

The effect of culture preservation techniques on patulin and citrinin production by *Penicillium expansum* Link

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2002/84: received 11 March 2002, revised 26 April 2002 and accepted 7 May 2002

I.M. SANTOS, L. ABRUNHOSA, A. VENÂNCIO AND N. LIMA. 2002.

Aims: To study the influence of culture preservation methods and culture conditions on the production of the mycotoxins patulin and citrinin by *Penicillium expansum*.

Methods and results: Ten strains of *Penicillium expansum* were preserved using subculture and maintenance at 4 °C, mineral oil, drying on silica gel and freeze-drying. Patulin and citrinin production was assessed on yeast extract sucrose agar (YES) and grape juice agar (GJ), using TLC before and after 0.5, 2–3, 6 and 12 months preservation. Citrinin was detected in all cultures for all preservation techniques on YES. The patulin profiles obtained differed with strain and culture media used.

Conclusions: Citrinin production seems to be a stable character for the tested strains. There is a tendency for patulin detection with time apparently more consistent for silica gel storage and freeze-drying, especially when the strains are grown on GJ.

Significance and Impact of the Study: Variability in the profiles of the mycotoxins tested seems to be more strain-specific than dependent on the preservation technique used.

INTRODUCTION

Retention of viability and of morphological and physiological stability is a fundamental trait of microorganism preservation. In this respect, it is essential to secure the long-term preservation of cultural characteristics and properties in culture collections through correct procedures (Santos and Lima 2001). Filamentous fungi, when grown in culture, exhibit a high tendency toward spontaneous change, either morphological (e.g. sectorization) or physiological (e.g. secondary metabolite production). Variants may be selected by the culture conditions and variability will also depend on the number of generations of growth. Culture collections have dealt with strain instability by developing empirical techniques with the aim of maximizing both longevity and stability of stock cultures. This can be accomplished by minimizing the number of subcultures and/or by reducing cellular activity. There are various methods available, depending on the nature of the organism and its intended future use (Ryan *et al.* 2000). Traditionally the routine assessment of fungal cultures after preservation

procedures involves the estimation of viability and the observation of morphological characters. An example is the study by Berny and Hennebert (1991) on the influence of the freeze-drying process on the stability of macroscopic and microscopic characters in *Penicillium expansum*. Smith *et al.* (1986) studied the changes in morphology and viability of hyphae of *P. expansum* during freezing and thawing. Recent investigation concerns optimization and the development of protocols for organisms considered recalcitrant to traditional techniques (Homolka *et al.* 2001; Ryan 2001) and also the maintenance of genetic, biochemical and metabolic stability (Sundari and Adholeya 2000; Ryan *et al.* 2001). Secondary metabolites have been used as chemotaxonomic criteria in the classification of terverticillate *Penicillia* (Frisvad and Filtenborg 1990). The mycotoxins patulin and citrinin have been reported as characteristic secondary metabolites of *P. expansum* (Frisvad and Filtenborg 1989), which is a common spoilage organism of fruits and vegetables. During a study of mould contamination of grapes and the assessment of the mycotoxin production capability of the isolates, Abrunhosa *et al.* (2001) isolated and screened 51 *P. expansum* strains. These strains exhibited different profiles according to the ability to produce patulin, citrinin or both in relation to the culture media used for growth and assessment. In order to evaluate the influence of different culture

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preservation techniques on the production of patulin and citrinin, we studied 10 representatives of the isolated strains before and after being submitted to subculture and maintenance at 4 °C, preservation under mineral oil, drying on silica gel and freeze-drying.

MATERIALS AND METHODS

Microorganism and culture conditions

The 10 *P. expansum* Link isolates (MUM 99-19, 99-20, 99-22–99-24 and 00-01–00-05), deposited in the Micoteca da Universidade do Minho (MUM) culture collection, were cultured on malt extract agar – Blakeslee's formulation (MEA) for 7 d at 25 °C for preservation. The preserved cultures were revived at defined time periods, of 0-5, 2–3, 6 and 12 months, using MEA, yeast extract sucrose agar (YES): 150 g l⁻¹ sucrose, 20 g l⁻¹ Difco yeast extract, 20 g l⁻¹ agar and grape juice agar (GJ): 1 kg of grapes were crushed in 1 l distilled water and boiled for 2 h. The volume was then brought to 1000 ml. The grape extract was filtered and sterilized at 110 °C for 15 min. Then, 20 g agar were dissolved in 800 ml distilled water and autoclaved. GJ agar was prepared by adding 200 ml of grape extract to the 800 ml agar solution. The medium was autoclaved at 102 °C for 5 min. After incubation for 7 d at 25 °C, the cultures were transferred to YES and GJ for mycotoxin detection.

Preservation procedures

Subculture and maintenance at 4 °C. The strains were subcultured onto fresh MEA slants every 2 months and maintained at 4 °C, in the dark.

Preservation under mineral oil. The strains were cultured on MEA slants, 30° to the horizontal, and grown at 25 °C for 7 d. They were then covered with sterile liquid paraffin, specific gravity 0-84–0-89, 10 mm above the agar surface and stored at room temperature in the dark.

Silica gel storage. Spore suspensions were prepared using cold skimmed milk 5% (w/v). The suspensions (approx. 1 ml) were added to precooled fine silica gel crystals, without indicator, in a bath of ice. The bottles were incubated at 25 °C with caps loose for 10–14 d until the crystals separated readily (Smith and Onions 1994). The cultures were then stored in the dark, at room temperature with the caps firmly closed, over indicator silica gel.

Centrifugal freeze-drying. The spore suspensions were prepared in a mixture of 10% (w/v) skimmed milk and 5% (w/v) inositol; 0-5 ml were transferred to glass ampoules and placed in the centrifuge container of a spin freeze accessory

of an Edwards High Vacuum, Modulyo drier. Primary drying proceeded for 3-5 h with centrifugation for the first 30 min. The chamber was then brought to atmospheric pressure and the ampoules were constricted with an air/gas torch. Secondary drying followed, for 17 h, over di-phosphorus pentoxide (Smith and Onions 1994). The ampoules were sealed with an air/gas torch and stored at room temperature, in the dark.

Mycotoxin detection

Mycotoxin profiles were analysed, before and after the application of the preservation techniques, using thin-layer chromatography (TLC) as described by Singh *et al.* (1991). Agar plugs were cut from 14-d-old cultures on YES and GJ, and applied to silica gel 60 TLC plates without indicator (Merck). The solvent system used was TEF (toluene : ethyl acetate : formic acid; 5 : 4 : 1, v/v/v). For patulin detection the plates were sprayed with 5 g l⁻¹ MBTH (3-methyl-2-benzothiazoline hydrazone hydrochloride), dried for 15 min and heated at 110 °C for another 15 min. Standards used were griseofulvin and patulin. GJ medium was also assessed for the mycotoxins, as a control.

RESULTS

Citrinin detection

Citrinin was easily recognizable as a yellow-green streak under ultraviolet light (366 nm) with a medium R_f value of 42. This metabolite was detected in all cultures by all techniques on YES agar but not repeatably on GJ. Before preservation, only strain MUM 00-01 produced citrinin on GJ, as observed previously by Abrunhosa *et al.* (2001). After preservation citrinin was detected on GJ also for strains MUM 99-19, 99-20, 99-23, 99-24 and 00-04, but citrinin production was not expressed consistently. The more steady behaviour seems to have been observed for the freeze-dried strains MUM 99-19 and 99-20. Strain MUM 99-19 failed to produce detectable levels of citrinin on YES after 12 months preservation by subculture and maintenance at 4 °C.

Patulin detection

Patulin exhibits a yellow spot under white light after treatment with MBTH. The recorded medium R_f value was 35. Three different profiles were encountered regarding to patulin production, represented in Table 1 by strains MUM 99-19, 99-23 and 00-05, respectively. Strains MUM 99-19, 99-20, 99-22, 99-24 and 00-01 were patulin-positive prior to preservation and this behaviour is maintained in a consistent manner throughout the experiment, regardless of the preservation technique or culture media used. Nevertheless,

Table 1 Detection of patulin for strains MUM 99-19, 99-23 and 00-05 before and after 0.5, 2–3, 6 and 12 months (M) preservation by subculture (SC), mineral oil (MO), silica-gel (SG) and freeze-drying (FD)

Strain	Time (M) Method	Culture medium																			
		MEA/YES					MEA/GJ					YES/YES					GJ/GJ				
		0	0.5	2*–3	6	12	0	0.5	2*–3	6	12	0	0.5	2*–3	6	12	0	0.5	2*–3	6	12
99-19	SC	+	nd	+	+	–	+	nd	+	+	–	+	nd	–	+	–	+	nd	+	+	–
	MO	+	+	–	+	–	+	+	–	+	–	+	+	+	+	–	+	+	+	–	–
	SG	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	FD	+	+	–	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
99-23	SC	–	nd	+	+	–	+	nd	+	+	+	–	nd	+	+	–	+	nd	+	+	+
	MO	–	–	–	–	–	+	+	+	+	–	–	–	+	+	–	+	+	+	+	–
	SG	–	–	+	+	–	+	+	+	+	–	–	+	+	+	+	+	+	+	+	+
	FD	–	+	+	+	+	+	+	+	+	–	+	+	+	+	+	+	+	+	+	+
00-05	SC	–	nd	+	+	–	–	nd	–	+	+	–	nd	–	–	+	–	nd	+	+	+
	MO	–	–	–	+	–	–	+	+	–	–	–	+	–	+	–	–	–	+	–	–
	SG	–	+	–	+	–	–	+	–	+	–	–	–	–	+	+	–	+	+	+	+
	FD	–	+	–	+	+	–	+	–	+	+	–	+	–	+	+	–	–	+	+	+

*Subculture only; nd: not determined.

silica gel and freeze-drying seem to give more stable results, particularly if the strains are grown on GJ for mycotoxin assessment. In the particular case of strain MUM 99-19 patulin was not detected in either culture media after 12 months of subculture and maintenance at 4 °C. A tendency towards non-detection of patulin also applies to storage under mineral oil. In contrast, strain MUM 99-23 (Table 1) was originally patulin-negative in YES medium, whereas in GJ patulin was always detected with the exception of 12 months' preservation under mineral oil. In YES medium the tendency is towards patulin detection with time. This is extremely consistent for the freeze-dried samples. Finally, in the case of strains MUM 00-05 and 00-02–00-04, patulin was not detected in either culture media before preservation. Patulin is, however, detected after preservation but with alternating patterns. A more homogeneous behaviour seems to be found when cultures are revived and cultured on GJ in the case of freeze-drying and silica gel storage. All the 10 strains studied failed to produce detectable levels of patulin after 12 months' preservation under mineral oil. Neither patulin nor citrinin were detected on the GJ control. Furthermore, all the strains were viable after 1-year preservation for all the tested methods.

DISCUSSION

After preservation of *P. expansum* using several techniques, citrinin production seems to be a stable character for the studied strains. However, loss of mycotoxin production is visible in strain MUM 99-19, preserved by subculture and

maintenance at 4 °C, for citrinin. The same happens for patulin when this strain is preserved by subculture and maintenance at 4 °C or under mineral oil. Loss of secondary metabolite production has been given as an example of strain instability in filamentous fungi (Shuba and Bennet 1992). After 6 and 12 months of subculture and maintenance at 4 °C, colonies on MEA also showed variation through sectorization and the development of a lanose texture.

Where patulin detection is concerned, three distinct profiles were observed according to strain and culture media used (Table 1). Both YES and GJ gave positive results for some strains, represented in Table 1 by strain MUM 99-19, but not for others. It is also clear that there was a tendency for patulin detection after preservation where production was not originally expressed. Secondary metabolite synthesis is often related to the depletion of nutrients (Griffin 1994) and with possible ecological roles in nature, as well as in, or at least coincident with, differentiation (Betina 1989). Here, mycotoxin synthesis seems to appear as a response to preservation. This may account for the fact that long-term deposits of *P. expansum* strains in culture collections, which have not become atypical with possible loss of mycotoxin production, are often regarded as patulin and citrinin producers.

Considering the three distinct behavioural groups found for patulin and the overall results obtained, variability in the profiles of the mycotoxins tested seems to be more strain-specific than dependent on the preservation technique used. Evidence supporting the assumption that response to preservation may be strain-specific was also found by Ryan *et al.* (2001). Nevertheless, and although there is no marked

evidence, higher consistency seems to be found for freeze-drying and silica gel storage, especially in the case of patulin, if the strains are cultured and/or revived on GJ. The best preservation method is considered to be the one where no growth and reproduction can take place, but where all the structural and functional characteristics are retained (Reusser 1963). While maintenance by subculturing and mineral oil storage allow for growth and metabolism to a higher or lesser degree, drying on silica gel, freeze-drying and cryopreservation below $-140\text{ }^{\circ}\text{C}$ are recommended as the most appropriate methods for the long-term preservation of sporulating filamentous fungi (Smith 1993). In this study, loss of secondary metabolite production occurred in subculture and maintenance at $4\text{ }^{\circ}\text{C}$ and storage under mineral oil, whereas silica gel storage and freeze-drying are the methods that present higher consistency in the cases where secondary metabolite production appears as a response to preservation. Taking into account the different responses that may be obtained when using different preservation techniques, it is always advisable to use more than one method, particularly if industrial or test strains are at stake.

The results also show that there is a potential for patulin detection on natural culture media. Some of the strains showed a marked preference for GJ. This is important when assessing the probability of mycotoxin production in natural substrates. It has been shown that grapes are able to support patulin production by natural fungal populations (Scott *et al.* 1977). Abrunhosa *et al.* (2001) reported on 65% *P. expansum* isolates positive for patulin on GJ against 20% positive on YES. The fact that citrinin is preferentially produced in YES is probably related to the composition of the culture media, as citrinin is not generally detected in grape products or is unstable (Scott *et al.* 1977).

Research activities often involve extensive isolation, identification and screening programmes. Immediate testing of all the isolates is not always possible. Isolated strains may be maintained as active cultures at $4\text{ }^{\circ}\text{C}$, transferred to fresh culture medium or preserved, before being tested. Also, rational screening programmes may be conducted on long-term deposits from culture collections. It is necessary to bear in mind that culture conditions, preservation procedures and the period of preservation may affect strain performance.

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