A simple mathematical model that describes the growth of the area and the number of total and viable cells in yeast colonies

E.-M. Rivas[1,2], E. Gil de Prado[1,2], P. Wrent[1,2], M.-I. de Sil´oniz[1,2], P. Barreiro[1,3], E.C. Correa[1,3], F. Conejero[4], A. Murciano[4,5] and J.M. Peinado[1,2]

Significance and Impact of the Study: Mathematical models used to predict microbial growth are based on liquid cultures data. Models describing growth on solid surfaces, highlighting the differences with liquids cultures, are scarce. In this work, we have demonstrated that a single Gompertz equation describes accurately the increase of the yeast colonies, up to the point where they reach their maximum size. The model can be used to quantify the differences in growth kinetics between solid and liquid media. Moreover, as all its parameters have biological meaning, it could be used to build secondary models predicting yeast growth on solid surfaces under several environmental conditions.

Abstract

We propose a model, based on the Gompertz equation, to describe the growth of yeasts colonies on agar medium. This model presents several advantages: (i) one equation describes the colony growth, which previously needed two separate ones (linear increase of radius and of the squared radius); (ii) a similar equation can be applied to total and viable cells, colony area or colony radius, because the number of total cells in mature colonies is proportional to their area; and (iii) its parameters estimate the cell yield, the cell concentration that triggers growth limitation and the effect of this limitation on the specific growth rate. To elaborate the model, area, total and viable cells of 600 colonies of *Saccharomyces cerevisiae*, *Debaryomyces fabryi*, *Zygosaccharomyces rouxii* and *Rhodotorula glutinis* have been measured. With low inocula, viable cells showed an initial short exponential phase when colonies were not visible. This phase was shortened with higher inocula. In visible or mature colonies, cell growth displayed Gompertz-type kinetics. It was concluded that the cells growth in colonies is similar to liquid cultures only during the first hours, the rest of the time they grow, with near-zero specific growth rates, at least for 3 weeks.

Introduction

Kinetic studies of growth on solid media are strongly needed in applied microbiology because predictive growth models have been based mainly on liquid cultures data, where micro-organisms have a planktonic growth in a higher homogeneous environment (Wilson *et al.* 2002). In solid cultures, the potential influence of the growth matrix structure on surface growth of micro-organisms, in terms of nutrient, water and oxygen distribution, has been remarked for many years (Hills *et al.* 2001; Wilson *et al.* 2002; Koutsoumanis *et al.* 2004). Different growth models of microbial colonies have been published. One of the first most used and cited was that published by Pirt in 1967 for bacterial colonies, based on an initial lineal increase of the radius with time, followed by a second

phase in which it is the squared radius or area which increases lineally (Cooper *et al.* 1968; Pirt 1975). More complex descriptions of the early development of colonies were published, mainly for bacteria (Palumbo *et al.* 1971; Reyrolle and Letellier 1979; Lewis and Wimpenny 1981; McKay and Peters 1995; Panikov *et al.* 2002; Theys *et al.* 2008, 2009) but also for yeasts (Gray and Kirwan 1974; Kamath and Bungay 1988; Vulin *et al.* 2014). Most of the works on yeasts were purely empiric, based only on colony size, without cell counting, made mainly with *Saccharomyces,* and using high inocula, although much lower contamination levels are actually found in natural or food environments. Their importance was also hindered by the short incubation times used, as compared with the extended period of colonies life (Francois *et al.* 2003). The goal of this work is the development of a mathematical model valid for the cell number and the area of the colonies that can be used for yeasts in general and able to quantify the differences between growth on liquid and solid media. To reach this goal and to overcome the experimental limitations of previous studies, we have analysed the growth in broth and on agar media of four yeast species with different oxygen requirements: *Saccharomyces cerevisiae,* purely fermentative, *Zygosaccharomyces,* with a mixed fermentative–oxidative metabolism, *Debaryomyces* that ferments glucose when oxygen is exhausted and *Rhodotorula,* a strictly oxidative basidiomycete. Four different inocula concentrations have been used and incubations of more than 500 h.

Results and discussion

Differences in cell growth kinetics between liquid and solid media

There were significant differences between the growth kinetics of viable cells, N_V, in liquid and solid cultures. Figure 1 depicts an experiment with *S. cerevisiae* growing in flasks and on plates, inoculated with a single cell. Initially, when the colonies were not or were hardly visible, both cultures grew exponentially as observed previously in bacteria (McKay and Peters 1995; Rodin and Panikov 1995; Wimpenny *et al.* 1995b) and in yeasts (Meunier and Choder 1999). The specific growth rate (μ) was equal and maximal (μ_{exp}) in the two types of cultures (Table 1) with very low inocula. The exponential phase, that took place when colonies were not, or hardly visible, was shortened with higher inocula as already observed by Meunier and Choder (1999). With an inoculum of 10^6 CFU, the initial μ_{exp} was lower than in liquid or low inocula solid cultures (Table 1). A lower μ in the exponential phase was also observed with bacteria colonies inside gel matrix, as compared with liquid cultures (Theys *et al.* 2008). After the exponential phase, when the colonies were already visible, a continuous decrease in μ was observed in the solid culture but not in the liquid one, in which the stationary phase was sharply reached. These results are in agreement with those observed in surface colonies of *Lactobacillus,* in which there was a growth rate

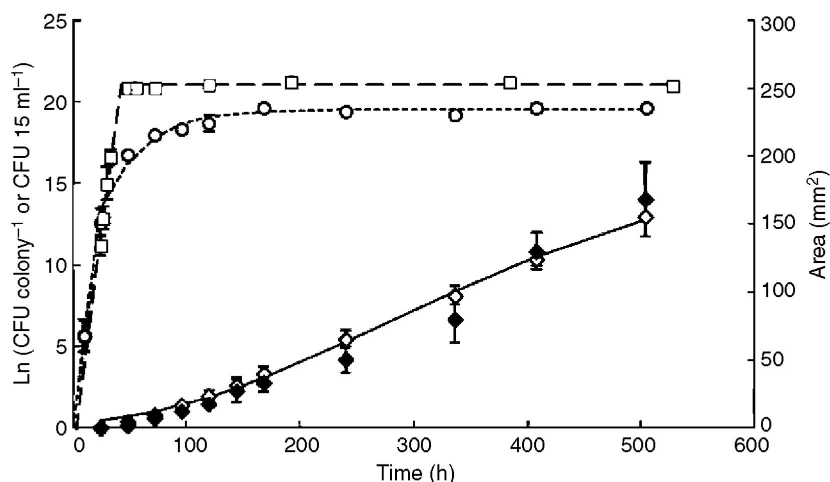

Figure 1 *Saccharomyces cerevisiae* viable cells growing in broth YMB (open squares) and YMA (open circles) mesured as CFU per 15 ml of liquid culture (the same medium volume of a plate) or per colony respectively (mean and standard deviation of three colonies or samples). Open diamonds show the mean value and standard deviation of the area of three undisturbed colonies from different plates. Closed diamonds show the mean value and standard deviation of the area of the three excised colonies whose counts are shown as open circles. The curves of YMB cultures were adjusted with Dmfit (discontinous line); for colonies, a linear fit was used for the first phase and the Eqn 7 for the second phase (dotted line). Equation 8 was used for the colony area (solid line).

Table 1 Kinetic parameters of the 1st (exponential) phases for viable cells of four yeast species, growing in broth or solid media, and of the 2nd phase (Gompertz) of solid cultures

	Exponential			Gompertz	
Strain	N_0	μ_{exp} (Broth) ± SD	μ_{exp} (Solid) ± SD	μ_i ± SD	$N_{V(max)}$ ± SD
Saccharomyces cerevisiae ATCC 7754	1	0·56 ± 0·017	0·54 ± 0·008	0·022 ± 0·012	$4{\cdot}97{\cdot}10^8 \pm 2{\cdot}17{\cdot}10^8$
	10^2	ND	0·47 ± 0·075	0·029 ± 0·011	$9{\cdot}15{\cdot}10^8 \pm 2{\cdot}33{\cdot}10^8$
	10^3	0·57 ± 0·029	0·46 ± 0·004	0·021 ± 0·012	$8{\cdot}44{\cdot}10^8 \pm 2{\cdot}78{\cdot}10^8$
	10^6	ND	0·32 ± 0·000	0·023 ± 0·014	$5{\cdot}92{\cdot}10^8 \pm 2{\cdot}14{\cdot}10^8$
Debaryomyces fabryii PR66	1	0·54 ± 0·010	0·50 ± 0·055	0·016 ± 0·008	$3{\cdot}43{\cdot}10^8 \pm 2{\cdot}16{\cdot}10^8$
	10^2	ND	0·51 ± 0·008	0·033 ± 0·013	$2{\cdot}87{\cdot}10^8 \pm 1{\cdot}04{\cdot}10^8$
	10^3	0·58 ± 0·056	0·48 ± 0·004	0·020 ± 0·011	$2{\cdot}49{\cdot}10^8 \pm 1{\cdot}05{\cdot}10^8$
	10^6	ND	0·19 ± 0·107	0·017 ± 0·005	$2{\cdot}49{\cdot}10^8 \pm 1{\cdot}34{\cdot}10^8$
Rhodotorula glutinis CECT 10145	1	0·51 ± 0·046	0·46 ± 0·006	0·012 ± 0·008	$9{\cdot}61{\cdot}10^8 \pm 2{\cdot}47{\cdot}10^8$
	10^2	ND	0·46 ± 0·043	0·035 ± 0·018	$1{\cdot}45{\cdot}10^9 \pm 5{\cdot}03{\cdot}10^8$
	10^3	0·48 ± 0·054	0·41 ± 0·039	0·009 ± 0·007	$1{\cdot}41{\cdot}10^9 \pm 1{\cdot}02{\cdot}10^9$
	10^6	ND	0·31 ± 0·000	0·012 ± 0·009	$9{\cdot}65{\cdot}10^8 \pm 4{\cdot}07{\cdot}10^8$
Zygosaccharomyces rouxii Bch	1	0·18 ± 0·008	0·20 ± 0·020	0·012 ± 0·006	$2{\cdot}64{\cdot}10^6 \pm 6{\cdot}48{\cdot}10^5$
	10^2	ND	0·17 ± 0·023	0·033 ± 0·012	$2{\cdot}93{\cdot}10^7 \pm 1{\cdot}51{\cdot}10^7$
	10^3	0·19 ± 0·029	0·19 ± 0·027	0·010 ± 0·004	$4{\cdot}32{\cdot}10^7 \pm 3{\cdot}32{\cdot}10^7$
	10^6	ND	0·08 ± 0·000	0·003 ± 0·001	$2{\cdot}83{\cdot}10^7 \pm 1{\cdot}73{\cdot}10^7$

N_0, inoculum (cells per flask or colony); μ_{exp}, maximum specific growth rate in broth or in colony in the initial exponential phase (h^{-1}); μ_i, specific growth rate at the inflexion point of the Gompertz curve; $N_{V(max)}$, maximal number of viable cells that the colony can reach under those experimental conditions; SD, standard deviation (0 values indicate that only two points could be used).

limitation when colonies reached a population of 10^5 CFU per colony (Malakar *et al.* 2002). In contrast, in others published results, obtained with submerged *Salmonella* in gelatine plates, that decrease in specific growth rate with time was not observed, and the Baranyi–Roberts model, with constant μ_{exp}, could be applied successfully in most of their growth curves (Theys *et al.* 2008). With our yeasts, both in liquid and solid culture and after some time that was always greater in the solid ones, N_V seemed to reach a maximal value, which implied that both cultures had entered into a stationary phase. However, the colony area continued to increase, indicating that, at least in solid culture, new cells were being incorporated into the colony. It is generally assumed that in yeasts, colonies growing cells coexist with G_0 or dying cells (Meunier and Choder 1999; Váchová *et al.* 2012). In our case, the increase of the area demonstrated that some cells were growing, but very slowly because the area increased only four times in about 300 h (see Fig. 1). This slow growth rate could not be detected in the semilog plot of viable cells due to the uncertainty linked to its determination. So, it could not be ascertain, with statistical significance, if the viable cells were maintained constant or they grew with a μ of about 0·005 h^{-1}. Clearly, the area provided a much more precise measurement of growth than when the number of cells was used. These results showed that mature yeasts colonies could be compared to continuous liquid cultures of at near-zero μ values, as those obtained with *S. cerevisae* in the retentostat (Boender *et al.* 2009). At very low growth rates, of the same order of those we have measured in the colonies, *Saccharomyces* energy metabolism shifts to mixed acid fermentation at μ values. These results are in accordance with the metabolic changes detected in colony cells, related with lactate transport, that have been described in *S. cerevisiae* colonies (Palková *et al.* 2002; Traven *et al.* 2012; Paiva *et al.* 2013). If these results are confirmed in other species, this would indicate that liquid and surface yeast cultures would be different not only in growth rates but also in their metabolism.

The decrease of the instantaneous μ values along time in microbial populations produces sygmoidal growth curves that can be described by a variety of models. Preliminary experiments showed that the use of both Logistic and Gompertz equations showed a reasonable goodness of the fit. We selected the Gompertz model, besides its reasonable fitting to experimental data, because of some additional advantages of the model that are described in the last section.

The Gompertz equation model (Eqn 5) could be used to describe cell growth in visible colonies also with higher inocula. The values obtained by the fitting are showed in Table 1. The statistical comparison of the parameters in Table 1 was hindered because the power of the analysis

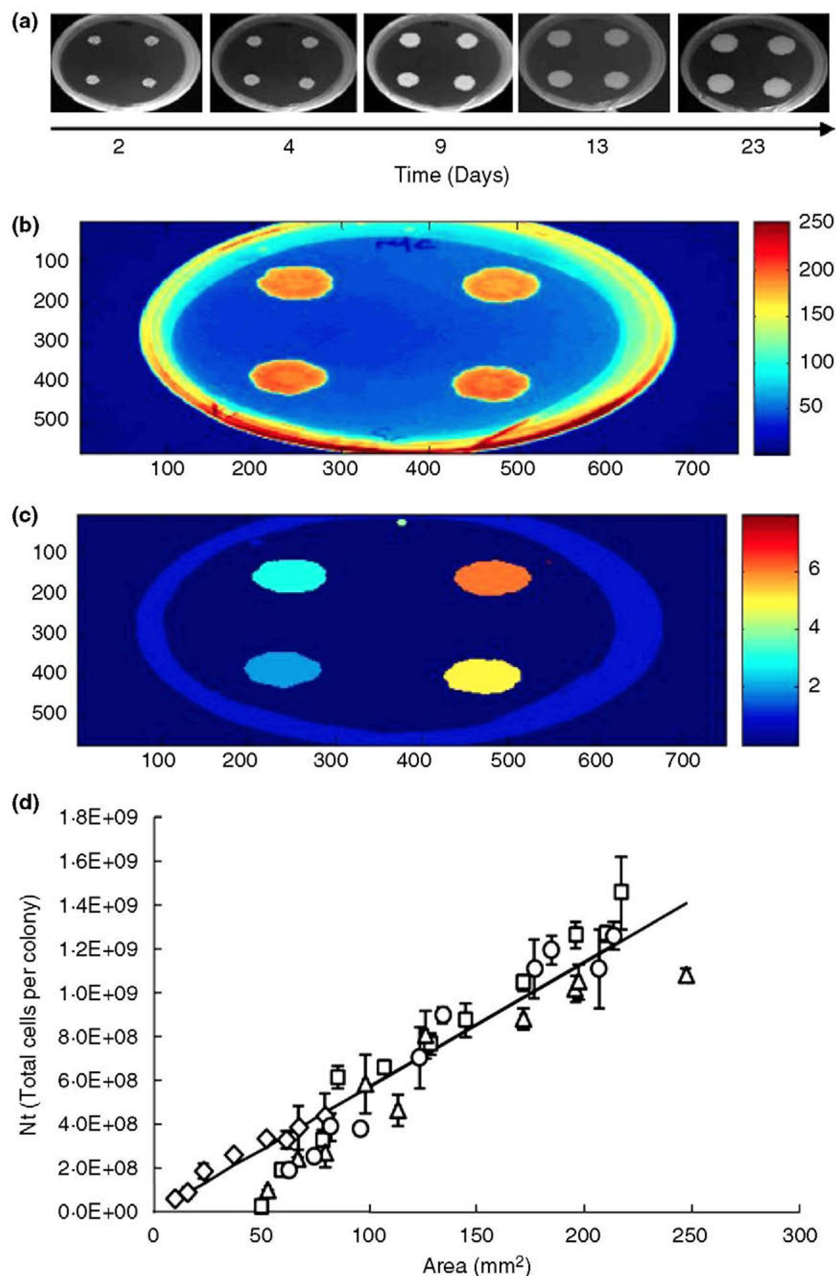

Figure 2 (a) Primary photos of colonies along time. (b) Image analysis (in colour in the online version): Colour segmentation of images depending on the grey segmentation threshold, self-adjusted for each image by application of Otsu's method. Bars indicate the colour allocated for each grey level. (c) Image analysis (in colour in the online version): Differentiation from the background. Bars indicate the colour allocated for each identified object of the image. (d) Correlation between the number of total *Saccharomyces cerevisiae* cells and the colony area. Inocula: 1 cell (diamonds); 100 cells (squares); 1000 cells (triangles); and 10^6 cells (circles). The slope of the linear regression line, crossing the origin, is considered the cell-surface coefficient (C_S).

was lowered by the high uncertainty linked to cell counting data. A more precise method to evaluate the cell number was desirable, and the area of the colony was selected for this purpose.

Relation between the area and the number of total cells of a colony

A good linear relation between the area, A, and the number of total cells, N_T, could be observed in colonies originated from one cell, as predicted by Eqn 2. Data from *S. cerevisiae* are depicted in Fig. 2d. With higher inocula, the initial areas were greater than expected when compared with the colonies from very low inoculum (see Fig. 2d), and this happened with the four species. When the colonies from higher inocula reached an area of about 80 mm^2, the ratio cells/area was the same as in the colonies from a single cell. This proportionality was observed in the four species, with R^2 values >0·95. The proportionality factor, C_s (cell-surface coefficient), was calculated as described in Materials and methods, and its value for each species was $5{\cdot}71{\cdot}10^6 \pm 1{\cdot}48{\cdot}10^5$ for *S. cerevisiae*,

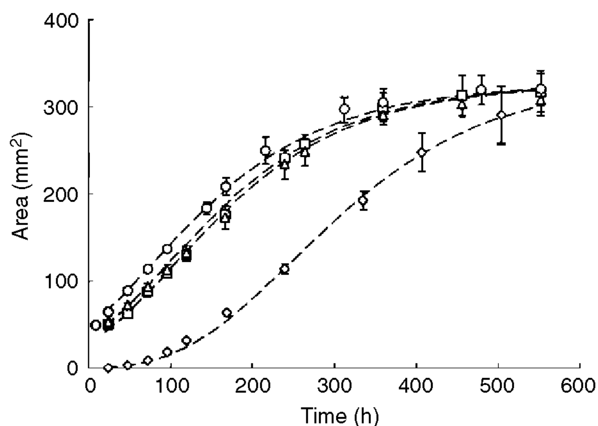

Figure 3 Kinetics of area growth in colonies of *Debaryomyces fabryi* originated from four different inocula. Fitting (discontinuous lines) corresponds to Eqn 8 with the parameters values of Table 2. Inocula: 1 cell (diamonds); 100 cells (squares); 1000 cells (triangles); and 10^6 cells (circles).

$5{\cdot}20{\cdot}10^6 \pm 1{\cdot}25{\cdot}10^5$ for *Debaryomyces fabryi*, $8{\cdot}22{\cdot}10^6 \pm 3{\cdot}85{\cdot}10^5$ for *Rhodotorula glutinis* and $2{\cdot}26{\cdot}10^6 \pm 9{\cdot}44{\cdot}10^4$ for *Zygosaccharomyces rouxii*, in cells mm^{-2}. Several types of equations, relating the logarithm of the number of cells and the area or radius of the colony, have been published for bacteria (McKay and Peters 1995; Harrison *et al.* 2000; Guillier *et al.* 2006; Skandamis *et al.* 2007). These are purely empirical relations because a mechanistic approach should relate directly to the number of cells contained in a colony with a determined volume. This has been our approach, as is described in Material and Methods and, to our knowledge, it is the first time applied to yeasts.

Growth Kinetics analysis of the colony area

The increase of the colony area could be accurately described by the Gompertz equation in all the cases (Eqn 8). Data corresponding to colonies of *D. fabryi*, together with their model curves, are showed in Fig. 3. The growth parameters values for all the species are in Table 2. Due to the higher precision of area measurements, the variability within each inoculum decreased strongly and the power of the statistical run test increased. This analysis supported that the Gompertz equation was appropriated to describe the area growth kinetics using, for the four inocula studied, the same maximum value of the area, $A_{(max)}$, and μ_i (the exponential decrease rate of the specific growth rate with time, as defined in Eqn 6). However, μ_i for total cells (area) were lower than those of viable cells (Tables 1 and 2). We submit that the higher value of μ_i for N_V, that is, the much more rapid decrease in μ values observed in the same colonies for N_V, as compared with N_T, is due to an inactivation process. Cell inactivation is present inside colonies of *S. cerevisiae,* as indicated by the appearance of apoptosis and G_0 markers in some cells (Meunier and Choder 1999; Váchová and Palková 2005, 2011). Inactivation is not an exclusive phenomenon of solid cultures. In stationary liquid cultures of *S. cerevisiae,* Allen *et al.* detected two types of cells: quiescent and non-quiescent. The first ones were unbudded daughter cells and they suggested they were in G_0 state. The second ones were composed of old replicative cells with more ROS and apoptosis signs (Allen *et al.* 2006). In fact, those apoptosis markers in liquid cultures have been detected by others authors in stationary phase. Chronologically aged cells showed DNA breaks, changes in chromatin condensation, ROS and caspase activation (Herker *et al.* 2004). The same markers have been described in cells growing in colonies. However, after some days, they were restricted to the cells in the colony centre. Those centre cells would release nutrients that would let the other cells to survive (Váchová and Palková 2005; Carmona-Gutierrez *et al.* 2010) explaining the high viability of colony cells. Palková also suggested the presence of viable but not cultivable cells inside the colonies (Palková *et al.* 2009).

Table 2 Kinetic parameters of the model equation for the area of four yeast species. Equation 8 for each species was tested for lack of fit applying run tests (Bradley 1968). The *P* values show that there is not a significant lack of fitness

Strain	$A_{(max)}$	Lower 95%	Upper 95%	μ_i	Lower 95%	Upper 95%	*P* value
Saccharomyces cerevisiae ATCC 7754	233·16	227·52	238·81	0·0070	0·0065	0·0075	0·15
Debaryomyces fabryi PR66	326·20	319·37	333·02	0·0085	0·0080	0·0089	0·38
Rhodotorula glutinis CECT 10145	296·11	281·95	310·28	0·0075	0·0065	0·0085	0·36
Zygosaccharomyces rouxii Bch	159·59	154·46	164·72	0·0055	0·0048	0·0061	0·73

$A_{(max)}$ (mm^2) is the maximal area that can be reached under the experimental conditions used. μ_i (h^{-1}) is the area specific growth rate at the inflexion point of the Gompertz curve.

Validation of the area as an estimation of total cells in surface colonies

The Gompertz equations for the area (Eqn 8) could be transformed into an equation to predict N_T in the colony. Assuming the range of values at which area and N_T are proportional, the area equations were converted into N_T equations multiplying them by their corresponding C_s values for each species (see Materials and methods). These model equations were validated comparing the N_T values predicted with the experimental values of N_T obtained in independent experiments. The bias and accuracy factors (Ross 1996), showed values close to 1, demonstrating that the area is an accurate and not biased method to estimate N_T in mature colonies.

Relevant properties of the Gompertz equation in primary and secondary modelling of the growth of yeasts colonies

The use of Gompertz model presents several advantages. The first is that one model equation describes the growth, previously modelled by two equations: the linear increase of the radius and then the linear increase of the squared radius (Pirt 1975) both for bacteria and yeasts (Kamath and Bungay 1988; Rodin *et al.* 1998; Salvesen and Vadstein 2000; Váchová and Palková 2005; Walther *et al.* 2011). The original Gompertz equation applied to cell number (not their logarithms) can be considered a mechanistic model (McKellar and Lu 2004), because all its parameters have biological meaning. In the model equations (Eqns 5, 8 and 9), the 'max' parameters ($N_{V(\max)}$, $N_{T(\max)}$, $A_{(\max)}$ and $r_{(\max)}$) estimate the maximal values that can be reached under the experimental conditions. The '0' parameters ($N_{V(0)}$, $N_{T(0)}$, $A_{(0)}$ and $r_{(0)}$) estimate the amount of cells or colony size at which growth limitation begin to be detected. The third parameter, μ_i, measures the rate at which the growth-limiting factors are affecting μ (Eqn 6). The greater its value, more rapidly the cells specific growth rate, μ, will decrease from its initial maximal value, at the end of the exponential phase, to near-zero values, due to growth limitations. The nature of the growth-limiting factors is not clearly known. Oxygen availability could be a relevant factor, but the similar results obtained with four species with so different oxygen requirements, do not support that relevance. The accumulation of toxic products inside the colony (Wimpenny *et al.* 1995a; Pipe and Grimson 2008) or its acidification (Wimpenny *et al.* 1995a; Walker *et al.* 1997) could be others. The diffusion of the limiting nutrient from the bottom agar through the colony is an obvious candidate (Walther *et al.* 2011). Our model can be a good help to identify those limiting factors and their effects, by analysing, in a quantitative way and after changing their concentrations, if they affect the biomass yield ($N_{V(\max)}$, $N_{T(\max)}$, $A_{(\max)}$ and $r_{(\max)}$), the growth restriction with time (μ_i) or both. It opens new possibilities for the development of secondary models once it had been validated in solid foods.

Another clear advantage of the Gompertz equation that has been relevant in our election of the model is that it has a useful property: its powers continue to be Gompertz equations with the same parameters. In this way, the equation for the area can be transformed easily in the equation for the radius with the same parameters (see Materials and methods). Although the measurement of the area by image analysis is much more accurate and do not depends on the shape of the colony, the simplification of circularity and the measurement of the radius, with a rule or micrometre, has been a very common method. The Gompertz equation permits to compare new and old published data based on the same parameters (maximal values and μ_i). For example, we have applied the model to the data on colony diameter of a not identified yeast, published by Gray and Kirwan (1974), obtaining a similar goodness of the fit and of the μ_i value ($0{\cdot}01\ h^{-1}$).

Taken together all these results, we conclude that mature colonies can be considered open cultures, which maintain a constant high density of viable biomass and grow at near-zero growth rates during many weeks, as long as substrate is available. In this work, we have demonstrated that those very low growth rates can be accurately measured by the colony area. The study of the pleiotropic effects of glucose limitation and the heterogeneity of the cells in the colony are the fields that we are now studying, but we think that our present quantitative results highlight the importance of the measured slow kinetics and the metabolic effects associated with it. They should be taken into account in basic experimental research using yeasts colonies and also in predictive solid food microbiology, once the model had been validated in solid foods.

Materials and methods

Strains and maintenance

The strains used are listed in Table 1. Spoiling strains (Bch and PR66) were isolated in our laboratory (Romero *et al.* 2005; Wrent *et al.* 2010). All were maintained at 4°C on Yeast Morphology Agar (YM-A) containing 0·5% (w/v) yeast extract (Difco Laboratories, Detroit, MI), 0·3% (w/v) proteose–peptone No.3 (Difco), 0·3% (w/v) malt extract (Difco), 1% (w/v) glucose (Panreac Quimica S.A., Barcelona, Spain) and 2% (w/v) agar.

Media and growth conditions in solid and liquid medium

Samples from slant cultures were inoculated into Erlenmeyer flasks containing 100 ml of Yeast Morphology Broth (YM-B) and incubated at 28°C with shaking overnight. Samples of these exponential cultures (15 h) were diluted in saline solution to prepare inocula. To produce colonies originated from a single cell, 50–100 μl, containing <10 cells, were spread over plates containing 15 ± 1 ml of YM-A. For higher inocula, four aliquots of 10 μl, with 10^2, 10^3 and 10^6 CFU, were inoculated as droplets ($6{\cdot}5 \pm 0{\cdot}5$ mm of diameter) in separate equidistant points. To analyse the growth in liquid medium, a minimum of three Erlenmeyer flasks containing 100 ml of YM-Broth, were inoculated with one to ten or 1000 cells approximately per flask from the appropriate dilution, as it was done for solid medium, and incubated with shaking at 120 rev min^{-1}. The incubation temperature was 28°C.

Count of viable and total cells in the colonies

To count the number of total (N_T) and viable cells (N_V) in the colonies along time, three selected colonies and their underlying agar were excised from each plate using a sterile alcohol-flamed spatula (Harrison *et al.* 2000). Each colony was resuspended separately in saline solution by shaking in vortex. N_T of the suspensions was calculated from the OD_{620} by the use of a calibration curve The N_V of the colony (CFU colony^{-1}) was estimated by Miles and Mishra method of plate count (Miles *et al.* 1938) inoculating four aliquots of 50 μl in a plate, from the same and proper ten-fold serial dilution. In liquid cultures, N_V of three separate batches was estimated using aliquots of 1 ml from each culture, sampled along time that, were conveniently diluted and inoculated, as with the samples from solid medium.

Measurement of colony area by image analysis

When colonies were visible (about 0·5–1 mm^2), digital pictures in greyscale of the colonies were taken (Fig. 2a). To automate the measurements of the area, an algorithm was programmed in Matlab ver. 7.0 (The MathWorks, Inc, Natick, MA) based on several image analysis routines from Image Processing Toolbox™. The first routine performed a segmentation of images according with a grey segmentation threshold, self-adjusted for each image by application of Otsu's method (Fig. 2b). Then, the objects of the images (yeast colonies) were differentiated from the background by colour (Fig. 2c). Using the function 'regionprops', several colony parameters, such as area and major and minor diameters, were calculated in pixels. We used a second routine to apply the first routine for the next images. The last routine changed the scale from pixels to millimetres. To validate this method, 130 values of radius measured and computed manually, by using rules, were compared with those obtained by the algorithm. The prediction errors in all cases did not exceed 3–4% of manual value.

Correlation between total cell number and the area of the colony along time

The relation cells area was analysed taking samples along the whole growth curves. Three colonies from different plates were selected each time. Their areas were measured and then they were excised and their cells resuspended to count N_T (cell groups per colony) and N_V (CFU per colony). Three other colonies from different plates, always the same along each experiment, were selected as control colonies and remained undisturbed to follow the growth of the colony area. To validate this method, the mean of the colony areas at the time of excision was compared with the mean area of the three undisturbed colonies at the same time. No significant differences in area among both types of colonies were found for any of the species and this is shown, representatively for *S. cerevisiae*, in Fig. 1. The experimental values of the areas correlated with the cells in Fig. 2d correspond to those of the excised colonies where the viable and total cells were estimated, also in *S. cerevisiae.*

Growth models and growth parameters calculation

Mature microbial colonies can be considered as cylinders of constant height h (in mm) (Pirt 1967; Kamath and Bungay 1988; Pipe and Grimson 2008). Consequently, N_T is related with the area, A (in mm^2), by the equation:

$$N_T = h\,\rho(N_T)\,A \tag{1}$$

$\rho(N_T)$ (cells mm^{-3}) is the density of total cells in the colony. Assuming that $\rho(N_T)$ is also a constant, then the relation of N_T with the area can be written as:

$$N_T = C_s A \tag{2}$$

We have called C_s at the cell-surface coefficient (cells mm^{-2}), whose value is equivalent to the height of the colony, multiplied by its cell density (Eqn 1). The value of C_s was calculated as the slope of the linear regression relating N_T with the corresponding values of area for each species.

With liquid cultures, the DMFit Web Edition (Institute of foof Research, Norwich, UK), based on Baranyi and

Roberts (1994), was used to estimate the growth parameters: initial and final value (Ln (CFU ml^{-1}), lag time (h) and maximum rate (h^{-1}).

In the solid growth curves in which the specific growth rate, μ, was not constant, its instantaneous value was estimated as the slope of the linear regression of the natural logarithm of the cell number vs time, in successive time intervals. This method was used for N_V, N_T and A. In the growth period when a direct proportionality between N_T and A exists (Eqn 2), the specific growth rates, μ (h^{-1}), of N_T and A are identical:

$$\mu(N_V) = \frac{\mathrm{dLn}(N_V)}{\mathrm{d}t} \quad (3)$$

$$\mu(N_T) = \frac{\mathrm{dLn}(N_T)}{\mathrm{d}t} = \frac{\mathrm{dLn}(A)}{\mathrm{d}t} \quad (4)$$

To describe the growth kinetics of N_V after the exponential phase, a Gompertz equation was used:

$$N_V = N_{V(\max)} \cdot \mathrm{e}^{-\left(\mathrm{Ln}\left(\frac{N_{V(\max)}}{N_{V(0)}}\right) \cdot \mathrm{e}^{(-\mu_i(t-t_0))}\right)} \quad (5)$$

in which $N_{V(\max)}$ (cells colony^{-1}) is the maximal number of viable cells that the colony can reach under the experimental conditions; $N_{V(0)}$ (cells colony^{-1}) is the number of viable cells at the beginning of restricted growth; t_0 (h) is the time at which this transition between exponential and restricted growth would occur, and μ_i (h^{-1}) is the specific growth rate at the inflexion point of the Gompertz curve. One property of the Gompertz model is that the instantaneous specific growth rate, $\mu(t)$, decreases exponentially with time:

$$\mu(t) = \mu_0 \cdot \mathrm{e}^{-\mu_i(t-t_0)} \quad (6)$$

This equation shows that μ_i can also be defined as the exponential rate at which $\mu(t)$ decreases from μ_0 (the value of $\mu(t)$ at t_0, that should be equal to $\mu_{\exp}$), down to near-zero values.

Cell numbers are more conveniently expressed as their natural logarithms, so, the logarithmic transformation of Eqn 5 has been used to fit the data in the semilog plots of N_V vs time:

$$\mathrm{Ln}(N_V) = \mathrm{Ln}(N_{V(\max)}) - \mathrm{Ln}\left(\frac{N_{V(\max)}}{N_{V(0)}}\right) \cdot \mathrm{e}^{-\mu_i(t-t_0)} \quad (7)$$

A similar Gompertz equation was used also to describe the area increase:

$$A = A_{(\max)} \cdot \mathrm{e}^{-\left(\mathrm{Ln}\left(\frac{A_{(\max)}}{A_{(0)}}\right) \cdot \mathrm{e}^{(-\mu_i(t-t_0))}\right)} \quad (8)$$

in which $A_{(\max)}$ (mm^2) is the maximal surface that the colony can reach under those environmental conditions, $A_{(0)}$ (mm^2) is the initial area value at t_0 and μ_i is the specific growth rate at the inflexion point of the area curve. With the image analysis used, the area can be measure with reliability even in very small (0·12 mm^2 area and 0·4 mm diameter).

The corresponding equation for the colony radius can be easily obtained from Eqn 8 because the radius is a power (0·5) of the area and the Gompertz equation has the property that their powers are also Gompertz:

$$r = r_{(\max)} \cdot \mathrm{e}^{-\left(\mathrm{Ln}\left(\frac{r_{(\max)}}{r_{(0)}}\right) \cdot \mathrm{e}^{(-\mu_i(t-t_0))}\right)} \quad (9)$$

in which $r_{(\max)}$ (mm) is the maximal radius that the colony can reach under those environmental conditions, $r_{(0)}$ (mm) is the initial radius at t_0, and μ_i is the same of Eqn 8.

Statistical analysis

C_s values, relating the number of cells with the colony area, were estimated by linear regression analysis. Gompertz equations parameters were estimated from experimental data (viable cells, area and radius) by nonlinear regression through the Marquardt algorithm. The goodness of fit of the selected models was analysed by means of run tests (Bradley 1968). Run tests examine the serial correlations of the residuals to detect lack of fit in the selected models. Stata 9.0 (Stata Corp. LP, College Station, TX) was used to perform the statistical analyses.

The performance of the area model (Eqn 8) to predict the values of N_T were statistically analysed as described in Ross (1996).

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Conflict of interest

No conflict of interest declared.

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