Effect of Inoculation Techniques and Relative Humidity on the Growth of Molds on the Surfaces of Yellow Layer Cakes

PATRICK FUSTIER,^{1*} ALAIN LAFOND,² CLAUDE P. CHAMPAGNE,¹ AND FRANÇOIS LAMARCHE¹

*Food Research and Development Center, Agriculture and Agri-Food Canada, St.-Hyacinthe,¹
and Culinar Inc., Ste. Marie de Beauce,² Quebec, Canada*

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Four inoculation techniques were compared for initiation of growth on cake surfaces: spot, air cabinet, spray (atomizer), and talc addition methods. Molds were isolated from commercial cakes and were identified as *Aspergillus sydowii*, *Aspergillus ochraceus*, *Penicillium funiculosum*, and *Eurotium herbariorum*. Cake surfaces were inoculated with mold spores and incubated under three equilibrium relative humidity (ERH) levels: 97, 85, and 75%. Random contamination by spores in a ventilated air cabinet was the simplest method of inoculation, but standard deviations in the inoculation rates (20% on a relative scale) were almost twice those observed with the other methods. The spot method was the most reproducible. Cake samples inoculated in the air cabinet had colony counts 10 times lower than those obtained for potato dextrose agar plates at 97% ERH, which was not the case with the spray and talc methods. Growth of molds was much slower in the samples incubated in 75% relative humidity, with all methods. Colony counts were generally similar in systems adjusted at 85 to 97% ERH but were lower for samples incubated at 75% ERH. In comparisons of the shelf life estimates obtained by the various inoculation methods, a correlation coefficient (r^2) of 0.70 was obtained between the spot method and the other methods of inoculation, while talc, air cabinet, and spray shelf life data were correlated better ($r^2 \approx 0.97$). The spot method appeared to be the method of choice in consideration of ease of use, precision, and the ability to enable the study of the effects of the environment on mold-free shelf life as well as on the rate of growth of molds on cakes.

Yellow layer cakes are bakery products made from wheat flour, water, vegetable oil, sugar, emulsifier, eggs, and baking powder. By definition, these cakes contain almost equivalent amounts of sugar and flour, while whole eggs and shortening represent 40 and 20 to 40%, respectively, of the content on a flour basis. Traditionally, these products are packaged in plastic film after baking, cooling, and the addition of fillings (e.g., vanilla) or topping (e.g., chocolate), and they are consumed within 1 month.

As products of intermediate moisture content with abundant nutrients, these cakes are prone to mold growth. Postprocess contamination is unavoidable (1, 12, 18), and a wide range of molds, such as *Penicillium*, *Aspergillus*, *Cladosporium*, and *Eurotium* species, gain access to the product surface prior to packaging. Sporadic appearance of white, grey, blue, and black molds may limit the product's shelf life to less than 2 or 3 weeks. Factors such as pH, nutritional profile, storage temperature, redox potential, addition of either propionate, sorbate, or benzoate (8), gas packaging involving a CO₂-N₂ gas mixture (11, 13), control of headspace oxygen via the use of oxygen absorbents (16), incubation in ethanol vapor (17), and the relative humidity (RH) of the atmosphere surrounding the product (3, 6, 14) all have a bearing on the mold-free shelf life. However, the merits and disadvantages of techniques for inhibition of mold growth need to be compared via a microbial-inoculation abuse study.

Mold-free shelf life can be assessed by adding a known number of spores to the product's surface prior to packaging and storage. At least three artificial inoculation techniques are known: (i) the spot method, which relies on the properties of

mold colonies to grow radially at a constant rate on solid media and was applied successfully to cheese for quantification of the kinetics of growth (19); (ii) the spray method, in which a spore suspension is vaporized on a solid medium by applying constant pressure on a manual piston (2, 4, 7); and the talc method, in which the colonization is carried out under a laminar flow hood with mold-contaminated talc (13, 15).

Although various techniques for inoculation of mold onto foods have been used, their efficiency or appropriateness in abuse studies of layer cakes has not been determined. Our objective was to examine various inoculation techniques and the role of environmental RH on the kinetics of mold growth in determining the mold-free shelf life.

MATERIALS AND METHODS

Mold cultures. Five mold strains were isolated from cakes provided by the quality control department of Culinar (Ste. Marie de Beauce, Quebec, Canada). One strain, a darkly pigmented *Mucor*-like culture, could not be identified and was coded CRDA-1. The four other strains were identified and coded as *Aspergillus sydowii* CRDA-16, *Aspergillus ochraceus* CRDA-8, *Penicillium funiculosum* CRDA-12, and *Eurotium herbariorum* CRDA-11.

The strains were propagated on acidified (pH 3.5; 10% tartaric acid) potato dextrose agar (PDA) (Difco, Detroit, Mich.). Although theoretically a high-pH, low-water-activity (low-a_w) medium would have provided a more representative growth medium, acidified PDA was used since it is recommended for general yeast and mold growth (9). Once abundant growth and spore formation were obtained, generally after 10 to 12 days at 24°C, 10 ml of a sterile 10% NaCl solution was added to the surface of the agar plates. The spores were recovered by gentle swirling of the saline solution over the mold colonies. Spore suspensions of approximately 10⁶ to 10⁹ CFU/ml were obtained by this procedure. The spore suspensions were stored in 10% saline and maintained at 4°C until used (within 7 days). No detectable mortality was observed during this storage period. The spore suspensions were quite stable, as mortality was limited to 0.5 log unit after 7 months of storage at 4°C.

Cakes. The intermediate-moisture cakes (IMC) used for this study were typical yellow layer cakes supplied by Culinar. Commercially, such cakes are used in combination with vanilla fillings and may be coated with a chocolate blend, but the cakes used for this study did not have such additions. The cakes were produced at a Culinar plant and shipped within 1 day to the Food Research and Development Center, Agriculture and Agri-Food Canada, St.-Hyacinthe, Que-

* Corresponding author. Mailing address: Food Research and Development Center, Agriculture and Agri-Food Canada, 3600 Casavant Blvd., St.-Hyacinthe, Quebec, Canada J2S 8E3. Phone: (514) 773-1105. Fax: (514) 773-8461. E-mail: FUSTIERP@EM.AGR.CA.

FIG. 1. Apparatus used for inoculation of sponge cakes with a spore-containing talc powder. The handle of the hammer (A) is pulled to a fixed level and released. The spring (B) propels the hammer head (C) on the top of the 60-cm² velvet support stand (D). A portion of the talc found on the velvet fabric base (E), which had previously been dipped in the mold-contaminated talc powder, then drops onto the cake or petri dish to be inoculated. A detailed plan of the design is available upon request.

bec, Canada. Ingredients included flour, water, vegetable oil, sugar, emulsifier, eggs, and sodium bicarbonate (see Table 1). Cakes B and C were chocolate flavored, while cake A was vanilla flavored. Round slices having 60-cm² surface areas were placed in sterile petri plates. Surface contamination was eliminated by exposing the cakes to UV light (three 6-W UV lights at 254 nm) during 30 min in a Cleansphere CA100 unit by Safetech Limited (model F-0400; National Labnet Co., Woodbridge, N.J.) under 85% RH (saturated KCl solution).

Inoculation of cakes. Each of the five spore suspensions was diluted to 10³ CFU/ml, and they were mixed in equal proportions. The inoculated cakes or petri plates were incubated at 21°C for up to 70 days. Four inoculation methods were examined.

(i) In the spot method (19), 10- μ l spots of the spore suspension were placed on the surface of a 60-cm² slice of cake having a thickness of approximately 1.5 cm. Growth was evaluated by measuring the radial extension of the colonies.

(ii) In the spray method (2, 4, 7), 200 μ l of the spore suspension was vaporized onto the cakes with a manual piston atomizer (IL-D Continental Industry, Oakville, Ontario, Canada). The distance between the spray nozzle and the cakes was maintained at 15 cm. Growth was evaluated by the time required to obtain visible (1-mm-diameter) colonies, as well as by the total number of colonies.

(iii) In the talc method (13), approximately 20 mg of culture-containing talc was added to the 60-cm² surface of the cakes. A sterile talc powder of calcium silicate (Aldrich, Milwaukee, Wis.) was mixed with an equal mass of the spore suspension, and the mixture was allowed to dry in a laminar flow cabinet at 25°C for 72 h. Following this drying step, the talc powder was estimated to contain 10³ CFU/g by plating on acidified PDA. The spore-containing talc was added to the cake with the apparatus shown in Fig. 1.

(iv) In the air cabinet method, a highly contaminated industrial environment is simulated. Five PDA plates colonized by sporulating mold cultures (one petri plate for each strain) were opened in a 0.53-m³ cabinet equipped with a domestic fan. The cakes were placed in the cabinet for 3 min and then removed. To show that this system distributed the spores, petri plates containing PDA were used in one experiment instead of cakes.

The a_w of acidified PDA was probably greater than 0.99, but the medium a_w was not adjusted at the various a_w levels to which the cakes were exposed. Rather, since all five molds grew well on this medium, the PDA plates served as a control for total populations in the samples.

TABLE 1. Compositions and properties of the layer cake products^a

Cake	Composition (g/100 g of cake)					a_w	pH
	Water	Carbohydrate	Protein	Lipids	Ash		
A	21.5–23.4	58	3.0	14.0	0.013	0.81	6.9–7.2
B	23.5–25.5	61	3.2	13.1	0.024	0.83	8.5–8.7
C	23.2–25.1	60	4.0	11.5	0.029	0.83	8.3–8.5

^a All the formulations contain 28 to 30 g of flour, 11 to 18 g of eggs, 25 to 27 g of sugar, 2.5 to 5 g of vegetable oil, and emulsifier, as well as 1.5 to 1.7% baking powder and 0.15% potassium sorbate, per 100 g (wet basis). Formulations B and C also contain cocoa powder.

Three plates or cakes were inoculated by each method, and three independent replicates (different product lot) of each assay were carried out.

Humidity-controlled packaging. The petri plates containing the inoculated cake slices were placed in a 1.8-liter thermo-formed polypropylene semirigid tray (model BQ180; InnovaPlast, Ville d'Anjou, Quebec, Canada). The RH level inside the systems was controlled by placing a thin layer of saturated solutions of NaCl or KCl in the bottom of the tray to generate RHs of 75 and 85%, respectively. An environment with 97% RH was achieved by adding pieces of water-soaked cotton inside small aluminum dishes with the petri dishes. Four petri dishes were placed in each tray. The trays were sealed with a Multipak 486 polyester-saran-polyethylene film with the following gas transmission rates: H₂O vapor transmission, 0.08 g/m²/24 h; 90% RH at 38°C; O₂, 9 ml/m²/24 h at 75% RH; and CO₂, 23 ml/m²/24 h at 75% RH. The sealed packages were stored at (21 $\pm$ 1)°C for up to 70 days.

Analyses. Viable spores in inoculum suspensions were estimated by plating the suspensions on PDA and incubating the cultures at 24°C for 5 to 7 days. The moisture content of the IMC was determined by gravimetry; the samples were dried for 16 h at 70°C under a 25-inch vacuum, according to Association of Official Analytical Chemists method 925.09 (5). An EEJA-3 unit (Novasina, Zurich, Switzerland) enabled the determination of a_w levels of the IMC. RH levels in the containers were verified with a Solomat MPM500e hygrometer equipped with a 355 RHX probe (DurPro, Longueuil, Quebec, Canada).

The numbers and the diameters of the mold colonies were determined without opening the container. No attempt was made to identify the species that were growing. Although this approach does not enable the study of individual species, the multiple-mold inoculum was made in order to reflect the phenomena that would occur under commercial conditions.

The pH of an aqueous slurry of 10 g of milled samples in 250 ml of deionized water was measured on an expanded-scale Corning 140 pH meter. The composition of the product was analyzed by standard methods (5).

Desorption curves were established by placing the cakes in sealed hermetic containers, to which saturated solutions generating RH levels of 0% (Drierite), 33% (magnesium chloride), 43% (potassium carbonate), 59% (sodium bromide), 65% (sodium nitrite), 75% (sodium chloride), 79% (ammonium chloride), or 85% (potassium chloride) were added. The system was incubated at 25°C for up to 20 days. At various times, samples were taken and analyzed for moisture content. Equilibrium was generally reached after 6 days of incubation.

FIG. 2. Desorption curve of the yellow layer cakes.

TABLE 2. Inoculation levels of the various systems with PDA-containing petri plates^a

Method	CFU/60 cm ²		
	Theoretical ^b	Exptl	SD
Spray	200	53	6
Talc	20	23	3
Air cabinet	NA	157	32

^a Data are from three independent replicates.^b Theoretical values determined from the known concentrations of spores in the inoculum and the amount of inoculum added (talc) or sprayed. NA, not applicable.

RESULTS

Composition and physicochemical characteristics of the cakes. The composition, a_w , moisture content, and pH of the yellow layer cakes are shown in Table 1. With abundant nutrients (Table 1), basic pH, and an a_w of 0.83, the cakes are good media for mold growth. Incorporation of 0.15% potassium sorbate did not inhibit the sporadic mold growth on the surfaces of cakes produced commercially. Since sorbate has little or no activity at this high pH, these results are understandable. Cakes B and C, containing cocoa powder, are apparently more

FIG. 3. Growth of a five-strain mixed mold population used to inoculate cakes by the spot method at 97% (A), 85% (B), and 75% (C) RH. As a control, growth on PDA at 97% RH is also shown (D). ■, cake A; ▲, cake B; ●, cake C.

FIG. 4. Growth of a five-strain mixed mold population used to inoculate cakes by the air cabinet method at 97% (A), 85% (B), and 75% (C) RH. As a control, growth on PDA at 97% RH is also shown (D). ■, cake A; ▲, cake B; ●, cake C.

susceptible to mold growth, as white or grey colonies are more noticeable on dark surfaces of cake within 2 weeks of storage at 21°C.

The desorption isotherm curve of the cakes (Fig. 2) has a very steep slope with increasing a_w beyond 0.60. This indicates that at an a_w above 0.60, the moisture content increases markedly and that lowering the moisture content and a_w as a means of controlling microbial proliferation is not practical because of detrimental organoleptic changes in the cakes.

Inoculation methods. To assess the number of spores delivered to the cake surface, plates containing acidified PDA were used as targets for the various inoculation systems. This experiment also served to determine the standard deviations of the methods. The highest inoculation levels were achieved with the air cabinet method (Table 2). By the talc method, experimental colony counts were in agreement with the inoculated populations (Table 2), despite our concern that lumps would occur and that CFU counts for the plates would not reflect the mold population of the powder. However, with the spray method, only a quarter of the expected population was obtained. It was not determined if this was because some droplets did not reach the plate or because droplets contained more than one spore.

Random contamination by spores contained in a ventilated air cabinet is an easy method of inoculation. However, this

FIG. 5. Growth of a five-strain mixed mold population used to inoculate cakes by the spray method at 97% (A), 85% (B), and 75% (C) RH. As a control, growth on PDA at 97% RH is also shown (D). ■, cake A; ▲, cake B; ●, cake C.

method had the highest standard deviation, 20% of the inoculation level (Table 2). The talc and spray methods were more consistent, as 13 and 11% variations, respectively, were recorded. Although the talc and spray methods used in this study were more predictable than the air cabinet system, care had to be taken in standardization of the various steps of the inoculation techniques. With the talc method, the critical points were appropriate mixing of talc dust and the spore suspension, pressure of the velvet disk on the talc powder, and distance of displacement of the hammer. With the spray method, the critical factors were distance between the nozzle and the cake and pressure applied on the spray handle.

The growth curve obtained by the spot method was reproducible on PDA petri plates (Fig. 3). The standard deviations of the colony surfaces varied between 5 and 17%, as a function of incubation time. Colonies were visible on PDA after 3 to 4 days of incubation at 25°C. This method was the easiest to reproduce.

The cake samples inoculated in the air cabinet and incubated at 97% equilibrium RH (ERH) had colony counts 10 times lower than those obtained for PDA plates (Fig. 4). This was not due to an inadequacy of the cakes to support growth of the molds, since PDA counts were only slightly superior to those obtained for the cakes by the talc and spray methods (Fig. 5 and 6).

Effect of relative humidity. a_w is the most important factor determining whether a mold will grow and the rate at which it will grow on intermediate-moisture foods (15). Mold growth was much slower in the samples incubated in 75% RH, with all inoculation methods (Fig. 3 to 6). With three inoculation methods (spray, talc, and air cabinet), the rate of colony appearance and the total number of colonies were affected by ERH. Colony counts for cakes were similar in systems adjusted at 85 and 97% RH but lower for samples incubated at 75% ERH (Fig. 4 to 6).

Shelf life. A cake's shelf life ended when a mold colony appeared. Regression analyses were performed using shelf life to compare the effects of the inoculation methods. Correlation coefficients (r^2) of around 0.70 were obtained between the spot method and the other methods of inoculation (Fig. 7). The shelf lives of the talc, air cabinet, and spray methods were much better correlated. As an example, the shelf life data for the spray and talc methods showed an r^2 of 0.97. The spot inoculation method was the easiest and most precise inoculation technique, but its shelf life data differed slightly from those obtained by talc, spray, and air cabinet inoculation.

DISCUSSION

Misleading results can be obtained in mold-free shelf life studies carried out with deliberate additions of spores to the

FIG. 6. Growth of a five-strain mixed mold population used to inoculate cakes by the talc method at 97% (A), 85% (B), and 75% (C) RH. As a control, growth on PDA at 97% RH is also shown (D). ■, cake A; ▲, cake B; ●, cake C.

FIG. 7. Regression analyses of shelf lives obtained for cakes inoculated with a five-strain mixed mold suspension between the spot method and the talc (■), spray (●), or air cabinet (○) method. r^2 values were 0.68, 0.73, and 0.69, respectively.

surfaces of products. The cultures used may not be representative of the ones in the plant and may not develop well at the particular ERH level of the product. Thus, as recommended by Seiler (15), we used a mixture of five molds isolated from commercial products. *Penicillium* and *Aspergillus* are frequent spoilage agents of cake (8, 12, 18), and *Eurotium* is frequently recovered in studies of the shelf life of bakery products, particularly in low-ERH environments (15). Thus, the cultures used in this study (*A. sydowii*, *A. ochraceus*, *P. funiculosum*, and *E. herbariorum*) are representative of cake spoilage molds. The use of a mixed culture instead of an individual culture provides a more realistic estimate of the effects of ERH and cake composition on the shelf lives of the cakes. However, with this approach, it is difficult to determine which species is present on the moldy cake and whether the experimental conditions alter the frequency of the strains that develop on the cakes. Cake composition had a significant effect on mold development, and this work obviously raises questions as to the effects of pH, cocoa, and initial a_w on the development of the molds.

We expected that colonies would appear more quickly with the spot inoculation method than with the other three methods, but this was not the case. This confirms the data reported by Seiler (15), in which differences in levels of mold contamination had a surprisingly small effect on mold-free shelf life.

The cake samples inoculated in the air had colony counts 10 times lower than those obtained for PDA plate controls. The reason for this discrepancy is unknown. These results suggest that even with identical exposures to air cabinet contaminations, the inoculation levels can vary greatly as a function of the type of substrate exposed to the air. The spot, spray, and talc methods are more reliable inoculation techniques for studies involving different food matrices.

Treatment of the conidia, dry in talc or wet in spray, did not influence the mold-free shelf life at 75 and 85% ERH. In these instances, the ERH was the determining factor in mold-free shelf life. However, colonies appeared faster at 97% ERH with the spray method. This suggests that mixing the spore suspension with talc may have generated some form of stress on the conidia.

With the spray, talc, and air cabinet inoculation methods, the total number of colonies and their appearance were af-

fected by ERH. This suggests that the conditions were selective for some mold strains. We expected that *A. sydowii*, *A. ochraceus*, and *P. funiculosum* would not grow as well at 75% ERH as *E. herbariorum*. Of all these species, *E. herbariorum* has the lowest published minimal a_w (12).

The selection of a particular method will depend on the aims of the study, the precision required, and the material constraints of the laboratory. The easiest methods to carry out in this study were the spot and air cabinet methods, but the air cabinet system suffers from wide variations in inoculation levels. Although all four methods can be used to evaluate the mold-free shelf life of cakes, the spot method was the most appropriate for study of the effect of the environment on the growth rate of the molds. Therefore, of the four methods tested, the spot method appears to be the method of choice for most applications.

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