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RAPID METHOD OF ESTIMATING VIABLE SPORES OF *ASPERGILLUS*

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SUMMARY

A rapid method of counting viable spores of spoilage fungi has been developed The method is based on the production of the enzyme pectinesterase during germination of the spores This enzyme hydrolyses the ester linkages of pectin to produce methanol which can be assayed by gas–liquid chromatography

A linear relationship can be established between the methanol produced and the spore concentration. The relationship is common to the six species of *Aspergillus* tested. Results obtained by this method in 19 h compare closely with plate counts of *Aspergillus* obtained at 48 h.

INTRODUCTION

Fungi of the genus *Aspergillus* are found widely in a variety of agricultural produce, where they are frequently present as dormant spores which may germinate under inadequate storage conditions and cause spoilage

Several types of spoilage may take place, the most important being the production of various toxins, especially the aflatoxins Non-toxigenic strains may also cause spoilage by production of heat leading to a loss of seed viability, alteration of flavour components and also by causing hydrolysis of triglycerides to free fatty acids. These are subsequently desaturated and subjected to autoxidation, thus leading to rancidity Considerable financial losses are caused by these fungi, especially in some developing countries where storage facilities and transport networks are inadequate and the ambient temperature and humidity are both high

Traditional methods for the estimation of fungi consist of plate counts, which may take 7 days or more depending upon the species[1] Such methods are of little value in a modern produce control laboratory, as toxigenic strains would have commenced toxin synthesis during this time period.

It is obvious that there is a great need for a rapid method of counting fungal spores and predicting whether or not they are viable

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Many species of the genus *Aspergillus* produce pectin degrading enzymes when grown in pectin-containing medium[2,3]. The enzyme pectinesterase (E C. 3.1.1.11) hydrolyses pectin to pectic acid and methanol. The enzyme may be measured by the titrimetric estimation of the carboxylic acid groups of pectic acid[4], or the methanol liberated may be measured either as formaldehyde[5] or by gas chromatography[6,7].

This work utilizes the activity of the pectinesterase found after 15 h germination to determine the number of viable dormant spores of six *Aspergillus* species known to cause spoilage.

MATERIALS AND METHODS

Organisms and media

The six species of *Aspergillus* used were obtained from the Commonwealth Mycological Institute (Ferry Lane, Kew, Great Britain) They were *A. flavus* 15959, 39178a, 86769, *A. niger* 31821, *A. nidulans* 16643 and *A. luchuensis* Inui 83356

Cultures were maintained by growth on slopes of the following medium; maltose, 38 g; neutralized soya peptone, 8 g; yeast extract, 2 5 g; malt extract, 2 g; Agar technical No. 3 (Oxoid), 20 g. These were dissolved in 1 l of distilled water and the pH adjusted to 5 4 before autoclaving Cultures were incubated at 30°C for 5 days and then stored at 4°C until required Subcultures were prepared at intervals of 4 weeks.

Organisms were grown on the following medium to obtain spores: KH_2PO_4, 2 0 g; KNO_3, 2 0 g; $CaCl_2$, 0 25 g; yeast extract, 5.0 g; glucose, 10.0 g; bactocasamino acids, 5.0 g; metals solution, 0.1 ml; distilled water, to 1 l. The medium was adjusted to pH 5 4 before autoclaving. The metals solution was made up as follows. $MgSO_4$, 0.5 g; $ZnSO_4$, 0.02 g; $FeSO_4$, 0 02 g; $MnSO_4$, 0 01 g; $CuSO_4$, 0.05 g; water, to 10 ml.

The medium for the assay of methanol was made up as follows: glucose, 4 g; pectin (citrus pectin, rapid set-type 104; Bulmer Ltd , Hereford, Great Britain), 1 g, soya peptone, 1 g; chloramphenicol, 100 mg; chlortetracycline, 100 mg; 1% phosphate buffer pH 7 0, to 100 ml.

Plate counts for viable spores were carried out on the following medium: maltose, 38 g; yeast extract, 2.5 g; mycological peptone, 8 g; malt extract, 2.0 g; agar, 20 g; distilled water, to 1 l. The medium was adjusted to pH 5.4 before autoclaving

Growth of spores

The sporulation medium (200 ml) was placed in Roux flasks, inoculated with a loopful of organisms from the slopes and incubated at 30°C for 3 weeks At the end of this period, a thick mycelial mat covered with spores was obtained. A spore suspension was obtained[8] and microscopic examination was carried out to ensure the absence of mycelial contamination.

The spores were stored as stock suspensions at 0°C until required, and to maintain a high level of viable spores fresh stock suspensions were prepared every 3 months.

Before use the spore suspensions underwent heat shock activation at 50°C for 25 min[9].

Production of methanol

Aliquots (100 ml) of the assay medium were placed in 500-ml Erlenmeyer

flasks. Fifty ml of spore inoculum were added and the flasks were incubated at 30°C on an orbital incubator at a speed of 150 rpm for 15 h. After this time, fungal germination and pectinesterase activity were stopped by placing the flasks in a water-bath at 80°C for 10 min Control flasks were inoculated with 50 ml of sterile distilled water.

The methanol produced was separated from the growth medium by dialysis across a Visking membrane using a modification of the dialysis cell method of Lee and Wiley[6] The dialysis cell consists of two poly(methyl methacrylate) half-cells separated by a Visking membrane. Each half-cell has an injection port and a cavity with a volume of 5 ml The contacting surfaces were smeared lightly with silicone grease and the front half of each cell was loaded with 3.0 ml of culture medium The rear half of each cell was loaded with an equal volume of distilled water and the injection ports were sealed The cells were then rotated on a revolving power unit at 70 rpm and 30°C for 4 h At the end of this time the concentration of methanol in the two halves of the cell is equal

Estimation of methanol

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

A sample of 10 μl was injected and the methanol was determined from peak areas calculated by triangulation[10]. An equal volume of a standard solution of purified methanol was injected into the column before and after each test sample, and the amount of methanol in the test sample was then calculated Each run lasts approximately 5 min

Experimental procedure

The master spore suspension for each organism was randomly diluted 10–$7 \cdot 10^5$ spores per ml Each spore suspension was then mechanically shaken for 20 min to disperse clumps Aliquots of each sample were subjected to the following sequence

(a) A total spore count using a Helber chamber.

(b) A plate count for viable spores using the medium described earlier

(c) A dry weight determination of the spores in each test sample

(d) Portions (50 ml) of the spore suspension were then inoculated aseptically into 100 ml of the methanol assay medkum and shaken at 30°C for 15 h

(e) An aliquot of (d) was taken and the dry weight of the growing mycelium after 15 h incubation was determined.

(f) An aliquot of (d) was taken and the methanol was determined after 15 h incubation. Methanol production could then be described as μg/ml per mg dry weight of mycelium

RESULTS

The production of methanol as a function of the size of spore inoculum for each strain is shown in Figs 1–6.

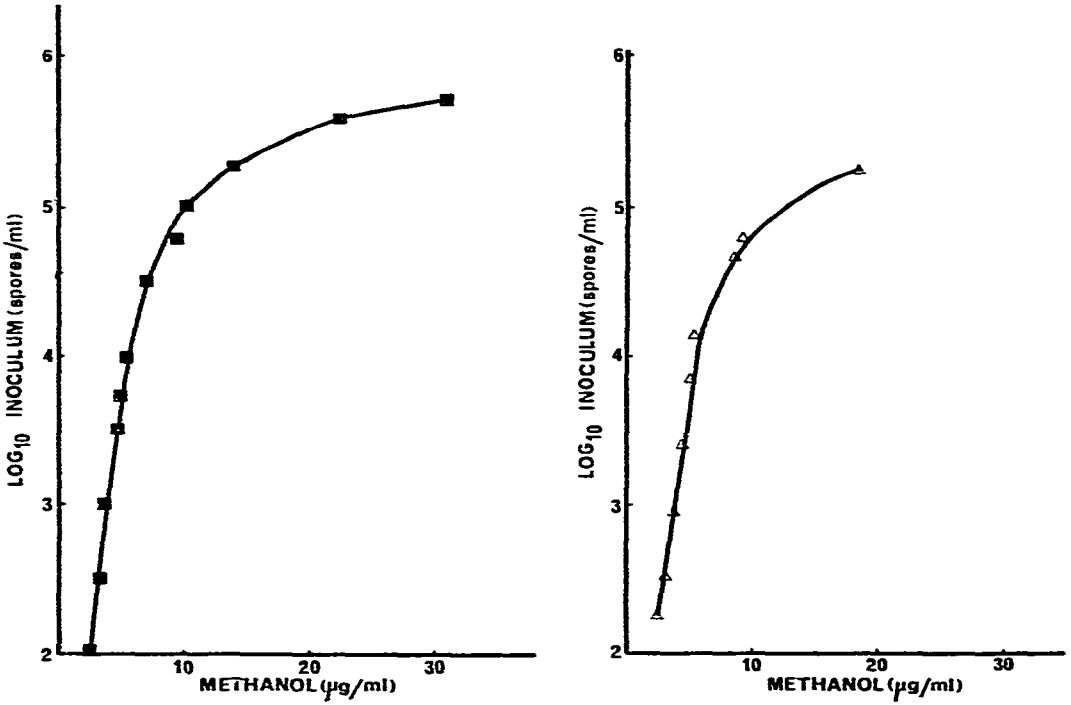

Fig. 1. The influence of inoculum size on methanol production for *A niger*. See text for experimental procedure.

Fig. 2. The influence of inoculum size on methanol production for *A flavus* 15959 See text for experimental procedure.

The results show that an increasing spore concentration liberates an increasing amount of methanol into the medium, reaching an upper limit when the spore concentration is infinitely large. As no methanol is produced when sterile water is used as inoculum, then the curve will pass through both axes.

The results shown in Figs. 1–6 can therefore be described by the equation[11]

$$y = Ce^{-\frac{B}{x}} \tag{1}$$

where $y = \log_{10}$ of the initial spore number per ml, x = amount of methanol released at the end of the incubation period (μg/ml) and C, e and B are all constants.

Eqn. 1 may be transformed to

$$\log_{10} y = \log_{10} C - \frac{B}{x} \tag{2}$$

and a plot of $\log_{10} y$ against $1/x$ should give a straight line. When this was done using the results shown in Figs. 1–6, straight line plots were obtained whose slopes and intercepts are shown in Table I. The six lines were almost parallel, the slopes having a

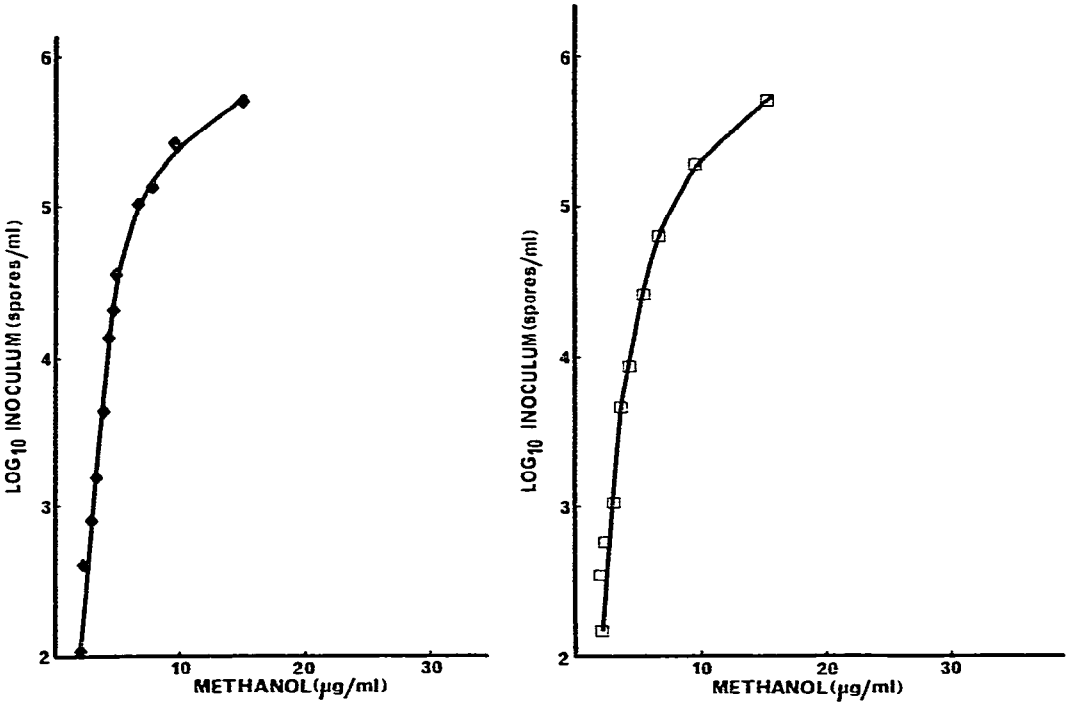

Fig. 3 The influence of inoculum size on methanol production for *A flavus* 86769 See text for experimental procedure

Fig 4 The influence of inoculum size on methanol production for *A flavus* 39178a See text for experimental procedure

standard deviation of $-1.2576 \pm 0\,063$. The intercepts were not identical and correspond to spore numbers ranging from $4\,7 \times 10^4$ to $7\,2 \times 10^5$

It was thought that the methanol production by various strains might be a more consistent property if it were related to some character indicating the germination rate of the spores, for example, protein content or mycelial dry weight at 15 h (the period of incubation).

This was carried out measuring methanol production per mg of dry weight of mycelium at 15 h incubation The results for individual species are shown in Table II using both $\log_{10}$ dry weight of spore material and $\log_{10}$ $\log_{10}$ spore numbers *versus* the reciprocal of methanol production.

The results were then pooled and plotted as a single line (Table II and Fig 7) for each set of data.

Once the level of methanol produced has been established these relationships may be used to determine spore numbers in an inoculum. The results obtained (Table III) are in good agreement with those obtained by standard surface plate methods which took 48 h.

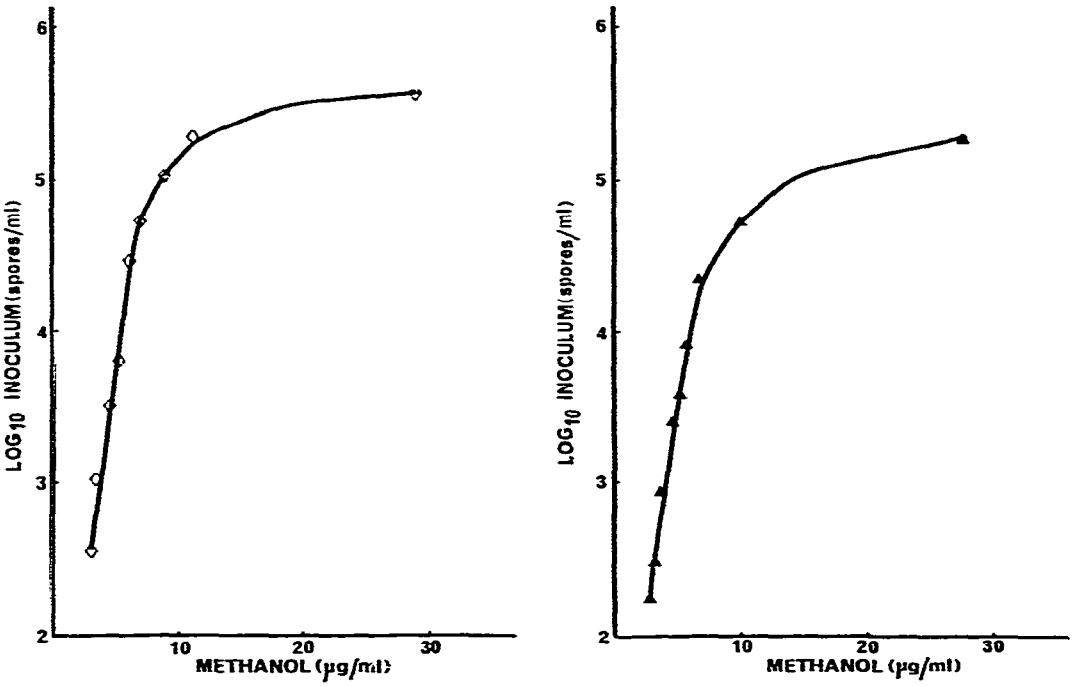

Fig. 5. The influence of inoculum size on methanol production for *A nidulans* See text for experimental procedure.

Fig. 6 The influence of inoculum size on methanol production for *A luchuensis* Inui See text for experimental procedure

DISCUSSION

The presence of spoilage fungi in grain and cereals represents a major problem for industries producing or using these commodities. Due to the toxic nature of some fungal metabolites, rapid analysis for the purpose of quality control is obviously highly desirable

One method for the rapid estimation of fungi is the determination of

TABLE I

COMPARISON OF THE RESULTS SHOWN IN FIGS 1–6 PLOTTED AS LOG_{10} LOG_{10} SPORE NUMBERS AGAINST THE RECIPROCAL OF METHANOL PRODUCTION USING EQN 2

Organism	*Slope*	*Intercept*	*Correlation coefficient*	*Standard deviation*
A. flavus 39178a	−1.228	0 883	−0 946	0 023
A. niger	−1 328	0 842	−0 952	0 021
A. flavus 15959	−1.305	0 851	−0 921	0 026
A. luchuensis Inui	−1.75	0 816	−0 914	0 016
A. flavus 86769	−1.249	0 913	−0.942	0 02
A. nidulans	−1.160	0 853	−0 937	0 011

TABLE II

COMPARISON OF THE RESULTS OF PLOTTING LOG_{10} LOG_{10} SPORE NUMBER OR LOG MYCELIAL DRY WEIGHT AGAINST THE RECIPROCAL OF METHANOL PRODUCTION

Organism	*log_{10} dry weight vs reciprocal methanol*			*log_{10} log_{10} (spore number per ml) vs reciprocal methanol*		
	Slope	*Intercept*	*Correlation coefficient*	*Slope*	*Intercept*	*Correlation coefficient*
A flavus 39178a	−6 62	1 034	−0 993	−0 741	0 701	−0 975
A niger	−6 64	1 098	−0 999	−0 725	0 706	−0 968
A flavus 15959	−6 41	1 076	−0 995	−0 721	0 704	−0 976
A luchuensis Inui	−6 55	1 094	−0 997	−0 712	0 704	−0 984
A flavus 86769	−6 82	1 127	−0 986	−0 733	0 703	−0 979
A nidulans	−7 06	1 106	−0 987	−0 745	0 710	−0 973
All six Organisms	−6 727	1 078	−0 988	−0.739	0 706	−0 963

chitin[12–15]. This method is rapid, requiring 4–5 h, but it suffers from several serious drawbacks High levels of contamination are required to produce sufficient chitin for analysis, and the method does not distinguish viable from non-viable spores. A further problem is that the chitin found in insects which frequently infest stored products would interfere with the use of chitin as an index of fungal contamination

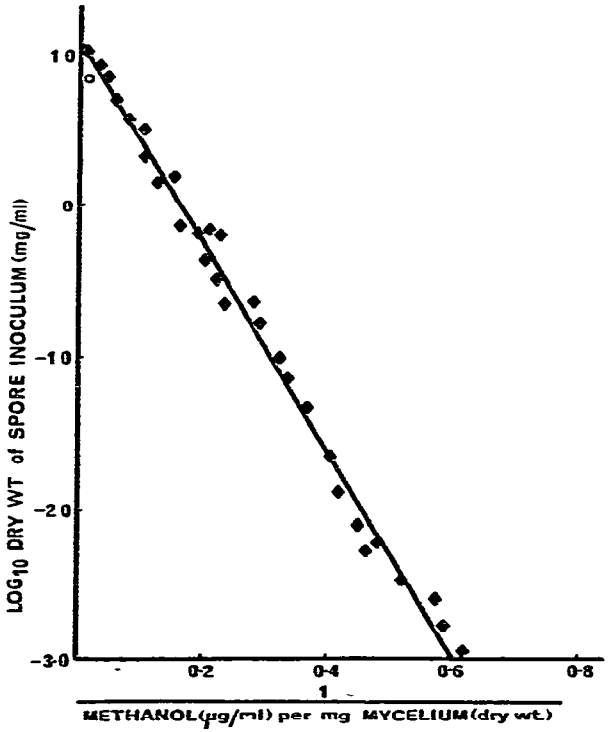

Fig. 7 Calibration curve obtained using data from all six species. The calibration curve is based on eqn 2.

TABLE III

COMPARISON OF SPORE NUMBERS IN SPORE SUSPENSIONS BETWEEN SURFACE PLATE METHOD AND USING DRY WEIGHT OF SPORES AND LOG_{10} LOG_{10} SPORE NUMBERS

Two suspensions of each of the six organisms were prepared by random dilutions. In addition two suspensions were prepared by mixing spores of all six organisms at random. When using the dry weight method a calibration curve of dry weight and viable spore number is necessary.

Organism		*Spore numbers per ml (mean ± standard deviation)*		
		Surface plate method	*Use of spore dry weight*	*Use of log_{10} log_{10} spore numbers*
A. flavus	1	950 ± 350	1123 ± 372	1386 ± 544
39178a	2	1100 ± 270	1002 ± 274	1295 ± 308
A. niger	1	190 ± 47	205 ± 53	312 ± 104
	2	4800 ± 420	5041 ± 480	4606 ± 608
A. flavus	1	11000 ± 2700	10217 ± 2729	9711 ± 3063
15959	2	600 ± 130	689 ± 160	874 ± 187
A. luchuensis	1	8600 ± 1100	9182 ± 1504	12248 ± 4338
Inui	2	38000 ± 6400	36815 ± 5775	42253 ± 6280
A. flavus	1	180 ± 100	270 ± 99	264 ± 119
86769	2	77000 ± 13800	91115 ± 8851	105056 ± 24630
A. nidulans	1	840 ± 152	915 ± 186	1243 ± 331
	2	570000 ± 56000	562500 ± 62250	510700 ± 46175
Mixture of all	1	47000 ± 11600	57915 ± 12777	64103 ± 12193
6 organisms	2	990 ± 320	1195 ± 308	1632 ± 546
Time		48 h	19 h	19 h

The method described in this paper, although not as rapid as the chitin method, has the advantage of distinguishing viable from non-viable spores, and as it measures dormant viable spores capable of germination it is also predictive, *i e.*, it can give an indication of possible sources of contamination in the future. A further advantage is that it is linear over a wide concentration range of spores, thus removing the need for numerous dilutions.

The results (Table II) show that there is a good correlation (−0.988) between inoculum size (in mg dry weight) and methanol production when all the results are pooled. Although the correlation is not so good for spore numbers (−0.963, Table II), it is more convenient as it gives a direct count of viable spores without the need for a calibration curve. It also allows the estimation of lower numbers of spores than the dry weight method due to the difficulties in obtaining accurate dry weight measurements when only a small number of spores are involved.

An examination of Table III shows that the results obtained by this method are in good agreement with those obtained by traditional plate counts

A useful point to make is that as the results for all six species of *Aspergillus* are so similar, it is therefore not necessary to identify the organism to species level when counting. A count may be made on a worst possible prediction basis, *i.e.*, the assumption can be made that all species are toxigenic and the material can be accepted or rejected on the basis of the spore count alone.

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