

# Effect of preincubation of fungal conidia in modified atmosphere on subsequent germination and growth on a solid medium

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## Abstract

The combined effects of relative humidity (R.H.), incubation time, oxygen and carbon dioxide concentration on conidia were studied using a method where conidia were incubated in small bags of cloth under modified atmosphere (M.A.) conditions in high gas barrier plastics. At regular intervals the bags were opened, conidia suspended and transferred to a glucose medium in order to determine the effect of incubation on germination and growth. Conidia from *Penicillium commune* were chosen for evaluating the experimental system as this fungus is a common contaminant on products stored under M.A. conditions. Growth examinations were carried out using impedance mycology on a Bactometer, with detection time and growth rate as responses.

Conidia survived and started to swell under all experimental incubation conditions resulting in a decrease in lagtime. This effect was somewhat reduced when incubation time was increased. Regression analysis of growth rates indicated that R.H. and O<sub>2</sub> were significant factors controlling growth of the conidia. After 16 days of incubation the growth rate was increased with increasing R.H. and CO<sub>2</sub>. These results are in agreement with results obtained for mycelial growth.

## Introduction

The filamentous fungi *Penicillium commune* is a very common contaminant on foodstuffs and has been isolated from especially cheese, nuts, and meat (Frisvad and Filtenborg 1989; Hocking and Faedo 1992). The presence of fungi on foodstuffs is a serious problem. In order to prevent the growth of these contaminants the use of modified atmosphere packaging (MAP) involving various gas mixtures of Carbon dioxide (CO<sub>2</sub>), Oxygen (O<sub>2</sub>) and Nitrogen (N<sub>2</sub>) has become increasingly popular as a way to extend both the chemical and microbiological shelf life of foodstuffs. The technique has specially been applied in the packaging of various meats, peanuts, bakery and cheese products. However, fungal spores may be located on the foodstuff before packaging and then start to germinate and cause deterioration when the package is broken and spores are introduced to a normal atmosphere.

Fungal spores represent a resting state of the fungal life cycle and are characterised by very low respiration, low metabolism and a dehydrated state (Smith 1978). Germination of the spores involves transformation of the spore from a state of low metabolic activity to one of high metabolic activity and, as in the case of *P. commune*, germination involves changes in morphology due to swelling of the conidia before formation of a germ tube. This will happen only when the external environ-

ment provides the necessary nutrients and when sufficient water is available. Most studies so far have dealt with the need for external factors for the proper germination and growth of fungal spores and there are very few reports on how these factors affect the later survival of the spores if growth is not sustained.

In an experiment by Deploey (1985) sporangiospores from the thermophilic fungus *Rhizomucor pusillus* were incubated in eight different nutrient solutions in atmospheres containing mixtures of N<sub>2</sub> and low levels of O<sub>2</sub>. If spores failed to germinate after incubation in some specific atmosphere, spore-liquid mixture were pipetted onto starch basal medium to determine if the spores had survived the experimental conditions. The results showed that none of the incubation conditions interfered with the survival of the spores. They did not investigate whether lag time or growth rate was affected, but by implementing impedance microbiology as a way to measure growth it is possible to detect even small changes in lag time and growth rates. The objectives of the study reported here were to develop a method which made it possible to investigate the influence of modified atmosphere, relative humidity and incubation time on conidia from *P. commune* and subsequently examine the effect of this incubation on germination and growth when transferred to a growth medium under normal atmospheric conditions. This was done using a mathematical modeling technique (Response Surface Modelling) in order to quantify the effect of several factors simultaneously. This paper describes the method used to incubate the conidia and the first results on how MAP of spores influences their ability to subsequently germinate and grow.

## Materials and Methods

### Experimental design

The experiment was designed as a multifactorial experimental design using a statistical program (MODDE Version 2.0, UMETRI AB, Umeå, Sweden). The factors and levels of each factor used in this study included relative humidity (40–100%), incubation time (4–21 days), and levels of O<sub>2</sub> (0.1–8.0%) and CO<sub>2</sub> (16–40%) in the package headspace (balance N<sub>2</sub>). The experiments were carried out as a central composite circumscribed (CCC) design with six starpoints and three centrepoints.

### Microorganism

*P. commune* IBT no 10253, isolated from Danish cheese was obtained from the IBT Culture Collection at the Department of Biotechnology, Technical University of Denmark.

### Media

Conidiated cultures were produced on Czapeck yeast extract agar (CYA) before use. Plates were spread-plate inoculated with 0.1 mL of a conidial suspension (10<sup>6</sup> conidia/mL).

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### Preparation of conidia for packaging

Conidia from two 7-days-old cultures were harvested by gentle scrape with a sterile scalpel and transferred to four layers of sterile gauze (size 100 × 100 mm). The gauze was closed with a rubber band and packaged in 200 × 250 mm Riloten/ × 40/70 bags (Otto Nielsen Emballage AB, Lyngby, Denmark) ( $O_2$  transmission rate 2.0 mL/m<sup>2</sup>/24 hours) as shown in Figure 1.

### Packaging

Sealing of packages was done using a Multivac type vacuum packaging unit (Model AG4, Sepp Haggenmuller KG Bohen/Allgau, Germany). The various gas atmospheres were combined in precise mixtures by a gasmixer (KM 60-30-MESO, Witt-Gastechnik GmbH, Witten, Germany) and added through a septum attached to each bag. In order to control the relative humidity in the plastic bags 50 mL saturated salt solutions giving the r.h. levels set by the design were added. The bags were incubated at 5°C.

### Chromatographic analysis

Shortly after packaging and just before harvesting, gas samples were withdrawn from the bags and analysed in a gas chromatograph (Hewlett Packard 5790A) equipped with a thermal conductivity detector and a Alltech CTRI column 6 ft × 1/4 in. o.d. packed with a molecular sieve and propak mixture was used. The carrier gas was helium at 65 mL/minute and the temperature 40°C.

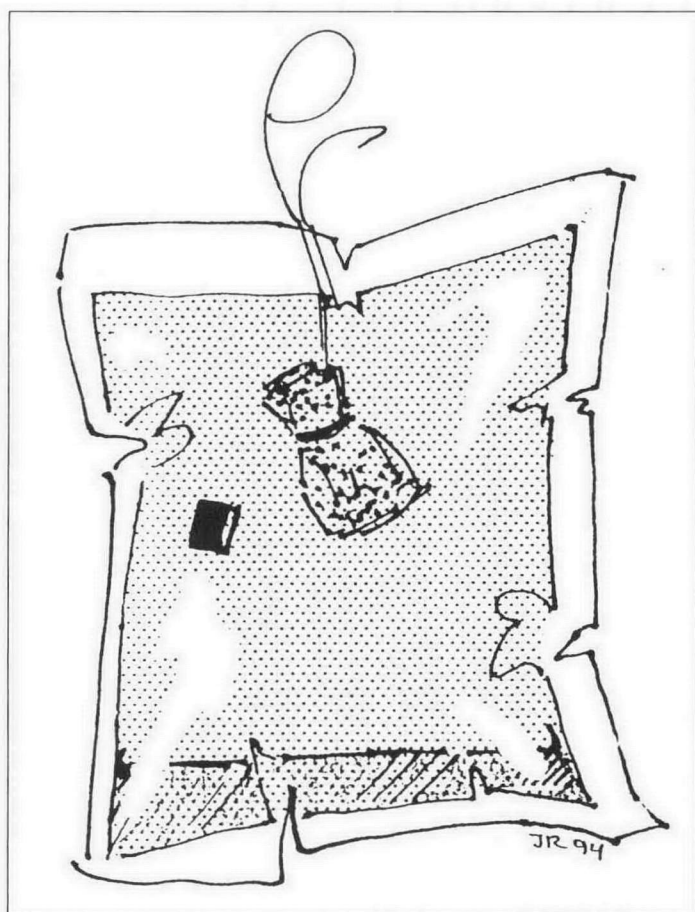


Fig. 1. Experimental system used to determine the effect of MAP on conidia from *P. commune*.

### Preparation of inoculum

Conidia from each gauze bag were suspended in 7 mL Peptonized water [0.1% Peptone (Difco)]. Suspensions were diluted to 10<sup>6</sup> conidia/mL as determined by counting chamber (Thoma haemocytometer). Four additional inocula were obtained by serial dilutions of the conidial suspension to 10<sup>5</sup>, 10<sup>4</sup>, 10<sup>3</sup> and 10<sup>2</sup> conidia/mL.

### Microbial monitoring system

The experiments were carried out in a Bactometer B123-2 (bioMérieux UK Ltd.) as described by Nielsen (1991).

### Bactometer medium

Medium consisted of (per L of distilled water) yeast extract (Difco), 7.5 g; glucose (anhydrous), 30.0 g; KH<sub>2</sub>PO<sub>4</sub>, 10.0 g; and agar, 20.0 g, pH 6.0. The media were poured into disposable Bactometer modules, 0.75 mL in each well.

### Inoculation procedure

Aliquots (100µL) of the conidial suspensions (10<sup>5</sup>, 10<sup>4</sup>, 10<sup>3</sup>, and 10<sup>2</sup> conidia/mL) were dispensed into prefilled wells. The number of conidia in the Bactometer measuring cells were consequently 10<sup>4</sup>, 10<sup>3</sup>, 10<sup>2</sup> and 10<sup>1</sup> conidia/well. Modules were incubated at 25°C for 100 hours in the Bactometer. Detection time (= time until growth was evident) was recorded by the Bactometer (one reading every 6 minutes).

## Results and Discussion

Although fungal spores do maintain some respiration (Sussman 1966) changes in headspace O<sub>2</sub> and CO<sub>2</sub> during the experiment were negligible (data not shown) resulting in only around 5% decrease and increase in CO<sub>2</sub> and O<sub>2</sub> levels, respectively. Conidia were readily removed from the gauze bags and conidial suspensions were made. Microscopic examinations of the conidial solutions revealed that all incubation conditions resulted in swelling of the conidia. No germtubes or mycelia were detected within the period of incubation. Deploey (1985) found that the spores from *R. pusillus* started to swell in atmospheres containing some oxygen, but no swelling of spores occurred when they were incubated in atmospheres of pure nitrogen.

When growth examinations were carried out in the Bactometer it was noted that the detection time was decreased for all incubated conidia compared with the unincubated control (Table 1). This is most probably due to swelling of the conidia, as swelling is the first step during germination (Gottlieb, 1978). Conidia are ready to produce germ tubes and start elongation when transferred to a growth supporting medium.

Table 1 Influence of incubation time on detection time.

Incubation time (days)	Detection time <sup>b</sup> (hour)
Control <sup>a</sup>	34.8
4	26.4
12	27.6
16	32.2
21	34.5

<sup>a</sup> As control unincubated conidia from 7-day-old cultures were used.

<sup>b</sup> Detection time was obtained by the Bactometer at 104 CFU/mL (data have been averaged).

The combined effect of relative humidity, incubation time and initial headspace O<sub>2</sub>/CO<sub>2</sub> concentration on detection time (DT, time before growth was detected by the Bactometer) and growth rate was analysed. The ANOVA for DT indicated that the model was significant with an R<sup>2</sup> value of 0.81. Examination of the fitted model indicated that incubation time and the level of carbon dioxide were significant factors (p<0.01) in controlling the lag phase of conidia from *P. commune* on a glucose medium. Irrespective of all other factors involved in this experiment the overall influence of incubation time on DT was significant. As shown in Table 1 the length of DT (obtained with 10<sup>4</sup>(FU/mL) increased with increasing incubation time, from 26.4 hours after 4 days of incubation to 34.5 hours after 3 weeks (which is comparable with the control).

Detection times were computed automatically by the Bactometer. Within a certain range a linear relationship exists between the logarithm of colony forming units and the detection time as measured by the Bactometer. This relationship was used to estimate growth rates ( $\mu$ ) of the organism as  $\mu = -\ln(10)/a$  where  $a$  is the slope of the regression line (Nielsen 1991).

When growth rate was used as the response, regression analysis of the model showed that the fitted model was significant, R<sup>2</sup> = 0.92. Examination of the fitted model indicated that the linear terms, relative humidity and O<sub>2</sub>, and several of the quadratic and cross product terms were significant.

The significant terms influencing growth rate were subsequently used to generate two-dimensional contour plots. An example of a plot of CO<sub>2</sub> versus relative humidity on the growth rate of *P. commune* with O<sub>2</sub> level held constant at 1% after 8 days of incubation is shown in Figure 2(a). This figure illustrates the growth rate decrease with increasing relative humidity and CO<sub>2</sub>. However, by incubating the conidia for 16 days instead of 8 days (Figure 2(b)) the picture is reversed. Growth rate is increased with increasing relative humidity and CO<sub>2</sub>. These results are in agreement with those of Megan and Lacey (1984). They investigated the effect of CO<sub>2</sub>, O<sub>2</sub> and  $a_w$  on growth of some *Aspergillus* and *Penicillium* spp. by incu-

bating inoculated petri dishes in experimental chambers. During incubation, lag phase and growth were measured visually. *Penicillium* spp. showed great tolerance of low O<sub>2</sub> concentrations when  $a_w$  was increased. At low levels of O<sub>2</sub> they observed a stimulatory effect of 5–10% CO<sub>2</sub> on fungal growth.

During the present experiment CYA-plates were routinely inoculated for evaluating the effect on colony diameter and on morphological characters. Generally, growth was not significantly affected when colony diameter was used as the response (data not shown) but the structure of some colonies appeared more dense than the control. However, these observations need more detailed study before any conclusion on the effects of MA incubation on later growth of fungal spores can be drawn.

The method described in this paper worked well. Furthermore, it seems as if the significant factors affecting growth of preincubated conidia in the present experiment after 16 days of incubation were comparable with observations on mycelial growth obtained by other workers.

## Acknowledgments

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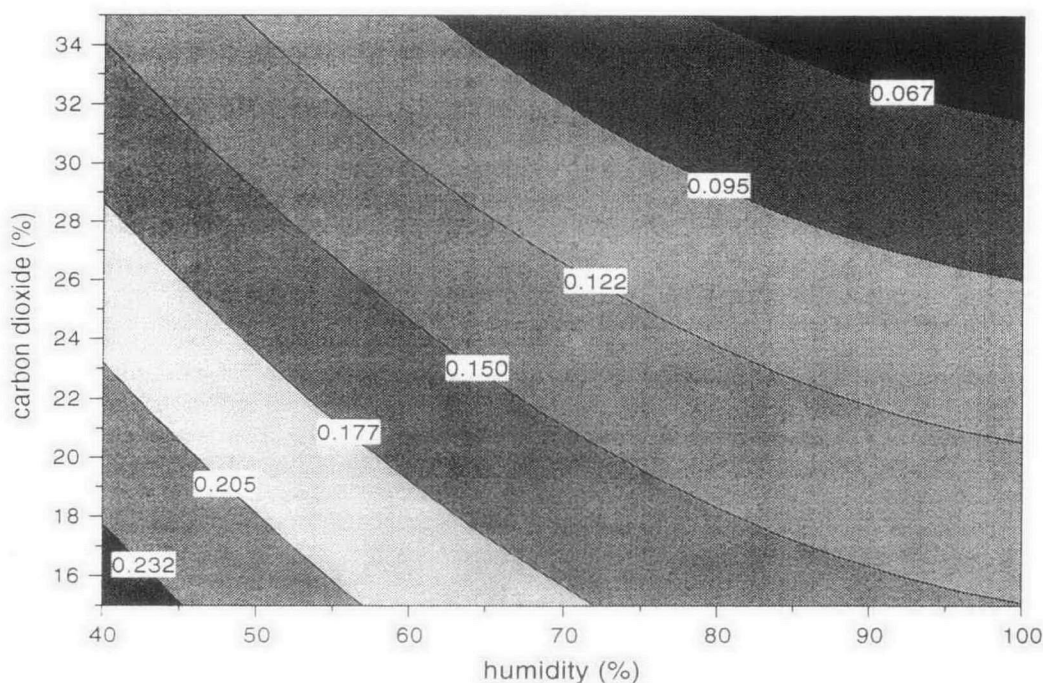
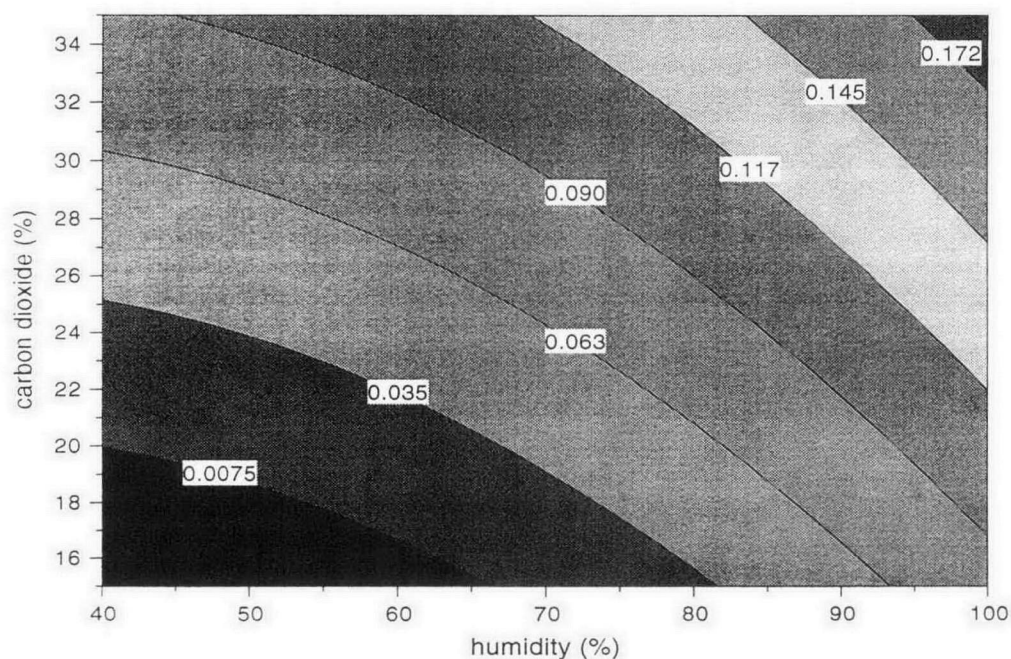


Fig. 2 (a). Two dimensional contour plot showing the effect of carbon dioxide and humidity on growth rate after 8 days of incubation. Oxygen held constant at 1%.





**Fig. 2 (b).** Two dimensional contour plots showing the effect of carbon dioxide and humidity on growth rate after 16 days of incubation. Oxygen held constant at 1%.

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