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Prediction of conidial germination of *Penicillium chrysogenum* as influenced by temperature, water activity and pH

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Aims: Conidial germination of *Penicillium chrysogenum* was carried out under operating conditions compatible with a pastries manufacturing process.
Methods and Results: A range, limited by two experimental values, was defined for each environmental factor tested: temperature (15 or 25°C), water activity (0·75 or 0·85) and pH (3·5 or 5·5). A closed device was made, which maintained an equilibrium between water activity of the culture medium and atmospheric relative humidity during 25 days, to follow spore germination. The combined effects of temperature, water activity and pH on spore germination were studied by applying factorial design methodology.
Conclusions: Higher rates of spore germination were associated with a high level of water activity. The incubation temperature also had a positive effect. A significant positive interaction between water activity and temperature was observed. Under these specific experimental conditions, pH did not have a significant effect on conidial germination.
Significance and Impact of the Study: A model describing the behaviour of fungal conidia is proposed.

INTRODUCTION

Under favourable environmental conditions, moulds can grow on a wide diversity of substrates. In general, fungal growth causes economic losses due to the appearance of visible hyphae and the production of unpleasant odours (Bullerman 1984). These disadvantages can be prevented by adding preservatives (Liewen and March 1985), but this practice has been strongly criticized by some authors (Sofos and Busta 1981). Literature data have emphasized the need to control factors such as temperature and water activity for the prevention of mould growth (Beuchat and Pitt 1990; Gibson *et al*. 1994; Cuppers *et al*. 1997; El Halouat and Debevere 1997). However, little information is available on the combined effects of moisture and temperature on spore germination. The objectives of the present study were to evaluate the germination of *Penicillium chrysogenum* conidia under environmental conditions compatible with mildly-processed food production. A factorial design allowed the study of the main effects of three environmental factors (temperature, water activity and pH) and their interactions on spore germination during 25 days.

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MATERIALS AND METHODS

Moulds and maintenance

Penicillium chrysogenum was isolated from spoiled pastry products and identified according to the descriptions of Samson *et al*. (1995). The basal medium used for spore production was MEA (Malt Extract Agar, BioMérieux). A conidial suspension was prepared by flooding the surface of the aerial fungal cultures with sterile saline solution containing Tween 80 (0·1% v/v). Spores were counted using a Malassez cell.

Spore germination

Germination media, prepared under aseptic conditions from YNB (Difco: 6·7 g l^{-1}) supplemented with glucose (20 g l^{-1}) and agar (15 g l^{-1}), were adjusted to the different pH values using a phosphate citrate buffer. Water activity (a_w) in these media was adjusted by substituting part of the water with an equal weight of glycerol (Gervais *et al*. 1988). Water activity measurements of the adjusted media were determined using

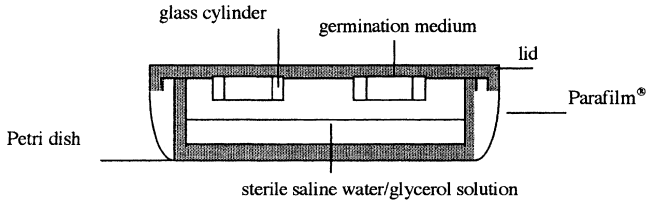

Fig. 1 Experimental device used to study spore germination of *Penicillium chrysogenum*

an Aqualab CX2T (Decagon Devices, Inc., Pullman, Washington, USA).

The device used in each experiment was made from a Petri dish. The sterile plastic Petri dish (diameter 90 mm) was opened within a laminar flow cabinet. Three, small, glass cylinders (diameter 16 mm) were placed on the internal side of the lid and filled with the appropriate sterile germination medium. After solidification, each surface was inoculated with 10 μl of a 10^6 spores ml^{-1} suspension. In order to equilibrate the relative humidity inside each device after inoculation, an appropriate sterile water/glycerol solution (15 ml) was poured into the Petri dish. The water activity of this solution was identical to that of the culture medium tacked to the lid. The devices, sealed with Parafilm® (Fig. 1), constituted closed chambers used for incubations at the two selected temperatures. Without opening the device, spore germination was observed daily under the microscope (×100 or ×400) through the Petri dish lid. Spores were considered to have germinated when the length of the germ-tube was equal to one half of the spore diameter (Paul *et al.* 1992).

Experimental design

The selection of a pre-defined design is based on the number of factors studied and the type of effects evaluated (main effects and interactions). Once the factors and the designs are selected, the coded values (X_i) of each factor given by the design can be expressed in experimental values (U_i).

Main effects and interactions of factors on spore germination. The factorial design 2^k is a two-level design (Box and Hunter 1961) used to evaluate the level of spore germination. This model is used to study the main effects of the three factors and their interactions with 2^3 (=8) experiments. An experimental range was defined inside lower (−1) and upper (+1) coded limits of the three factors. The corresponding experimental values (U_{min} and U_{max}) of these factors (Table 1), compatible with mildly-processed food production, are: temperature (15 or 25°C), a_w (0·75 or 0·85) and pH (3·5 or 5·5). One of the features of this factorial design is the uniform distribution of the experiments at the eight corners of a cube in a three-dimensional space (Fig. 2).

Table 1 Experimental values of the factorial design 2^3 defining temperature (T °C), water activity (a_w) and pH conditions

Factors	Experimental values	Lower limit U_{min}	Upper limit U_{max}
Temperature (T °C)	U1	15	25
Water activity	U2	0·75	0·85
pH	U3	3·5	5·5

Analysis and interpretation of the results. All trials were performed in triplicate. Multiple regression analysis was performed by the least square method using Nemrod software (LPRAI, Marseille, France). The linear combination of the parameters allowed estimation of the percentage of spore germination after 25 days by a polynomial equation 1 with eight coefficients:

$$Y = b_0 + b_1X_1 + b_2X_2 + b_3X_3 + b_{12}X_1X_2 + b_{13}X_1X_3 + b_{23}X_2X_3 + b_{123}X_1X_2X_3 \quad (1)$$

X_1, X_2 and X_3 are the coded values of the three factors, ranging from –1 to +1, according to:

$$X_i = [2 \cdot U_i - (U_{max} + U_{min})]/(U_{max} - U_{min}) \quad (2)$$

and the coefficients of the model (b_0, b_1, b_{12},…,b_{123}) were calculated by multiple regression analysis. Interpretation of the data was based on the signs (positive or negative effect on the experimental responses) and on the statistical significance of the coefficients. Interactions between two factors could be expressed as an antagonistic effect (negative coefficient) or a synergistic effect (positive coefficient).

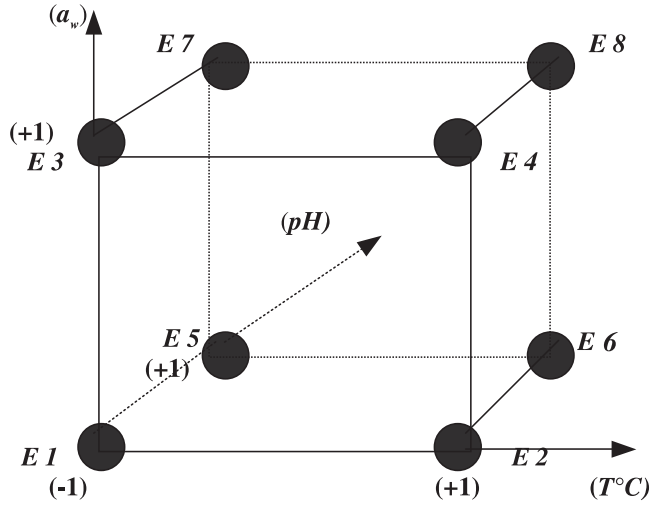

Fig. 2 Cubic representation of the factorial design 2^3

RESULTS

According to the factorial design, the level of germinated spores *vs* three environmental factors was studied. The effects of these factors (temperature, a_w and pH) during the incubation period are reported in Table 2. From the eight results (responses Y_1 to Y_8) obtained and the numbers of spores inoculated, rates of germinated spores are categorized into three groups: five responses with 10% or less, two responses with 90% or more and one response with an intermediate value ($Y_7 = 41{\cdot}5\%$ of the initial spores germinated after 25 days; Table 2, experiment 7). The higher rates of spore germination are associated with a high level of a_w.

Using glycerol as an a_w depressor, the coefficient values of the model equation (Table 3), given by Nemrod software, illustrated this predominant effect of water activity (b_2; $P < 0{\cdot}001$). The incubation temperature (b_1; $P < 0{\cdot}01$) also had a positive effect and was accompanied by a significant ($P < 0{\cdot}05$) positive interaction (b_{12}) when combined with water activity. The corresponding model giving the rate of conidial germination after 25 days was a polynomial equation 3:

$$Y(\%) = 32{\cdot}56 + 19{\cdot}06\ X_1 + 27{\cdot}19X_2 + 6{\cdot}19X_3 + 16{\cdot}19X_1X_2 - 2{\cdot}81X_1X_3 + 4{\cdot}81X_2X_3 - 3{\cdot}19X_1X_2X_3 \quad (3)$$

Table 2 Coded values and responses of the factorial design

Experiments	X_1 Factor 1 (T °C)	X_2 Factor 2 (a_w)	X_3 Factor 3 (pH)	Response: % germination
E1	− 1	− 1	− 1	$Y_1 = 1{\cdot}5$
E2	+ 1	− 1	− 1	$Y_2 = 6{\cdot}5$
E3	− 1	+ 1	− 1	$Y_3 = 7{\cdot}5$
E4	+ 1	+ 1	− 1	$Y_4 = 90$
E5	− 1	− 1	+ 1	$Y_5 = 3{\cdot}5$
E6	+ 1	− 1	+ 1	$Y_6 = 10$
E7	− 1	+ 1	+ 1	$Y_7 = 41{\cdot}5$
E8	+ 1	+ 1	+ 1	$Y_8 = 100$

Table 3 Model coefficients of the factorial design

Factors	Coefficients	Regression coefficients
Response means	b_0	32·56***
Temperature (T °C)	b_1	19·06**
Water activity (a_w)	b_2	27·19***
pH	b_3	$6{\cdot}19^{N.S.}$
T° · a_w	b_{12}	16·19*
T° · pH	b_{13}	$-2{\cdot}81^{N.S.}$
a_w · pH	b_{23}	$4{\cdot}81^{N.S.}$
T° · a_w · pH	b_{123}	$-3{\cdot}19^{N.S.}$

***Significant ($P < 0{\cdot}001$); **Significant ($P < 0{\cdot}05$); *Significant ($P < 0{\cdot}01$); ^{NS}Not Significant.

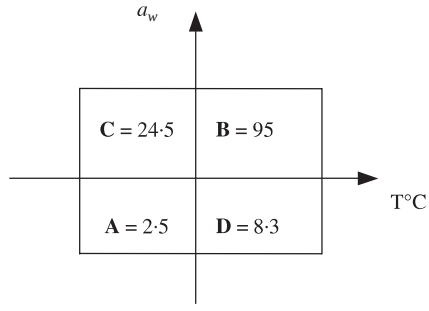

Fig. 3 Interaction diagram between temperature and water activity

To investigate the interactions between temperature and water activity, and leaving the pH influence, a diagram was constructed (Fig. 3). Each quarter of the figure indicates the mean result of the two experiments run under the same conditions of temperature and water activity; quarter A presents the results of experiments E1 and E5 performed at the lowest limits of a_w and temperature. It can be seen that at low temperature (15°C), when the water activity increases (from 0·75 to 0·85), the rate of germination increases 10-fold. This observation was confirmed at the higher temperature (25°C). Likewise, the effect of temperature on spore germination was more pronounced at the higher a_w value (0·85) than at 0·75 (Fig. 3). Under these particular experimental conditions, pH showed no significant effect (b_3, Table 3) on conidial germination after 25 days.

DISCUSSION

Development of food spoilage fungi on mildly-processed foods depends on many factors, such as biomass quality, environmental moisture or ambient temperature (Conner and Beuchat 1987; Pitt 1993; Gibson *et al.* 1994). The combined effects of these factors have also been studied (Skirdal and Eklund 1993; El Halouat and Debevere 1997; Betts *et al.* 2000). Invariably, fungal growth involves two steps: spore germination and hyphal elongation to form a filamentous colony. In order to investigate the first step, a factorial design was developed to elucidate the relationships between experimental responses and several factors. Statistical analysis of these experimental responses showed that among the three factors tested, water activity had the main influence on conidial germination of *P. chrysogenum*. These results are in accordance with the data of Ayerst (1969) and Seiler (1976) on moulds growing on mildly-processed foods. Nevertheless, at a lower level, the effect of the environmental temperature, alone or in interaction with water activity, was also noticeable. In this experimental domain, the effect of pH remained low.

After 25 days, the small proportion of germinating conidia of *P. chrysogenum* observed at $a_w = 0{\cdot}75$ is in accordance

with data obtained by Fustier *et al.* (1998) from five mould species growing on the surface of cake, and with the observations of Smith *et al.* (1988) on *Aspergillus niger* strains isolated from crumpets. This study has proposed a model to describe the behaviour of fungal conidia on laboratory medium (YNB), which should now be validated on foods with intermediate moisture levels.

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