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PRODUCTION OF CITRININ BY VARIOUS SPECIES OF *MONASCUS*

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SUMMARY

The production of citrinin by various *Monascus* species was determinated using various culture mediums and conditions. The maximal production was obtained in fermentor using *M. ruber* with concentrations of 380 mg/l. Since citrinin is a toxic product, it is essential that the production of red pigments as food additives from *Monascus* sp. avoid the occurrence of citrinin; so, we argue that some nitrogen sources are unfavorable to the production of citrinin.

INTRODUCTION

The fungal genus *Monascus* was discovered by van Tieghem (1884) and was known in Europe as a contaminant on cereals, starch and silage. In Asia, *Monascus* is used to prepare red mold rice as a natural food coloring matter and also as a food disinfectant (Went, 1895).

Several authors (Wong and Bau, 1977; Wong and Koehler, 1981; Fink-Gremmels *et al.*, 1991) have shown the occurrence of antibacterial activities among crude pigments while others such as Ober and Kunz (1989) argue that some strains are unable to produce antibacterial compounds. Investigating the purification and identification of *Monascus* pigments regarding their acceptability as food additives by European Union, we have isolated monascidin A in various species of *Monascus* and identified it as citrinin (Blanc *et al.*, 1995 in press), a nephrotoxic metabolite produced by various fungi (Wu *et al.*, 1974). This was the first report of the occurrence of citrinin among metabolites produced by *Monascus*. This work has contributed to the characterization of the chemical structure of monascidin A and to identify one compound among the pigments of *Monascus*.

As *Monascus* is being used as a food additive, it might be necessary to focus the investigations either on non citrinin producing species (Ober and Kunz, 1989) or on culture conditions unfavourable to citrinin production or at last, on detoxication of the pigments which could occur naturally (Barber *et al.*, 1988) as really, some commercial colored preparations that we have analyzed didn't contain citrinin.

MATERIALS AND METHODS

Microorganism

Species of *Monascus*, according to the new taxonomy proposed by Hawksworth and Pitt (1983) were used : *Monascus ruber* ATCC 96218, one albino strain isolated from the previous one, 3 obtained from culture collections namely *Monascus purpureus* CBS 109.07, *Monascus pilosus* CBS 286.34 and *Monascus purpureus* DSM 1379 and one mutant strain isolated from *Monascus purpureus* CBS 109.07. Purity and identity of subcultures were verified.

Media

The strains were kept on slant culture composed of potato dextrose agar (PDA) Difco. Spores of the strains were prepared by growth on PDA slants for 10 days at 28°C.

Culture conditions were established to analyze the effects of submerged and solid-state cultures on the production of citrinin. Three culture media were used :

-the synthetic medium classically used for the production of red pigments (Fabre *et al.*, 1993) composed of monosodiumglutamate (MSG), 5 g; K_2HPO_4, 5 g; KH_2PO_4, 5 g; $MgSO_4.7H_2O$, 0.5 g; $CaCl_2$, 0.1 g; $FeSO_4.7H_2O$, 0.01 g; $ZnSO_4.7H_2O$, 0.01 g; $MnSO_4.H_2O$, 0.03 g; ethanol or glucose 20 g (unless otherwise indicated) per litre deionized tap water. The initial pH of the medium was adjusted to 6.5 with ammonia. This medium was incubated with agitation at a temperature of 27 °C until exhaustion of carbon substrate. Cultures were performed either in agitated Erlenmeyer flasks (baffled or not) or in a 20 litres SGI fermentor.
-a modified YES medium, specifically used for the production of fungal toxins and composed of yeast extract 40 g and sucrose 160 g/l deionized tap water. The YES medium was incubated at a temperature of 27 °C without agitation for two weeks.
-a solid-state medium, traditionally used for various commercial productions of red mold rice (named anka in Asia) and composed of wet rice (50 % of water w/w) was incubated at a temperature of 27 °C for two weeks.

Analytic methods

Dosage of citrinin from submerged cultures of *Monascus*

Citrinin was determinated by HPLC on a C18 Nucleosil column using a separation gradient composed of methanol/water. The concentration of citrinin was then measured on culture supernatant.

Dosage of citrinin from solid-state cultures of *Monascus*

Fungal cultures on rice were extracted with acetonitrile. The filtered extract was twice defatted with isooctane. After adding an equal volume of water and acidification to pH 4.5 with H_2SO_4 (50:50, v/v), the extract was partitioned with $CHCl_3$. The lower phase was evaporated to dryness; it was then dissolved in methanol and analyzed by HPLC.

Measurement of optical density

Pigment production was spectrophotometrically determined by measuring the absorbance at 480 nm.

RESULTS AND DISCUSSION

The concentration of citrinin was quantified using several species of *Monascus*, several substrates at various initial concentrations and different conditions of cultures.

1) Effect of carbon source (2 litres agitated Erlenmeyer flasks) on various species on citrinin production

Species	Citrinin production (mg/l)*				
	Synthetic medium (glucose 13 g/l)	Synthetic medium (ethanol 9 g/l)	Synthetic medium (acetate 10 g/l)	YES medium (static) .	Rice powder (static)
M. purpureus CBS 109.07 (wild)	5	9	3	240	100
M. purpureus CBS 109.07 (mutant)	0	3	0	-	-
M. purpureus DSM 1379	-	-	-	20	-
M. ruber (wild)	18	59	19	370	300
M. ruber (albino)	0	0	0	-	-
M. pilosus	0	0	0	-	-

* (- = not done)

2) Effect of nitrogen source (cultures of *M. ruber* strain on ethanol in agitated Erlenmeyer flasks) on citrinin production

Nitrogen source	Citrinin production (mg/l)
Urea	17
NH_4NO_3	100
NH_4Cl	42
Monosodium glutamate	120
Methionine	0

Urea and methionine unfavoured both citrinin and pigments production while growth was not affected.

3) Effect of the initial carbon source concentration (cultures of *M. ruber* strain on ethanol or glucose in 50 ml. agitated Erlenmeyer flasks) on citrinin production

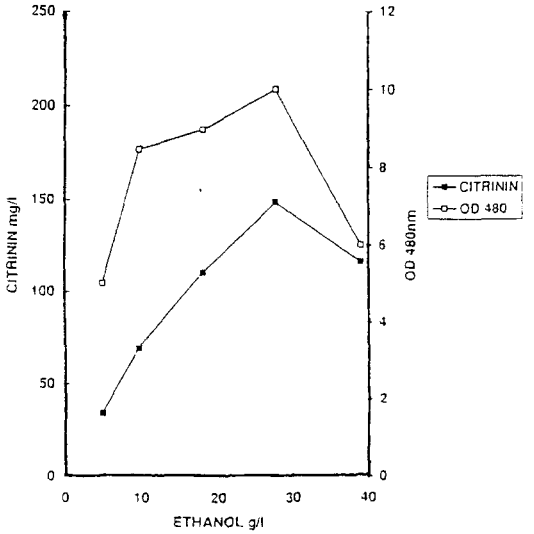

Figure 1. Effect of initial ethanol concentration on O.D. and citrinin production

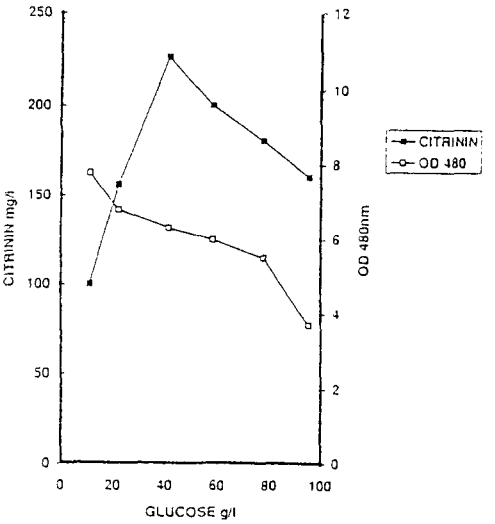

Figure 2. Effect of initial glucose concentration on O.D. and citrinin production

Figures 1 and 2 showed maximal citrinin concentrations of 226 mg/l obtained on glucose (initial substrate concentration of 45 g/l) and 136 mg/l obtained on ethanol (initial substrate concentration of 28 g/l). Ethanol inhibited growth at concentrations higher than 45 g/l.

4) Effect of the conditions of culture (*M. ruber* strain on synthetic medium (ethanol as carbon source)) on citrinin production

Conditions	Citrinin production (mg/l)
Agitated Erlenmeyer flasks	136
Fermentor	380

The maximal citrinin concentration of citrinin was obtained using *M. ruber* cultured on ethanol in fermentor, very close the one obtained in static cultures using YES medium (370 mg/l).

5) Effect of the addition of inhibitors on the production of citrinin

It was reported that cerulenin and ethionin are specific inhibitors of both citrinin (Betina and Binovska, 1979) and pigment (Lin and Demain, 1993) biosynthesis. Their effect was tested on *Monascus* cultures. The results obtained showed that both growth and metabolite biosynthesis was inhibited; it was not possible to prove any specific inhibitory effect of these molecules on the citrinin production.

CONCLUSION

According to our work, citrinin was produced by *M. ruber* and *purpureus*. With *M. purpureus*, the maximal productions were obtained in submerged cultures (YES medium) with a citrinin concentration of 240 mg/l or in solid state culture with a citrinin concentration of 100 mg/kg dried matter. The maximal productions were obtained using cultures in fermentor of *M. ruber* on ethanol with a concentration in citrinin of 380 g/l or in solid state culture with concentration of 300 mg/kg dried matter. The use of methionin or urea as nitrogen source instead of MSG were unfavourable to the production of citrinin but also the pigment production. Nevertheless, the concentrations of these nitrogen sources and the ways to add them to the culture medium have to be optimized.

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