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The inhibition of the development of the spores of a spoilage mould by chemical preservatives

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The swelling characteristics of developing spores from a variety of spoilage moulds have been examined using the Coulter Counter. *P. spinulosum* spores began swelling earliest and had the most rapid swelling rate. For these reasons these spores were the most suitable for rapid electronic sizing and were used to assess preservative efficiency. The preservatives examined were propylhydroxybenzoate, Phenonip (a mixture of esters of hydroxybenzoic acid and β-phenoxyethylalcohol, Nipa Laboratories Ltd.) and benzoic acid. The first two preservatives reduce the rate of swelling of germinating spores at its onset while benzoic acid increases the time before swelling begins. Suppression of spore swelling provides early indication of preservative potential and the type of swelling curve produced indicates the mechanism of fungistasis.

The swelling of mould spores during germination and immediately preceding emergence is characteristic of most species, an exception being the *Erysiphaceae* (Brodie & Neufeld, 1942). This early development is suppressed by antimicrobial agents and measurement of changes in volume of spores has been used for rapid assessment of fungitoxicity by Mandels & Darby (1953). They determined spore volume using haematocrit tubes. Controlled conditions of deposition were essential because errors could result when the spores packed unevenly, a situation exacerbated by the production of germ tubes. Volume changes of spores during germination were determined with greater sensitivity using a microscope (Barnes & Parker, 1966).

Unlike the volume, the average diameter of developing spores increases linearly with time in agreement with the theory of mould growth kinetics of Emerson (1950), and this linear increase allows statistical and comparative estimations of fungistatic activity to be made. A refinement of the measuring technique was introduced by Barnes & Parker (1967), who used the Coulter Counter for automatic size analysis of spores.

Our previous work, confined to *Trichoderma* species, indicated that the type of swelling curve obtained in preservative systems provided insight into the mechanism of fungistasis. We now describe the swelling characteristics of spores of several species of spoilage mould and the use of one of these for fungitoxic assays. The effects of some preservatives on the chosen spore have been examined.

EXPERIMENTAL

Moulds. These were isolated from a variety of spoiled cosmetic and pharmaceutical preparations and characterized as *Penicillium spinulosum*, *Penicillium roqueforte*, *Aspergillus versicolor*, *Syncephalastrum racemosum*, *Mucor spinosus* and *Trichoderma viride*.

Spore suspensions were prepared by the method of Barnes & Parker (1968) from the moulds grown on Oxoid malt extract agar for 21 days at 25°.

Methods

Sizing of spores. Spores developing in static malt broth cultures at 25° were sized, using a Coulter Counter Model B (with a Model J plotter), as described by Parker, Barnes & Bradley (1966).

Linear growth determinations. The method of Mandels & Darby (1953) was used; the inoculum placed centrally on the surface of a wort agar (Oxoid) plate. Plates were incubated at 25° and periodic measurements of the developing colonies were made using calipers to gauge two diameters at right angles. Three replicate plates were used for each determination.

Preservatives. Solutions were prepared in sterile water of benzoic acid B.P., propylhydroxybenzoate B.P. and Phenonip (the trade name for an undefined mixture of esters of hydroxybenzoic acid and β-phenoxyethylalcohol, Nipa Laboratories Ltd.).

To measure their effects upon spore swelling, solutions of preservatives at double the overall required concentrations were mixed with aliquots of double strength malt broth as previously described (Parker & others, 1966). The pH of all reaction mixtures was measured.

To measure preservative effects upon linear growth, double strength solutions were mixed with double strength wort agar.

RESULTS

The rate of swelling of the various mould spores in static malt broth cultures is shown in Fig. 1.

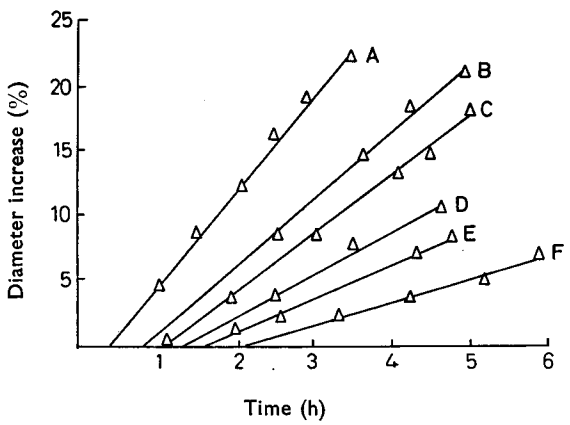

FIG. 1. Rates of spore swelling in malt broth measured with the Coulter Counter.
A, *P. spinulosum*; B, *S. racemosum*; C, *T. viride*; D, *P. roqueforte*; E, *A. versicolor*; F, *M. spinosus*.

P. spinulosum was chosen as the test organism for fungitoxic determinations because its spores showed the greatest size increase in the shortest time.

The effects of preservatives at various concentrations upon the swelling of *P. spinulosum* spores are shown in Figs 2A, B and 3. The pH values of the preservative–broth systems were all in the range 4–4·5.

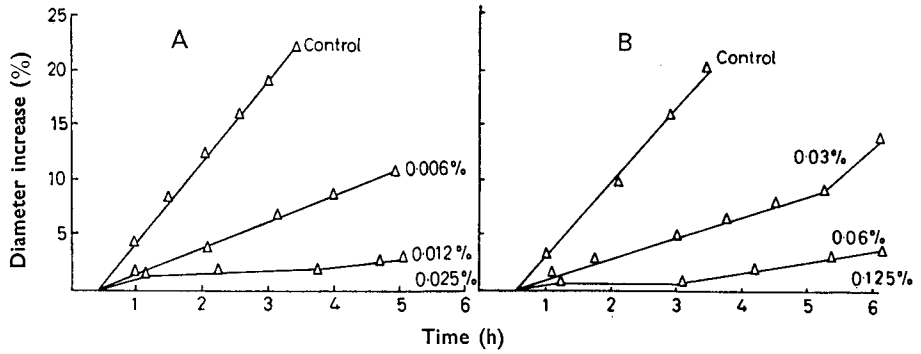

FIG. 2. The effect of (A) propylhydroxybenzoate and (B) Phenonip upon the metabolic swelling of *P. spinulosum*. Concentrations of preservative expressed as % w/v.

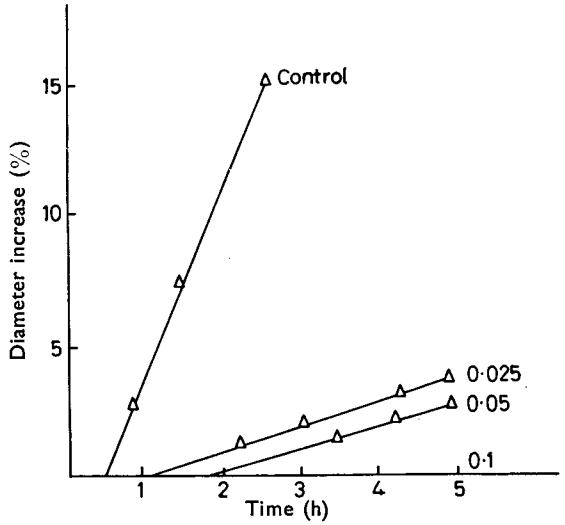

FIG. 3. The effect of benzoic acid upon metabolic swelling of *P. spinulosum*. Concentrations of preservative expressed as % w/v.

Linear growth rates of colonies from the fastest (*P. spinulosum*) and the slowest (*M. spinosus*) developing spores are shown in Fig. 4. The effect of preservatives upon linear growth rate of *P. spinulosum* is shown in Fig. 4b–d.

DISCUSSION

The spores of all the moulds examined showed a linear increase in diameter with time. Such swelling when measured optically is detected immediately (Barnes & Parker, 1966), but when using the Coulter Counter there is some delay before it is apparent. The duration of this delay ranges from 25 min for spores of *P. spinulosum* to 120 min for *M. spinosus* (Fig. 1).

It has been suggested that there is a period of spore swelling in the malt broth when the osmotic pressure of the spore contents is attaining a value isotonic with the saline electrolyte used in electronic sizing. Until this isotonicity is reached, swelling will not be detected electronically (Barnes & Parker, 1967). That this is purely an osmotic phenomenon, is indicated by the fact that the primary swelling is not suppressed by

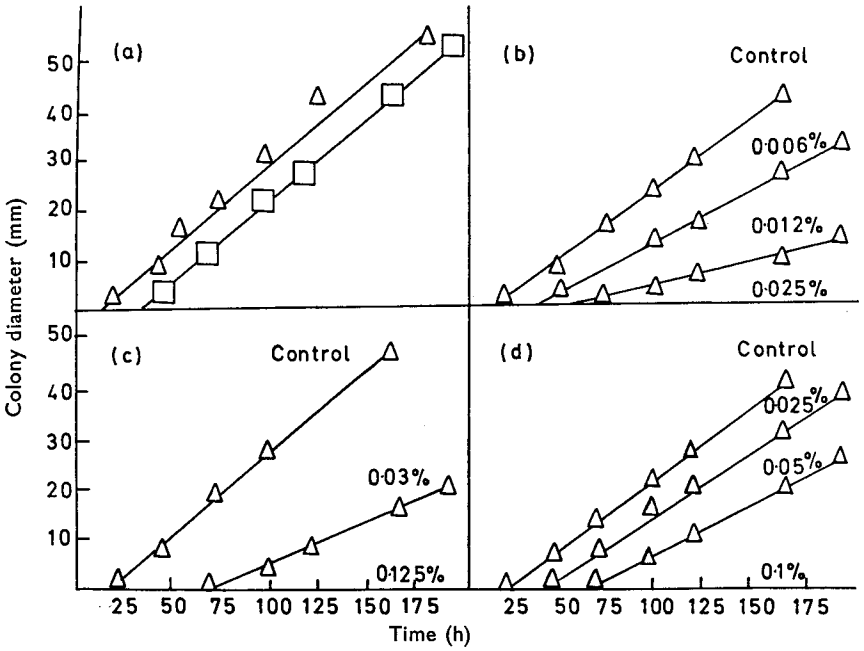

FIG. 4. Linear growth rate of colonies on wort agar of (a) *P. spinulosum* △ and *M. spinosus* □, (b) *P. spinulosum* in the presence of propylhydroxybenzoate, (c) Phenonip and (d) benzoic acid. Concentrations of preservative expressed as % w/v.

preservatives as is the subsequent swelling measured here with the Coulter Counter (Barnes & Parker, 1966). It is interesting to compare the swelling process in mould and bacterial spores, for although in the latter an osmotic phenomenon of the type described does not occur, there is an initial phase in the swelling which is insensitive to preservatives (Parker, 1969).

Since we are concerned here with the swelling phase that is susceptible to preservative action, it will be referred to as "metabolic swelling" to distinguish it from the early non-susceptible phase.

The shorter the lag period before the onset of metabolic swelling the greater the rate of the swelling (Fig. 1). The duration of this period of osmotic adjustment may reflect some efficiency of adaptation to nutrients available since in subsequent linear growth there is a time lag of some 20 h between onset of visible growth from the fastest and slowest swelling spores (Fig. 4a).

In choosing one of the spoilage moulds as a test organism, the spores most suited to the technique will be those which show swelling detectable with the Coulter (metabolic swelling) as early as possible and then swell at a maximal rate. Apart from these criteria, based upon expediency, evidence has been cited that indicates that when moulds are developing at their optimal rate fungicides have least effect. Conversely, if any of the factors which influence growth rate are changed to lessen the rate, then the resistance of the mould is also decreased (Tomkins, 1929). *P. spinulosum* was chosen as the test organism for fungitoxic estimations since it best meets the criteria required.

In general the effect of a preservative is to depress the rate of metabolic swelling and if present in sufficient concentration to suppress it completely.

Propylhydroxybenzoate depresses the rate of swelling at 0·006% and prevents it completely at 0·025% (Fig. 2A). The intermediate concentration (0·012%) depresses the rate of swelling and imposes an apparent lag, during which no swelling is detected, before some partial recovery occurs. Any detectable swelling, however slight, will result in eventual outgrowth and colony formation on solid medium. Thus, linear growth determinations confirm that concentrations of the ester below 0·025% allow colony formation (Fig. 4b). The depression of the swelling rate of the spore is followed by the delayed appearance of mycelial development in much the same way that slow swelling spore species lag behind faster swelling forms.

Phenonip may impose an early lag in swelling (0·06%), or at a lower concentration (0·03%) it initially reduces the rate of swelling but with an indication of later recovery (Fig. 2B). Linear growth measurements again confirm that the concentration sufficient to prevent swelling (0·125%) allowed no colony formation (Fig. 4c).

Benzoic acid differed from the other preservatives in that it extended the lag period before which the onset of metabolic swelling was detected with the Coulter. Swelling then followed at a reduced rate depending upon the ambient concentration of the preservative (Fig. 3). The pH of the reaction mixture (4·2) allowed some 50% of acid to be available as the active (undissociated) molecule. Linear growth was prevented by a concentration of 0·1% and was delayed by lower levels of the preservative (Fig. 4d).

Both propylhydroxybenzoate and phenonip, which contains parabens, reduce the rate of metabolic swelling at its onset. At concentrations of these agents which almost completely suppress this swelling, the rate is so reduced that apparent lags are imposed before size increase of spores can be detected (Fig. 2A,B). This may reflect a difference in action against different phases in the swelling process (Barnes, 1968) or some degree of adaptation to the preservative.

Benzoic acid, in prolonging the period before metabolic swelling commences, delays the initiation of the animate phases of germination. After this delay, however, the subsequent vegetative growth rate is little affected by the preservative (Fig. 4d). Increased concentrations of the acid will, of course, completely suppress metabolic swelling and vegetative growth.

The method of fungitoxic assay described will rapidly provide information of effective preservative levels and can be adapted to predict effects of extraneous additives such as non-ionic (Parker & others, 1966) and anionic (Parker, McCafferty & MacBride, 1968) surfactants.

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