0.86
M. purpureus 109.07	10.3	0.57	1.08	0.79	0.73
M. purpureus 285.34	9.0	0.47	1.27	0.89	0.70
M. purpureus 94–5	8.7	0.48	1.29	1.06	0.82
M. purpureus 1603	7.5	0.4	0.88	0.76	0.86
M. purpureus 1604	8.0	0.42	0.57	0.34	0.60
M. purpureus AKC	9.3	0.49	3.39	3.6	1.06
M. ruber 62478	6.0	0.32	0.24	0.17	0.71
M. purpureus M_{12}	11.0	0.57	–	–	–
M. ruber 1561	10.5	0.56	0.43	0.28	0.65
M. ruber 291.34	8.1	0.45	0.25	0.18	0.72
M. purpureus 288.34	11.3	0.55	0.58	0.40	0.69
M. purpureus 94–1	10.9	0.57	–	–	–

* All experimental data presented are mean values of at least three individual measurements.

pH of the nutrient medium defined the colour of the pigments (Carels and Shepherd, 1977).

Biosynthesis of citrinin

Simultaneous pigments and citrinin biosynthesis was analysed in accordance to the above described cultivation conditions and strains. The results are represented in Table I. It is shown that strains *M. pilosus* C_1, *M. purpureus* R_{13}, M_{12} and 94–1 are not able to synthesize this mycotoxin. All other strains – *M. purpureus* N8, 109.07, 285.34, AKC, 94–5, 1603, 1604, 288.34, *M. ruber* 62478, 1561, 291.34 and *M. pilosus* 286.34 – are able to produce citrinin and their values vary between 57 and 74 μg/ml culture broth. All these data indicate that the possibility for synthesis of citrinin is strongly strain-dependent. Even if the chemical and physical conditions of growth are maintained constant for all strains under investigation, they indicate different growth characteristics, pigments synthesis and citrinin production. Thus, besides some evidence for citrinin biosynthesis by strains of *Monascus purpureus* and *Monascus ruber* stated by some authors (Wong and Koehler, 1981; Hajjaj *et al.*, 1999b; Pastrana *et al.*, 1996; Blanc *et al.*, 1995), this event could not be treated as a common feature of these fungi. It could be concluded that the citrinin biosynthesis is not obligatory, in spite of the observation that the postsynthetic formation from polyketides already accumulated in the cells takes place. Our experiments indicate that there are citrinin-free strains possessing efficient pigment production capacity, which could contribute to reabilitation of the already compromised *Monascus purpureus* based technology. It is clear that profound investigation of citrinin-free strains in respect to the gene regulation of this process is necessary in order to verify the valuable properties of this friendly for human nutrition fungus, object of application in food technology for centuries.

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