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Inoculation of airborne conidia of *Penicillium chrysogenum* on the surface of a solid medium

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ABSTRACT

To reproduce a fungal contamination of food products by airborne conidia, a method to inoculate a few number (in the range 1–9) of conidia on the surface of agar media was developed. This technique would allow to determine accurately the time to detection of fungal colonies, then the mould free shelf-life of food products by using dry conidia. The method was based on dry-harvesting the conidia in the lid by gently taping the bottom of the dishes where sporulating mycelium was grown, retaining the conidia on glass beads, and, aseptically transferring the beads to successive Petri dishes to "dilute" the samples. Among the eleven factors tested by means of an experimental design, the most important factors were the incubation time of the sporulating mycelium, the resting time to dislodge as many conidia as possible from the lid, the number of beads and the number of successive dishes. The other factors were method used to produce conidia, the number of taps to remove as many conidia as possible from the sporulating culture before harvesting, the drop height of the harvest device, the number of taps to detached the harvested conidia from the lid, and the mixing times to attach conidia from the lid to the beads, and to detach conidia from the beads to the agar media. Decimal "dilutions" were achieved by transferring 10 beads to the successive dishes with a mixing time of 10 s. It was shown for *Penicillium chrysogenum* that an average of 3 colonies per dish were counted on the fourth of the successive dishes, for 3 days incubation time at 25 °C, 24 h resting time, and 10 beads.

1. Introduction

Many food products can be contaminated by moulds, such as cereals, meat, milk, fruit, vegetables, nuts, fats and products of these (Filtenborg et al., 1996). Mould spoilage is of great concern for many industries, such as dairies and bakeries. For dairy products in general, processing equipment, air, packaging material, starter cultures and fruit pulps are said to be the most frequent sources of contamination (Rohm, 1991a,b). Mold spores are generally killed by the baking process in fresh bread and other baked products (Knight and Menlove, 2006). Therefore, bread must be contaminated either from the air, bakery surfaces, equipment, food handlers or raw ingredients after baking during the cooling, slicing or wrapping operations (Saranraj and Geetha, 2012). Mould spores present in the air are a major source of contamination of dairy (Salustiano et al., 2003) and bread (Legan, 1993) products. Consequently, air quality is routinely controlled in these industries. Typical airborne mould and yeast counts were 60–1310 cfu/m^3 and 50–2000 cfu/m^3 for a Brazilian dairy (Salustiano et al., 2003) and Canadian bakeries (Ooraikul et al., 1987), respectively.

The mould-free shelf-life of food products can be assessed by adding a known number of spores to the product's surface prior to packaging and storage, and observing the time required for mycelium formation (Fustier et al., 1998). These authors compared four inoculation techniques for initiation of growth of *Aspergillus sydowii*, *Aspergillus ochraceus*, *Penicillium funiculosum* and, *Eurotium herbariorum* on cake surfaces, i.e., spot, air cabinet, spray (atomizer), and talc addition methods. The easiest methods to carry out were the spot and air cabinet. Based on precision, the spot method based on inoculating a spot of a calibrated spore suspension appeared to be the method of choice. Accordingly, the spot technique is widely used for studies examining the effect of

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environmental factors on germination, growth, and inactivation of fungal spores (Dantigny et al., 2006). The air cabinet method reproduced a real contamination by airborne conidia. A highly contaminated industrial environment was simulated by opening plates colonized by sporulating cultures in a cabinet equipped with a domestic fan. The cakes were placed in the cabinet for 3 min and then removed. The air cabinet method had the great advantage of using non hydrated spores, but this method could not be standardized and the risks of contamination of the laboratory were high. Depending on the experimental conditions, shelf-lives of 26 and 32 days; 38 and 20 days were described for the spot (hydrated conidia) and the air cabinet technique (non-hydrated conidia), respectively.

The physiological state had a great effect on the inactivation and the germination of fungal conidia (Blaszyk et al., 1998; Dao and Dantigny, 2009; Dantigny and Nanguy, 2009). In particular, the hydration of the spore, even for a short period of time, i.e., 20 min, had a significant effect of the germination time (Nanguy et al., 2010). Therefore, the differences between the shelf-lives obtained by the two techniques may be partly due to this effect.

The objective of the present study was to design a simple method capable of inoculating a few number of dry conidia, thus, simulating a real contamination. A protocol based on inverting a Petri dish culture, then tapping gently the bottom to harvest a high number of dry conidia in the lid was already developed in the laboratory (Dao et al., 2010). The present study was concerned with developing a method to dilute high numbers of conidia.

In a summary of 19 random surveys of moulds in atmospheric air, five genera accounted for almost 70% of the total mould count: *Cladosporium* 29%, *Alternaria* 14%, *Penicillium* 9%, *Fusarium* 6%, and *Aureobasidium* 5% (Al-Doory and Domson, 1984). The present study focused on *Penicillium chrysogenum*. This species isolated from food pastries in the laboratory, was consistently found in indoor environment of dairies in Norway (Kure et al., 2004) and bakeries in Belgium (Deschuyffeleer, 2012).

2. Material and methods

2.1. Mould and medium

P. chrysogenum isolated from a spoiled pastry product was identified according to Samson et al. (1995). Conidia were suspended in aqueous solutions 15% w/w glycerol, 0.05% vol/vol Tween80 (Merck, Fontenay sous Bois, France) and stored at −80 °C in 2 ml Eppendorf® tubes. The media for preparing sporulating cultures and inoculating conidia was potato dextrose agar, PDA (bioMérieux, Marcy l'Etoile, France). Plates were spread with 0.1 ml, or 3 spots inoculated with 10 µl of thawed conidial suspension. Incubation was 25 °C for 3, and 7 days.

2.2. Experimental factors

Eleven factors, k = 11, were tested, each factor X_i was characterized by two levels (−1, +1):

X_1, the inoculation technique (3 spots, spread);
X_2, the incubation time of the sporulating culture (3, 7 days); after this time, dishes were closed with Parafilm®.
X_3, the taps on the bottom (0, 100); after this operation, a sterile lid was substituted for the lid that contained the conidia and the dishes were closed with Parafilm®.
X_4, the drop height (5, 20 cm), 90 mm diameter plastic Petri dishes (Grosseron, Saint Herblain, France) were placed with the lid on a polystyrene board, a ruler was vertically placed on the dish. A 90 mm paper roll placed around the ruler was dropped along the ruler.
X_5, the number of taps on the lid (0, 100); after this operation a sterile dish bottom was substituted for that which contained the sporulating culture and the dishes were closed with Parafilm®. The lid was gently tapped to remove as many conidia as possible from the lid.
X_6, the resting time (0, 24 h); during this time, conidia felt down on the bottom of the dish. 24 h was sufficient to allow conidia present in the atmosphere of the Petri dish to be deposited on the bottom. Only the conidia remaining in the lid will be attached to the beads.
X_7, glass beads on the lid (10, 40); under a laminar flow cabinet, sterile glass beads were poured into the lid. A maximum number of 40 was fixed to allow movements of the glass beads.
X_8; shaking time step 1 (10, 60 s); this step allowed conidia present in the lid to adhere to the glass beads. The shaking procedure was analogous to that one which is used to pour autoclaved medium in Petri dishes, i.e., tracing X crosses with the Petri dishes.
X_9; glass beads on the agar (1, 10); the maximum number of glass beads transferred to the agar could not exceed the minimum number of glass beads on the lid. Up to 10 glass beads can be transferred easily, whilst greater numbers require the use of steel forceps.
X_{10}; shaking time step 2 (10, 60 s); this step allowed conidia attached to the glass beads to be deposited onto the surface of the agar.
X_{11}; number of dishes (1, 4); glass beads were transferred successively to Petri dishes, then the glass beads were discarded.

2.3. Screening matrix

A screening matrix proposed by Plackett and Burman (1946) was used to determine the coefficients of the linear effects of the factors on the colony counts. The number of experiments, n, is always a multiple of 4, and the maximum number of factors, k, that can be examined was: k = n−1 (Sergent et al., 2013). For k = 11 factors, twelve experiments were carried out in triplicate, Table 1.

2.4. Colony counts

All dishes inoculated by means of the glass beads were incubated for 7 days at 25 °C. Visible colonies were counted. When the number N of colonies exceeded 100 per dish, dishes were separated into 2, 4, 8, 16, 32, and 64 sectors until the number of colonies per sector was less than 100. Colonies were counted for one sector, then multiplied by the total number of sectors. The maximum number of colonies that could be counted this way was 64,000.

2.5. Statistical analysis of the results

Multiple regression analysis based on the latest square method was performed using Nemrodw software (LPRAI, Marseille, France). A linear polynomial equation was used to describe the main effects of the factors, on the colony counts.

$$N = b_0 + b_1 \cdot X_1 + b_2 \cdot X_2 + b_3 \cdot X_3 + b_4 \cdot X_4 + b_5 \cdot X_5 + b_6 \cdot X_6 + b_7 \cdot X_7 + b_8 \cdot X_8 + b_9 \cdot X_9 + b_{10} \cdot X_{10} + b_{11} \cdot X_{11} \quad (1)$$

The significance of the coefficients was evaluated by multiple regression analysis based upon the *F*-test with unequal variance.