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Estimation of growth parameters of six different fungal species for selection of strains to be used in challenge tests of bakery products

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ABSTRACT

The objective of this study was to estimate the growth rate, lag time and the times for the appearance of visible mycelium (t_v) of six fungal species (*Paecilomyces variotii*, *Penicillium paneum*, *Penicillium polonicum*, *Penicillium spinulosum*, *Penicillium citrinum* and *Aspergillus sydowii*). The growth parameters were determined at 20 °C, 25 °C and 30 °C and in two culture media (18% Dichloran Glycerol Agar - DG-18 and Dichloran Rose Bengal Chloramphenicol Agar – DRBC) aiming the selection of most appropriate strains for use in challenge tests and for the development of predictive models. Seventy-two growth curves were obtained after measuring the diameter of the colonies for 14 days at 20 °C, 25 °C and 30 °C. Values of growth rate between 1.4 and 11.1 (day^{-1}) were obtained in this study. Faster growth rates (> 7 day^{-1}) were observed for *Pa. variotii* (LMQA-001) and *P. paneum* (LMQA-002) at 25 °C. The lag times varied between 0.7 and 4.2 days, with 50% of the values being as short as 0.7 − 1.4 day. The variability between the species was analyzed and coefficients of variation (CV) of 44.2% and 41% for the growth rate were observed at 30 °C. At 20 °C and 25 °C, CV varied from 28.3% to 32.9%, respectively. Given these results, *Pa. variotii* and *P. paneum* could be considered for further use in microbiological challenge tests aiming to assess the robustness of bakery products formulations.

1. Introduction

Whole bread, as many other bakery products, is highly prone to fungal spoilage (Legan, 1993; Smith, Daifas, El-Khoury, Koukoutsis, & El-Khoury, 2004). Due to its intrinsic characteristics, such as slightly acidic pH (5 − 6), moisture content around 40%, water activity (a_w) between 0.93 and 0.96, bread is an excellent substrate for the growth of a wide variety of fungal species as it is usually stored at 20–30 °C. Apart from causing losses due to changes in the sensory characteristics of the products before the end of the shelf life, various fungal species also produce mycotoxins (Berenguer, Calderon, Herce, & Sanchez, 1991; Hill, Hocking, & Whitfield, 1995).

The main genera related to the spoilage of bakery products include *Penicillium*, *Aspergillus* and *Eurotium* (ICMSF, 2005; Pitt & Hocking, 2009). The predominance of a determined genus and species is mainly related to how the fungus respond to some factors, such as the a_w range, growth temperature, and tolerance to oxygen tension and preservatives (Pitt & Hocking, 2009). Fungal species, such as *Paecilomyces variotii*, *Penicillium paneum*, *Penicillium polonicum*, *Penicillium spinulosum*, *Penicillium citrinum* and *Aspergillus sydowii* have been isolated from cereals and cereal-based products (Lõiveke, Ilumäe, & Laitamm, 2004; Pitt & Hocking, 2009; Samson, 1974; Santos et al., 2016; Wigmann, Moreira, Alvarenga, Sant'Ana, & Copetti, 2016). *P. paneum*, *P. polonicum*, *P. citrinum* and *A. sydowii* show optimum growth temperatures between 20 and 30 °C, which is the temperature range that bread is commonly stored and commercialized (Pitt & Hocking, 2009; Samson, 1974). On the other hand, *P. variotii* presents optimal growth temperature between 35 and 40 °C, being able to grow up to 48 °C. The psychrophilic species *P. spinulosum* can grow at low temperatures, with a minimum of 0 °C and maximum of 30 °C (Pitt & Hocking, 2009). In addition, most of these species are xerophylic, and can grow at a_w values as low as 0.78 (Pitt & Hocking, 2009).

The spoilage of bread by fungi occurs after contamination by fungal spores, when favorable temperature and moisture conditions are available. Under these conditions, fungal spores can germinate and produce visible mycelium, thus leading to the product' spoilage (Dagnas & Membré, 2013). The rate of spore germination and mycelium size increase are dependent not only on some intrinsic and extrinsic factors of a food formulation but also on how fungi respond to these conditions. The output of these interactions are expressed in terms of growth parameters, which are characterized by some variability.

Variability represents the heterogeneity regarding the responses of a

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microbial cell or population. Depending on the heterogeneity observed, a data set can be spread or closely grouped. Variability in microbial data is currently a topic of major interest (Aryani, den Besten, Hazeleger, & Zwietering, 2015; Koutsoumanis & Lianou, 2013). The knowledge on the variability of growth parameters of fungi can be highly relevant for the formulation of products, challenge tests and predictive modelling studies, which aim to increase the microbiological stability of foods. Therefore, the current study aimed to assess variability among different fungal strains and to estimate their growth rate, lag time and the times for the appearance of visible mycelium. This information is highly useful as it further allows the selection of strains to be used in challenge tests and allows the development of predictive models aimed to improve the robustness of bakery products formulations.

2. Material and methods

2.1. Strains

The following fungal strains were used in the current study: *Paecilomyces variotii* (LMQA-001) *(E_1)*, *Penicillium paneum* (LMQA-002) *(E_2)*, *Penicillium polonicum* (LMQA-003) *(E_3)*, *Penicillium spinulosum* (LMQA-005) *(E_4)*, *Penicillium citrinum* (LMQA-006) *(E_5)*, and *Aspergillus sydowii* (LMQA-004) *(E_6)*. These strains were isolated from commercial whole multigrain bread (Santos et al., 2016) and were maintained on sterile 0.5% agar slopes (Acumedia, Lansing, Michigan, USA) until used in the experiment.

2.2. Culture media, inoculation and storage conditions

Using a sterile platinum needle, fungi strains were inoculated individually at the center of Petri plates (90 × 15 cm) containing 20 mL of 18% Dichloran Glycerol Agar (DG-18, Acumedia, Lansing, Michigan, USA) (a_w = 0.952; pH = 5.6) and Dichloran Rose Bengal Chloramphenicol Agar (DRBC, Merck, Darmstadt, Germany) (a_w = 0.994; pH = 5.6). After inoculation, the plates were wrapped using a polyethylene film and incubated at 20 °C, 25 °C or 30 °C. Each plate was prepared in duplicate and the experiments were repeated twice.

2.3. Growth assessment

The radial mycelial growth of the fungi colonies were monitored daily for 336 h (14 days). The diameter (mm) of the colonies was measured in two perpendicular directions using a digital pachymeter with a resolution of 0.01 mm (Digimess − 100.175BL, São Paulo, Brazil) (Silva, Sant'Ana, & Massaguer, 2010).

2.4. Mathematical modelling

The values of growth rate (μ_{max}, day^{-1}) and lag time (λ, days) were estimated by fitting the Baranyi & Roberts model (Baranyi & Roberts, 1994) (Eqs. (1) and (2)) using the software DMFit.

$$D = \mu_{max}A - \ln\left[1 + \frac{\exp(\mu_{max}A) - 1}{\exp(D_{max})}\right] \quad (1)$$

$$A = t + \left(\frac{1}{\mu_{max}}\right) ln\left[exp(-\mu_{max}t) + exp(-\mu_{max}\lambda) - \exp(-\mu_{max}t - \mu_{max}\lambda)\right] \quad (2)$$

where $D_{(t)}$ is the diameter of the colony as a function of time (mm), μ_{max} is the maximum growth rate (day^{-1}), λ (days) is the time obtained from the intersection and D_{max} is the maximum diameter of the colony.

For each fungal strain, the time (t_v) for the appearance of visible colony was calculated using Eq. (3):

$$t_v = \lambda + \frac{D_3}{\mu_{max}} \quad (3)$$

Where D_3 was the minimum diameter for the appearance of visible colony (3 mm).

2.5. Statistical analysis

The results were assessed using the software Statistica 7.0 to assess the difference between the means. The Scott-Knot test was used, taking on $p < 0.05$ (Granato, Calado, & Jarvis, 2014; Nunes, Alvarenga, Sant'Ana, Santos, & Granato, 2015). Frequency distribution graphs were generated by Microsoft Excel version 2010, and coefficients of variation (CV) between the strains were obtained for the growth kinetic parameters, using the following equation: $CV\ (\%) = (SD/Mean) \times 100$. In this equation, SD refers to the standard deviations between the strains for the parameters for the same treatment, divided by the means of the parameters between the strains for the same treatment.

3. Results and discussion

A total of 72 growth curves were obtained using the data of colony diameter (mm) as a function of time (days) for *Pa. variotii*, *P. paneum*, *P. polonicum*, *P. spinulosum*, *P. citrinum*, and *A. sydowii*. The growth of these strains was compared for two different culture media that are commonly used for enumeration of fungi, i.e., DRBC and DG-18 agars (Hocking & Pitt, 1980; King, Hocking, & Pitt, 1979). These media were used to mimic a_w values of breads as it is known that whole grain breads present a moisture content of approximately 40% and a high a_w (0.94–0.96) (Smith et al., 2004). While DG-18 is recommended for the quantification and selection of fungi in products with a_w values < 0.95 (Hocking & Pitt, 1980; Samson, Hocking, Pitt, & King, 1992), DRBC is recommended for foods with a_w values > 0.95 (King et al., 1979).

The six strains studied were isolated from raw materials, final products and/or air of a bakery industry (Santos et al., 2016). Among the strains, *P. paneum* and *P. polonicum* are of special interest as these species were isolated from 20% and 24% of samples of final products (breads), respectively (Santos et al., 2016). This suggest that these species are able to overcome the hurdles imposed by the formulation of breads, such as a_w, pH, preservatives, further growth and spoil the product. *Pa. variotii, P. spinulosum*, *P. citrinum*, and *A. sydowii* were selected for this study because of their high prevalence in the air of processing environment, mainly after the baking step (Santos et al., 2016).

The growth of the abovementioned strains was assessed by measuring the colony diameter in the culture media incubated at 20 °C, 25 °C and 30 °C (Table 1). These temperature values were selected based on the storage conditions in which whole grain breads are exposed throughout shelf life. All the growth curves were linear after the initial lag period and, in some cases, upper asymptotes were observed. The upper asymptotes were observed as a result of growth limitation by the diameter of the plate (80 mm). The λ was estimated for all the fungi species studied with the exception of *P. citrinum* at 20 °C and 25 °C, as it was not possible to obtain this parameter after fitting to the model.

Table 1 shows the growth kinetic parameters (μ_{max} and λ) estimated after fitting the Baranyi model to the data (Eq. (1)). In DG-18, the growth rate of *P. paneum* and *P. polonicum* was significantly faster at 25 °C ($p < 0.05$), while *A. sydowii* presented a faster growth rate at 30 °C ($p < 0.05$). *P. paneum* and *P. polonicum* have been found to grow under stressful conditions. For instance, *P. paneum* can grow well at low temperatures, acidic conditions, and in the presence of high concentrations of carbon dioxide (Chitarra, Abee, Rombouts, Posthumus, & Dijksterhuis, 2004), whereas *P. polonicum* isolated from spoiled chilled products (Saccomori, Wigmann, Bernardi, Alcano-González, & Copetti, 2015) are able to survive after thermal treatments (Wigmann et al., 2016). The faster growth rate values obtained for *P.*

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