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RAPID DETERMINATION OF SPOILAGE FUNGI

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SUMMARY

Mixed cultures of viable spores of fungi of the Aspergillaceae were determined rapidly by gas–liquid chromatography of the methanol released from pectin by the enzyme pectinesterase. Results obtained in 19 h were comparable with plate counts taking 48 h. Attempts to count spoilage yeasts using this method were not successful.

INTRODUCTION

Spoilage fungi of the Aspergillaceae may be detected by plate counts taking several days[1] or by chitin analysis, which does not distinguish between viable and non-viable spores[2].

The method of Offem and Dart[3] utilized the enzyme pectinesterase (E.C. 3.1.1.11), which is found widely in spoilage fungi[4]. The methanol formed from pectin by this enzyme can be assayed by gas–liquid chromatography[5] and used to determine viable spores of the genera *Aspergillus*[3] and *Penicillium*[6]. The work has been extended to cover mixed cultures of the Aspergillaceae and also a number of yeasts found in spoilage situations.

EXPERIMENTAL

Organisms and media

The organisms *Aspergillus flavus* 15959, 39178a and 86769, *A. niger* 31821, *A. nidulans* 16643, *A. luchensis* Inui 83356, *Penicillium expansum* 39761, *P. lividum* 99648, *P. brevicompactum* 92034, *P. chrysogenum* 26211 and *P. martensii* 91020 were obtained from the Commonwealth Mycological Institute, Kew, Surrey (Great Britain).

The yeasts *Saccharomyces fragilis* 587, *Cryptococcus albidus* 445, *Endomycopsis chodati* 440, *Pichia farinosa* 386, *Candida pseudotropicalis* and *Hansenula anomala* 432 were obtained from the National Collection of Yeast Cultures, Brewing Research Foundation, Nutfield, Redhill, Surrey (Great Britain).

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The maintenance of cultures of the Aspergillaceae, their growth and the preparation of spores has been described by Offem and Dart[3].

The yeasts were maintained at 4°C on slopes of the following medium: malt extract 3 g, yeast extract 3 g, glucose 10 g, neutralized soya peptone 5 g, agar 20 g and distilled water to 1 l (pH 5.4).

Yeast cells were cultivated on 250-ml portions of the same medium in Roux bottles for 3 weeks. The cells were harvested by washing the medium with sterile water, centrifuging and resuspending the cells at 4°C until required.

Production and determination of methanol

The assay medium for the production of methanol has been described earlier[3]. Methanol produced during the incubation period was separated from the germinating spores by a modification of the dialysis cell method of Lee and Wiley[5].

Methanol was determined by gas–liquid chromatography on a Pye 104 chromatograph using a hydrogen flame-ionization detector. The column (180 cm × 6 mm I.D.) was packed with Porapak Q (80–100 mesh). The column was operated isothermally at 110°C with the detector system at 140°C using nitrogen (flow-rate 20 ml/min) as the carrier gas.

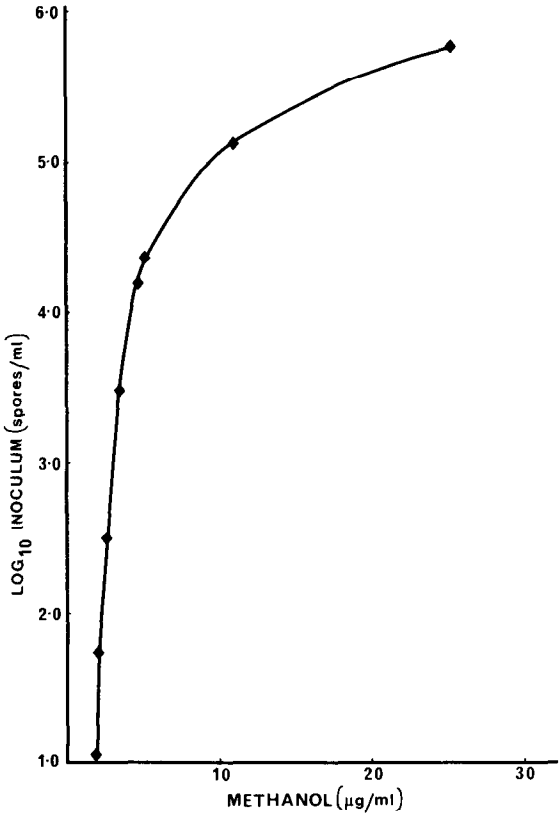

Fig. 1. Influence of inoculum size on methanol production in *Penicillium brevicompactum*.

Methanol was determined by triangulation[7] of the test peak compared with that of a standard sample.

Procedure

Spore suspensions of each species of the Aspergillaceae were prepared in the range 10–7 × 10^5 spores/ml by random dilutions of a master spore suspension and were then shaken mechanically for 20 min to disperse clumps. Yeast cell suspensions in the same range were prepared by diluting the master suspension.

A portion of each sample was then subjected to the procedure previously described[3], and the methanol concentration obtained was expressed as micrograms per millilitre of assay medium per milligram dry weight of mycelium.

RESULTS

Results of the type shown in Fig. 1 were obtained for ten species of Aspergillaceae. These results were converted into calibration graphs for *Aspergillus*[3] and *Penicillium*[6] and can be used to give a good estimate of the number of viable spores. The species not conforming to this pattern was *P. martensii*, which produced methanol and a second peak tentatively identified as ethanol. The two sets of data for *Aspergillus* and *Penicillium* appeared similar and a statistical examination by the principle of comparison of several regression lines[8] indicated that the ten species of Aspergillaceae gave results which are estimates of the same straight line (Table I). The data obtained for these ten species were therefore pooled and plotted as one calibration graph (Fig. 2), which could be expressed by the equation $y = 1.079 - 6.86x$ with a correlation coefficient of -0.96. This calibration graph was then used to count random dilutions of mixed spore suspensions, and the results obtained are shown in Table II.

Three of the yeast strains (*Saccharomyces fragilis*, *Cryptococcus albidus* and *Endomycopsis chodati*) produced methanol from pectin. The other three yeasts did

TABLE I

COMPARISON OF RESULTS OBTAINED FROM SIX SPECIES OF *ASPERGILLUS* AND FOUR SPECIES OF *PENICILLIUM* BY PLOTTING LOG (DRY WEIGHT OF SPORE INOCULUM) AGAINST RECIPROCOL OF THE CONCENTRATION OF METHANOL PRODUCED

The species not used was *P. martensii*

Organism	*Slope*	*Intercept*	*Correlation coefficient*
P. expansum	−7.39	1.08	−0.96
P. chrysogenum	−7.29	1.15	−0.91
P. lividum	−7.23	0.99	−0.94
P. brevicompactum	−7.54	1.13	−0.93
A. flavus 39178a	−6.62	1.03	−0.99
A. flavus 15959	−6.41	1.076	−0.99
A. flavus 86769	−6.82	1.127	−0.98
A. niger	−6.64	1.098	−0.99
A. luchuensis Inui	−6.65	1.094	−0.99
A. nidulans	−7.06	1.106	−0.98
All ten organisms	−6.86	1.079	−0.96

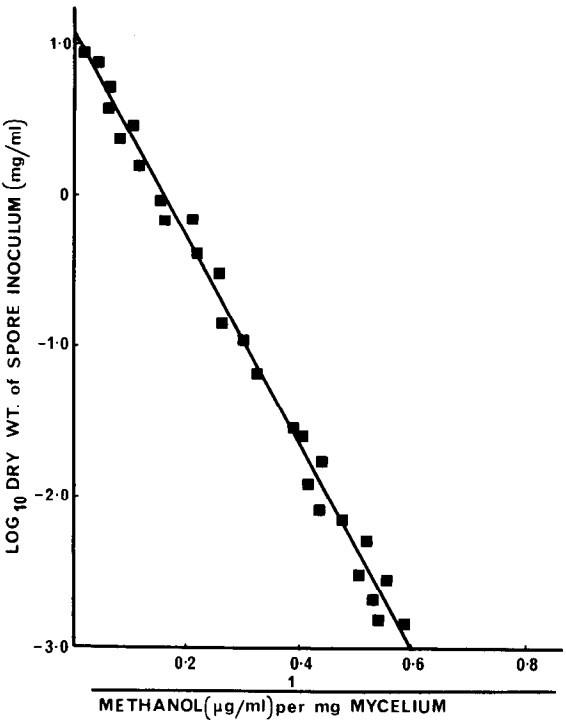

Fig. 2. Calibration data obtained using data from all species except *P. martensii*.

TABLE II

COMPARISON OF ESTIMATES OF SPORE NUMBERS IN MIXED CULTURES OF *ASPERGILLUS* AND *PENICILLIUM* BY THE SURFACE PLATE METHOD AND THE METHANOL METHOD

Each set of results is the mean of five determinations on the same sample.

Composition of spore mixture	*Sample No.*	*Number of spores/ml (mean ± S.D.)*	
		Surface plate count	*Methanol method*
A. flavus 15959	1	240 ± 14	240 ± 22
and	2	1440 ± 39	1514 ± 90
P. chrysogenum	3	15,700 ± 220	15,402 ± 305
All six *Aspergillus*	1	142 ± 14	150 ± 16
strains plus	2	1100 ± 31	1094 ± 103
P. chrysogenum	3	57,400 ± 190	57,662 ± 658
Four *Penicillium*	1	288 ± 11	282 ± 12
strains plus	2	2490 ± 90	2471 ± 48
A. flavus 15959	3	32,500 ± 220	32,665 ± 400
(*P. martensii* was omitted)			
Four *Penicillium*	1	104 ± 14	101 ± 17
strains plus all	2	5100 ± 50	5071 ± 47
six *Aspergillus* strains	3	74,200 ± 3300	41,158 ± 9694
(*P. martensii* was omitted)			

not produce methanol. The three strains producing methanol did not obey the relationship determined for the Aspergillaceae. No relationship of any type could be found between yeast cell numbers and the amount of methanol produced and there was no similarity between the methanol production of the three strains.

DISCUSSION

The pectinesterase method developed by Offem and Dart[3] gave significant results when utilized for a single species or a mixture of species from one genus. This, however, is a situation unlikely to be found *in vivo* and when it was realised that most of the earlier results could be represented by a single calibration graph, it was decided to repeat the work using mixed cultures from different genera.

The results obtained (Table II) indicate that the calibration graph (Fig. 2) may be utilized to count mixed cultures and at low concentrations the results are comparable to those obtained by plate counts taking 48 h. At high spore counts, some discrepancies between the two methods are found that do not occur when pure cultures are used[3,6]. The reason for these discrepancies was not established but it may be due to members of one genus producing chemicals inhibitory to the germination of the other genus.

As most of the species of *Penicillium* and *Aspergillus* tested obey the relationship determined, it is not necessary to grow the organisms for a sufficiently long period to determine which genus of species is present. The method could be related to a worst prediction basis, that is, the assumption could be made that all the species are toxigenic.

Only three of the six yeast species tested produced methanol from pectin and even these three did not follow the relationship determined for the Aspergillaceae. One possible explanation is that the methanol produced is metabolized by the yeast. However, it has been reported that yeast species are not a significant factor in the deterioration of stored grain although they are of importance in the deterioration of fruit[9].

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