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Relationship between time-to-detection (TTD) and the biological parameters of *Pichia anomala* IG02; modelling of TTD as a function of temperature, NaCl concentration, and pH and quantification of their effects

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Abstract

The time to detection (TTD) for *Pichia anomala* IG02 was defined, for inoculum sizes lower than $6\log_{10}$ cfu/ml, as the time elapsed from inoculation to the moment at which an OD of 0.12 was reached. In other cases, TTD can be estimated by interpolation within the time elapsed from the previous readings below OD = 0.12 and the next above it. A linear relationship, which depended on the inoculum size, between ln TTD with $\ln\lambda$ and $\ln\mu_m$ was found. These relationships can be used to estimate the biological parameters of cultures with low inoculum levels. In addition, TTD for *P. anomala* IG02 could be modelled as a function of environmental conditions. The model can also be applied to λ and μ_m through their relationships with TTD. The effects of temperature, NaCl content and pH were quantified by the generalized *z*-values. An increase of 5.97 in NaCl concentration, a decrease of 1.97 units of pH, or a decrease of 6.08 °C doubled the TTD or caused a 2.53-fold increase in λ and a 2.56-fold decrease in the μ_m.

Keywords: *Pichia anomala*; Optical density; Microbial modelling; Table olives

1. Introduction

Yeasts are a group of microorganisms which are highly relevant to the food industry. They are responsible for numerous fermentations although they can also spoil some foods (Loureiro, 2000; Praphailong and Fleet, 1997). Yeasts play a critical role in all olive fermentations (Garrido Fernández et al., 1997), especially in directly brined green and naturally black olives (about 1,400,000 metric tones/year). Representative yeasts of naturally black olives are *Saccharomyces cerevisiae, Pichia anomala, Debaryomyces hansenii, Candida diddensii, Torulopsis candida* and *Pichia membranaefaciens* (González Cancho et al., 1975). In aerobic naturally black olive processes, there is a simultaneous coexistence of fermentative and oxidative yeasts. *P. anomala* has also been identified in this process (García García et al., 1985). Recently, Tassou et al. (2002) followed the yeast population in naturally black olives. Panagou et al. (2002) studied the yeast population in dry-salted olives of the Thassos variety and followed the evolution of the microbiological characteristics (yeasts included) in packed untreated green olives of the Conservolea cultivar (Panagou, 2004). Development of models for *P. anomala* IG02, a common yeast in table olives, as a function of environmental factors is of interest to control its presence during fermentation and storage.

In the emerging field of predictive microbiology, only a few studies are related to yeasts (Passos et al., 1997;

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Sorensen and Jakobsen, 1997; Tchango et al., 2000; López-Malo et al., 2000; Veiga and Madeira-Lopes, 2000; Betts et al., 1999, 2000; Jenkins et al., 2000) and none of them is on specific yeasts isolated from table olives. The only model in this field was developed by Panagou et al. (2003), who investigated the combined effect of temperature, pH and a_w on the growth rate of *Monascus rubber*, a heat resistant fungus isolated from green table olives.

Viable count enumeration(VCE), expressed as cfu/ml, is the most common procedure for an accurate estimation of the biological parameters of microbiological growth. However, the method requires extensive labour and consumables. Optical density (OD) is an alternative procedure that is also used to follow microbial growth (McClure et al., 1993). It is easily applicable, inexpensive, and may be applied for continuous monitorization of cultures. This potent tool has a serious drawback: the narrow interval of population concentrations within which it can be applied, usually the range where a linear relationship between OD and microbial growth is observed (approximately, in the range of 6–8 $\log_{10}$ cfu/ml for yeasts and 7–9 $\log_{10}$ cfu/ml for bacteria). Therefore, OD growth curves from cultures inoculated with less than 6 $\log_{10}$ cfu/ml can hardly be used for the estimation of their biological parameters. Lag time (λ) of these curves includes a real lag time and a period during which the culture is growing, but is not detected because its OD is below the detection limit of the equipment. The use of equipment with automatic monitorization of OD changes has stimulated the use of time of detection (TTD) to overcome such difficulties (Zhao et al., 2000; Baranyi and Pin, 1999; Zhao et al., 2002). This is not a biological parameter but can be used to estimate maximum specific growth (μ_{max}) (McKellar and Knight, 2000; Baranyi and Pin, 1999) and give information on lag phase (λ).

In this work, we evaluate the use of the TTD for the estimation of the biological parameters of microbial growth when cultures are inoculated with initial populations below 6 $\log_{10}$ cfu/ml, provided previous calibration relationships between λ and μ_m with TTD are established. Modelling of TTD as a function of the temperature, NaCl concentration, and pH and the quantification of their effects throughout the generalized z value is also studied.

2. Material and methods

2.1. Yeast strains

The yeast strain used in this study was *P. anomala* IG02, which is usually present in olive fermentation brines (Garrido Fernández et al., 1997). It was identified according to the taxonomic criteria of Looder (1970), Barnett et al. (1990) and Kurtzman (1998). Its traditional nomenclature in table olives (van der Walt, 1970) was *Hansenula anomala*; but, it was recently named *P. anomala* by Kurtzman (1998). The strain belongs to the current collection of this Department.

2.2. Preparation of inocula

One single colony from pure cultures of the strain was inoculated into 5 ml of YMGP (Yeast–malt–glucose–peptone) broth (Difco™YM Agar, Becton, Dickinson and Company, Sparkes, Maryland, USA) and then incubated at 30 °C for 48 h. After this period, tubes were centrifuged; the pellet was washed with saline (0.9% NaCl, w/v) and re-suspended again in sterile saline to obtain a concentration of about 7 $\log_{10}$ cfu/ml.

2.3. Inoculation and incubation

YMGP broth was conditioned (pH and NaCl adjusted) and 100 ml aliquots were dispensed into 250 ml bottles and sterilized at 115 °C for 10 min. Bottles containing YMGP broth were inoculated with 10 μl of the saline suspension of the yeast strain to obtain a concentration of about 3 $\log_{10}$ cfu/ml. The bottles were incubated at the different temperatures required by the experimental design.

2.4. Cell growth monitoring

Yeast growth was followed by simultaneous determination of OD (mean of three readings) and VCE. OD was measured in a spectrophotometer (Bio-Rad Smart-Spec TM 3000) at 600 nm, using uninoculated liquid YMGP as blank. To determine cfu/ml counts, the culture was diluted in saline solution (0.8% NaCl) and surface spread on YMGP agar plates, using a spiral plate maker (Spiral System®, Interscience, St Nom la Breteche, France). The cell concentration on plates was estimated by automatic colony counter equipment (IUL-COUNTERMANT, Barcelona) and expressed as cfu/ml. All plates were incubated aerobically at 30 °C for 48 h.

2.5. TTD definition and estimation

TTD has not been defined uniformly. McKellar and Knight (2000) defined TTD as the time elapsed from inoculation to the moment that an increment of 0.05 units of OD was observed. Baranyi and Pin (1999) defined it as the time needed to reach a turbidity equivalent to an $OD_{det} = 0.15$. Jenkins et al. (2000) defined it as the time elapsed from inoculation to the moment that an OD of 0.30 was reached. TTD in this work was deduced from independent growth curves of *P. anomala* IG02, using low inoculum levels. Linear