J Ind Microbiol Biotechnol (2011) 38:153–165
DOI 10.1007/s10295-010-0840-4

ORIGINAL PAPER

Analysis of the effect of inoculum characteristics on the first stages of a growing yeast population in beer fermentations by means of an individual-based model

M. Ginovart · C. Prats · X. Portell · M. Silbert

Received: 25 March 2010 / Accepted: 26 July 2010 / Published online: 3 September 2010

Abstract The yeast *Saccharomyces cerevisiae* has a limited replicative lifespan. The cell mass at division is partitioned unequally between a larger, old parent cell and a smaller, new daughter cell. Industrial beer fermentations maintain and reuse yeast. At the end of fermentation a portion of the yeast is 'cropped' from the vessel for 'serial repitching'. Harvesting yeast may select a population with an imbalance of young and aged individuals, but the output of any bioprocess is dependent on the physiology of each single cell in the population. Unlike continuous models, individual-based modelling is an approach that considers each microbe as an individual, a unique and discrete entity, with characteristics that change throughout its life. The aim of this contribution is to explore, by means of individual-based simulations, the effects of inoculum size and cell genealogical age on the dynamics of virtual yeast fermentation, focussing on: (1) the first stages of population growth, (2) the mean biomass evolution of the population, (3) the rate of glucose uptake and ethanol production, and (4) the biomass and genealogical age distributions. The ultimate goal is to integrate these results in order to make progress in the understanding of the composition of yeast populations and their temporal evolution in beer fermentations. Simulation results show that there is a clear influence of these initial features of the inocula on the subsequent growth dynamics. By contrasting both the individual and global properties of yeast cells and populations, we gain insight into the interrelation between these two types of data, which helps us to deal with the macroscopic behaviour observed in experimental research.

Keywords Yeast population · Yeast inoculum · Fermentation · Individual-based modelling · Serial repitching

This article is part of the BioMicroWorld 2009 Special Issue.

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Introduction

Microbiology has traditionally been concerned with and focussed on studies at the population level. Information on how cells respond to their environment, interact with each other, or undergo complex processes such as cellular differentiation or gene expression has been obtained mostly by inference from population-level data. Nevertheless, individual microorganisms, even those in supposedly 'clonal' populations, may differ widely from each other in terms of their genetic composition, physiology, biochemistry, or behaviour [1, 31]. This heterogeneity has important practical consequences for a number of human interests, including, among others, the productivity and stability of industrial fermentations. New appreciation of the existence

and importance of cellular heterogeneity, coupled with recent advances in technology, has driven the development of new tools and techniques for the study of individual microbial cells. As a result, scientists have been able to characterize microorganisms and their activities at unprecedented levels of detail [1]. Because observations made at the single-cell level—individual-based observations—are no longer subject to the 'averaging' effects characteristic of bulk-phase (population-level) methods, they offer a unique possibility to observe discrete microbiological phenomena that is unavailable using traditional approaches. This type of information could also be useful in advancing the development of more accurate models applied to the prediction and control of fermentative processes.

The yeast *Saccharomyces cerevisiae* has a limited replicative lifespan, i.e. each cell within a population is capable of only a finite number of divisions prior to senescence and death. Replicative ageing depends on the number of divisions experienced by each cell, and can be determined by counting the number of bud scars on the wall of the mother cell. As a consequence of senescence, yeast cells are subject to morphological, metabolic and genetic modifications [24, 25, 31]. However, the attempt to ensure both the production of quality beer and the maintenance of yeast vitality exposes yeast cells to a great deal of stress [8]. Towards the end of beer fermentation, yeast begins to form large clumps of cells or 'flocs' and subsequently sediments and collects within the fermenter cone. Industrial fermentation to produce beer is unique within the alcoholic beverage industry in the sense that the yeast is not discarded after use, but is maintained and reused a number of times in a process termed 'serial repitching'. Serial repitching, whereby yeast cropped at the end of fermentation is reused in subsequent fermentations, is nowadays a process ripe for study. The rate at which each cell sediments is believed to vary according to its replicative age. Older cells tend to sediment faster and accumulate at the bottom of the cone, leading to stratification by genealogical age. So, sedimentation results in the formation of zones enriched with cells of a particular age. Typically, the yeast removed ('cropped') from the fermentation vessel to be used for serial repitching is the centre-top portion, theoretically comprising middle-aged and virgin cells. However, yeast is increasingly removed earlier, in order to decrease process time via a 'warm' or 'early' cropping regime. This facilitates removal of the lower portion of the crop, comprising a greater proportion of aged cells. Harvesting yeast may therefore select for a population with an imbalance of young and aged individuals, depending on the cropping mechanism employed [20, 24, 25, 30].

For the budding reproduction of yeast cells, the cell mass at division is partitioned unequally between a bigger, old parent cell and a smaller, new daughter cell. After each yeast cell birth, the cell size increases up to the critical threshold required to enter the budded phase. This threshold value is modulated according to the genetic background, the genealogical age of the cell and the physiological growth conditions. Studies in this context would bring significant advantages to industrial fields in which budding yeast is widely used. In fact, the output of a bioprocess is strictly dependent on the physiology of each single cell in the population, on the distribution of the cells throughout the cell cycle, and on the effects of environmental conditions on the population [31]. Understanding the behaviour of budding yeast populations at the single cell level, and monitoring and even controlling bioprocesses by using mathematical models, represents a future that, while not yet close, is extremely challenging and promising [2, 18, 23].

Flow cytometry is a powerful technique for the rapid analysis of single cells in a mixture, and cell characterisation is possible by means of both scattering and fluorescence signal measurements in order to determine intrinsic or extrinsic cell parameters [3]. *Saccharomyces cerevisiae* has been used as a template microorganism in a large number of this kind of experimental study. In biotechnology, the power of this method lies both in the possibility of determining a wide range of cell parameters at the single cell level, and in the ability to obtain information about their distribution within cell populations, providing valuable information for bioprocess design and control [4]. For instance, this technique has been used for the identification of different daughter and parent subpopulations in asynchronously growing *S. cerevisiae* populations [22], for the physiological analysis of yeast cells during 'serial repitching' of low-malt beer fermentation [21], and for age assessment of a yeast population [20].

Unlike continuous models, individual-based modelling (IbM) is a bottom-up approach, meaning that it considers each microbe as an individual, a unique and discrete entity, with more than one characteristic that changes throughout its life. IbMs explicitly simulate individuals, and population-level behaviour emerges from their cumulative behaviour and interaction. In ecological modelling, IbM constitutes a well-established alternative to the traditional population-level approach, and most applications of IbM to date have been geared to high trophic levels [15]. However, advances in microbiology and biochemistry have stimulated an increase in the application of IbMs to microbes. Thus, IbMs are increasingly being established to deal with diverse microbial communities [19], and their use is also becoming more widespread in food microbiology [7]. In addition, and in contrast to population-based models, IbM outcomes can be checked against results proceeding from the above-described experimental techniques that involve individual-based observations.

Of the microbial IbMs available at the present time (for a recent review, see [19]), we use INDISIM, the simulator developed by our group [9], which has already been used to study different features of bacterial growth [26–28], providing an ample pool of interesting results. A short review and selected applications of INDISIM can be found in [6]. INDISIM-YEAST constitutes the adaptation of INDISIM to the study of the specific characteristics of the yeast cell cycle, and is designed to deal with yeast populations growing in liquid media [11, 12]. Recently, this simulator was used to study the influence of cell ageing and inoculum size on the lag phase and first division time of a yeast culture [13]. The aim of this contribution is to explore, by means of the simulator INDISIM-YEAST, the effects of inoculum size and cell genealogical age on the dynamics of yeast fermentation, focussing on: (1) the first stages of yeast population growth, (2) the mean biomass evolution of the population, (3) the rate of glucose uptake and ethanol production, and (4) the biomass and genealogical age distributions, in order to be able to integrate these results and to advance understanding of the composition of yeast populations and their temporal evolution in beer fermentations.

Materials and methods

For this study, we used INDISIM-YEAST as the individual-based simulator, which is based on the generic simulator INDISIM [9, 11–14]. Although the reader can refer to some previously published papers for a description of different parts of INDISIM-YEAST [11, 12], a formal and brief description of this simulation model is presented below in order to make this study more self-contained and autonomous. Moreover, in this outline description we have adopted the ODD (overview, design concepts, and details) standard protocol established by Grimm and co-authors [16]. This protocol is widely accepted for describing IbMs, and contributes to the further unification of the formulation and implementation of IbMs. This protocol combines a general structure for describing IbMs (making the description of the model independent of its specific structure, purpose and form of implementation) and the language of mathematics (separating verbal considerations from a mathematical description of the equations, rules, and schedules that constitute the model).

Description of INDISIM-YEAST according to the ODD protocol

The sequence used in the presentation of this yeast IbM following this ODD protocol consists of seven elements: Purpose, State variables and scales, Process overview and scheduling ('O: Overview'), General concepts underlying the design ('D: Design concepts'), Initialization, Input, Sub-models ('D: Details').

O: Overview

Purpose

This rule-based model was developed for analysing the dynamics of populations of generic single-species of yeast, and the collective behaviour that emerges from inocula that is affected mainly by intra-specific diversity and variability at an individual level in a liquid medium.

State variables and scales

This model has two hierarchical levels: individual yeast cells and simulated area. Each yeast (E_i) is defined by a vector that contains its individual characteristics and variables: $e_1(t)$, $e_2(t)$ and $e_3(t)$, identify its position in the spatial domain; $e_4(t)$, its biomass, which is related by the model to spherical geometry in order to evaluate its cellular surface; $e_5(t)$, its genealogical age as the number of bud scars on the cellular membrane (Figs. 1, 2); $e_6(t)$, the reproduction phase in the cellular cycle in which the cell currently is, namely the unbudded or budding phase, i.e. Phase 1—the unbudded phase, when the cell gets ready to create a new cell (the bud), and Phase 2—the budding phase, in which the daughter cell-genealogical age 0 (virgin cell) grows until it separates from the parent cell, leaving behind another scar (Fig. 1); $e_7(t)$, its "start mass", the mass required to change from the unbudded to budding phase; $e_8(t)$, the minimum growth of its biomass for the budding phase; $e_9(t)$, the minimum time required to complete the budding phase; $e_{10}(t)$, its survival time without satisfying its metabolic requirements. Let $N = N(t)$ denote the total number of individual yeast cells at time t. Then

$$P_N(t) = \left\{E_i\left[e_1^i(t), e_2^i(t), \ldots, e_{10}^i(t)\right]\right\}_{i=1,2,\ldots,N} \quad (1)$$

denotes the matrix that describes the state of the population at time t and identifies each individual yeast cell (i) in the system.

The simulated area is a cube, with liquid medium and yeast, subject to appropriate boundary conditions, and divided into spatial cubic cells ($Sxyz$), each described by a vector that stands for the instant values of characteristic variables, such as the main nutrient or glucose particles $[s_1(t)]$ and excreted ethanol particles, as the only end product $[s_2(t)]$. The three dimensional grid composed of 1,000 spatial cells is then described as a whole by:

$$D(t) = \left\{S_{xyz}\left[s_1^{xyz}(t), s_2^{xyz}(t)\right]\right\}_{x=1,\ldots,10;\, y=1,\ldots,10;\, z=1,\ldots,10} \quad (2)$$

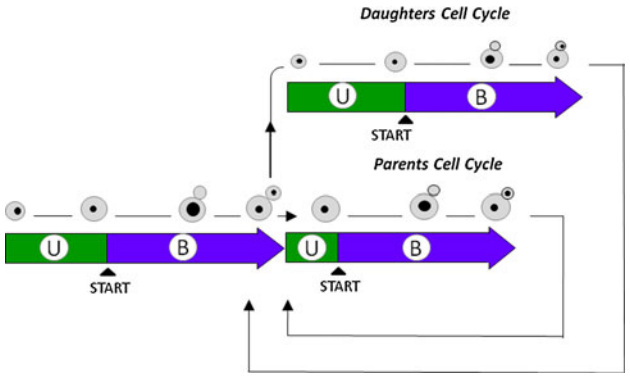

Fig. 1 Scheme of the budding reproduction model implemented at individual level yeast cell. *U* Phase 1 or the unbudded phase, *B* Phase 2 or the budding phase. Adapted from Hatzis and Porro [18]. During the unbudded phase the yeast cell grows and gets ready for budding. It enters the budding phase once it has attained a minimum cellular mass, the 'start mass', and a minimum increase in biomass. Budding phase lasts until two conditions are fulfilled: a minimum time interval must go by to allow for DNA replication, and a minimum amount of biomass must be synthesised

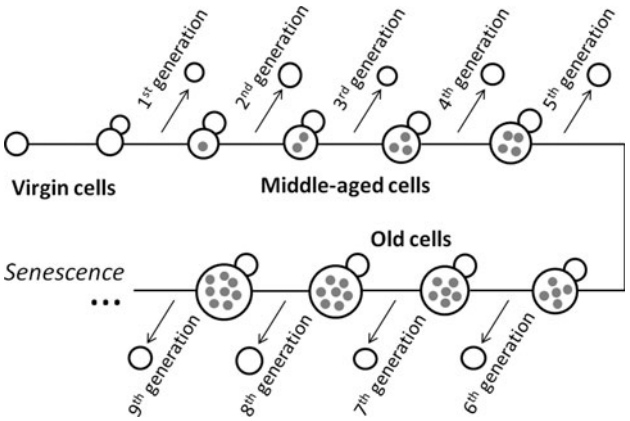

Fig. 2 Yeast model illustrating the progression from a newly emerged daughter cell (virgin cell) to senescence, moving through the different genealogical ages, which allows classification of the parents cells into middle-aged cells (or young parent cells) and old cells (or old parent cells). When yeast cell division takes place, a daughter cell is formed. The parent cell genealogical age is updated, and the number of bud scars on its cellular membrane is increased by one

The temporal evolution of the population is divided into equal intervals associated with computer or time steps. The scales covered by INIDISIM-YEAST range from 1 to 60 min for the time step and comprise cell sizes from several microns to millimetres, while the extent of the model may reach the centimetre scale.

Process overview and scheduling

The sets of rules governing the behaviour of each yeast cell are in the following categories or sub-models: glucose uptake, cellular maintenance, new biomass production, ethanol excretion, budding reproduction and cell viability. The biological sub-models are based on information from the specialised literature and other models. The environmental impact of yeast activity and extracellular phenomena are also locally described. At each time step, yeast cells act or perform their set of actions sequentially, and once this is finished, processes concerning configuration of the environment are taken into account. Because we assume that glucose fermentation takes place in a closed environment, there is neither entry nor exit of yeast cells, nutrient particles or end particles (batch culture). Stirring the culture permits the exclusion of local diffusion limitations, thus there is a periodic redistribution of yeast cells and particles within the system. The global scheduling of the simulation model is made up of several elements: (1) initialisation of the system, in which the input data is entered and the initial configuration of the population and environment is determined; (2) the main loop (time step), in which all the rules for each yeast cell and the medium are implemented and repeated until the end of the simulations; and (3) the output of results and their storage, including the information obtained from each yeast cell at the end of each time step. This makes it possible to obtain the results of simulations both at the level of individual cells and at the level of the yeast population throughout the fermentation profiles. Flowcharts of the computer code can be found in a previous publication [12].

D: Design concepts

The design concepts most carefully studied are the effects on the global behaviour of the diversity and variability in the yeast population, and the stochasticity of individual and local processes. Understanding of the emerging phenomena arising from these complexities at an individual and local level is the main aspiration of this kind of model.

Stochasticity is introduced into the model when setting some characteristics of individuals using a Gaussian distribution around an expected mean value. This distribution remains the most commonly encountered distribution in nature and statistics, and reflects range in the population. Randomness is also considered when the rules are applied to individuals and to spatial cells by using probabilistic distributions to deal with or manage individual events. This represents the uncertainty in these processes and reflects the high variety of mechanisms that underlie the irregularity observed in natural processes and biological materials. Finally, the sequence of actions of individuals changes randomly at each time step, in order to avoid privileging first-acting yeast cells.

Individual adaptive behaviour, the further interactions of the different biotic and/or abiotic elements of the system,

and optimization of fitness and the individual's sensitivity, which can modulate such optimization, may be addressed under other conditions and uses of the model.

This simulator continuously records the state variables. The simulation output can be separated into data related to the global properties of the system and data concerning the properties of individual yeast cells. The former includes information on the temporal evolution of the number of nutrient particles (glucose), the number of metabolites (ethanol particles), the average nutrient consumption (defined as the number of nutrient particles metabolised at each time step divided by the number of viable cells), the number of viable yeast cells, the number of non-viable yeast cells, viable yeast biomass, non-viable yeast biomass, heat dissipation of the system, maintenance energy expended by the yeast population (defined as the number of metabolised nutrient particles not used in the production of new biomass), and the mean biomass of the cell population (defined as the viable biomass in the culture divided by the number of viable cells). In addition, microscopic population parameters, namely distributions of variables controlled at the individual level, such as the distributions of genealogical age and of the biomass of the populations (Fig. 2), are considered among other potential individual properties. These distributions are related mainly to the cellular cycles of budding reproduction, and reflect the state of the yeast population at given times in the fermentation process. Because the simulator saves information about every cell at each time step, this recorded information makes it possible to construct bar charts and histograms to represent the latter distributions. These simulations results allow us to observe the time evolution of the system and the structure of the population throughout the virtual fermentation process. The preceding separation of output simulation results mirrors the classification of experimental techniques used to study those properties.

D: Details

Initialisation

Initialisation and input parameters are obtained from experimental measurements or from accepted values found in the literature. This means that most of the values that are used to characterise the system at a cellular level of description are inferred from information obtained at a system level (see, for instance, the study of Ginovart et al. [10]).

The initial number of yeast cells that constitute the inoculum and the glucose particles distributed uniformly in the spatial domain are input parameters of the simulation (see the section on "virtual experiments" below). In order to test population dynamics under different initial conditions, to compare these evolutions among themselves, and to identify tendencies and qualitative behaviours found in experimental research, it seems reasonable to initialise this IbM with initial conditions independent of any specific set of experimental data. In all the work discussed below we use dimensionless units. We assume that units of length are given in terms of the length of a spatial cell, units of time in terms of program steps, and units of mass in terms of the critical mass of reproduction. Actually, one unit of simulated mass (usm) has been chosen to be 0.7% of the value of the critical mass. Quantitative comparisons with sets of experimental data will constitute further studies.

Input

Input parameters account for the external manipulation of the culture system. Periodically, at each time step, the system (yeast and abiotic particles) will be stirred, and therefore a redistribution of these elements takes place.

Sub-models

Specific rules designed for sub-models have been discussed in previous publications related to this model [11, 12, 14], but in order to give a precise idea about how these rules work, the budding reproduction (Fig. 1), which is the most relevant sub-model for this study, is detailed here as follows:

The simulator simplifies the yeast cell cycle by assuming that a new cell cycle is allowed to begin only after the preceding cycle is completed, and that the model for the cellular cycle involves only two clearly differentiated phases. Phase 1, or unbudded phase $[e_6(t) = 1]$, covers most of phase G1 and a very small fraction of phase S in the traditional division of the cell cycle [18, 31]. Phase 2, or budding phase $[e_6(t) = 2]$, covers a small fraction of G1, most of S and all of G2 and M [18, 31]. We assume that, in the unbudded phase, the yeast cell is getting ready for budding. The change to the budding phase takes place only if, at the end of Phase 1, the following conditions are satisfied: (1) the cell has attained a minimum stochastic cellular mass m_S, the start mass, related to the constant m_C, the critical mass; (2) the cell has achieved a minimum growth of its biomass, Δm_{B1}.

When Phase 1 begins, a value $e_7(t) = m_S$ is chosen randomly for each cell in the manner described below. m_S is the minimum mass the cell must attain during this first phase for the process of the phase change to take place, and its value is also a function of the individual cell properties. We denote by m_{in} the value of the mass of a yeast cell at the beginning of Phase 1. The following checks are made at each time step and for each yeast cell. If $m_{in} \leq m_C - \Delta m_{B1}$ then the start mass assigned to the cell

is $m_S = m_C + z_4$; otherwise, if $m_{in} > m_C - \Delta m_{B1}$ then the start mass assigned to the cell is $m_S = m_{in} + \Delta m_{B1} + z_4$ [z_j are random draws from a normal distribution with mean equal to 0 and standard deviation σ_i, $Z(0, \sigma_i)$]. Hence, whenever the mass of a yeast cell is bigger than m_S, the cell enters Phase 2. Note that, within our model, Phase 1 does not need to be completed in a given time interval. The check in our model is whether an individual cell has reached a start mass, irrespective of its original value and growth rate. The budding phase is the least flexible in the cellular cycle as it requires both temporal and growth checks. Within our model, two conditions must be satisfied for the initiation of cell division. These are: (1) a minimum growth of biomass $e_8(t) = \Delta m_{B2}$; and (2) a minimum time interval $e_9(t) = \Delta T_2$. As in Phase 1, we appeal to random variables. A yeast cell will complete its cellular cycle when: (1) it reaches a minimum growth Δm_2 of its biomass, given by $\Delta m_2 = \Delta m_{B2} + z_5$, and (2) it has remained in Phase 2 for a minimum time interval given by $\Delta t = \Delta T_2 + z_6$. The first condition is necessary because a yeast cell must have a minimum number of molecules and satisfy minimum structural requirements in order to function as an independent entity. In a culture starved of glucose, or subjected to other inhibitory effects, the growth rate will be slower. On the other hand, the bud growth, even under optimal growth conditions, has to be completed within a minimum time interval; this is what the second condition requires. The budding phase is completed with the cell division, a daughter cell and a parent cell, with a total combined mass $m_T = m_1 + \Delta m_2$. The mass of the daughter cell is given by $m_D = q \ \Delta m_2 + z_7$, where q denotes a percentatge that ensures both that the parent cell will experience growth during this Phase and that the daughter cell is a fraction of Δm_2. The mass of the parent cell is given by $m_P = m_T - m_D$. We note that the reproduction rules in INDISIM-YEAST are implemented every time a new yeast cell appears. Hence, different yeast cells in the culture need not have the same mass when the reproduction process begins. Moreover, the yeast cells involved in the reproduction process remain active, and the local environmental conditions, in turn, indirectly affect the overall yeast growth rate.

The different sub-models, glucose uptake, cellular maintenance, new biomass production, ethanol excretion, budding reproduction, and cell viability allow the integration, among other things, of some important aspects for this study: (1) the scars left on the parent cells, as they affect the cellular membrane and therefore the nutrient uptake and the possibility of new reproduction cycles; (2) the increase in volume, or biomass, of parent cells, as they increase their genealogical age; and (3) the varying duration of the reproduction cycles, unbudded and budding phases, since these phases require a minimum time interval of stasis as well as a minimum growth of biomass in order to move from one phase to the other.

The web page https://aneto.upc.es/simulacio/hoja-portada presents a basic version of INDISIM-YEAST, which allows for simulations of fermentation processes, and graphically represents a few of the variables controlled by the simulator [12].

Virtual experiments

We used the INDISIM-YEAST simulations in order to check the influence of the age and size of the inocula on the subsequent increase in population and the rates of glucose usage and ethanol production, as well as the internal structure of the population. This type of study can offer new mechanistic insights into the topic, suggest new experiments to correlate and corroborate empirical knowledge and biological principles on the subject [8, 21, 24, 25, 30], and can give ideas of how to design new strategies for ethanol production after testing the hypotheses highlighted in these virtual experiments.

The study was developed by following the growth of a virtual population of the yeast *S. cerevisiae*, up to the point when the production of ethanol has flattened out. Cells of different genealogical ages are then virtually cropped. Thus, this simulator provides a complete virtual characterisation of a pre-inoculum to be used in subsequent virtual experiments (fermentations). From this we chose the inocula, combining different sizes (i.e. 1, 2, 5, 10, 50, 100 and 1,000 cells) and genealogical age distributions (i.e. virgin or daughter cells with 0 scars, young parents or middle-aged cells with one–five scars, and old parent cells with more than five scars, exemplified in Fig. 2). A series of simulations was performed, selecting inocula that combined these two factors. We also simulated the growth of heterogeneous inocula of different sizes.

Results and discussion

In order to present and discuss the qualitative behaviour of the virtual yeast system, we have split the outcomes of the simulator for this study into different parts, collecting global and individual properties with a view to connecting them afterwards. Such a division of simulation results is somewhat arbitrary, as both parts stem from the collective behaviour of the yeast cells and their interaction with their environment.

Initial growth of the yeast population

Special attention is paid during the first stages of the development of culture until the population reaches the

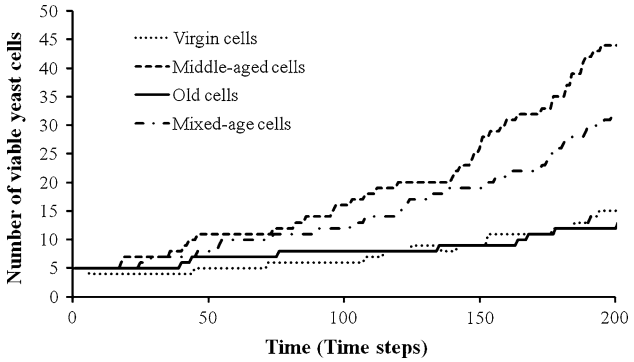

Fig. 3 Details of the initial growth before moving into the exponential phase of the number of viable cells in the simulated yeast cultures from the four different small inocula corresponding to the four age groups: virgin or daughter cells with no scars, young parents or middle-aged cells with one to five scars, old parent cells with more than five scars, and mixed-age cells ($N_0 = 5$ cells)

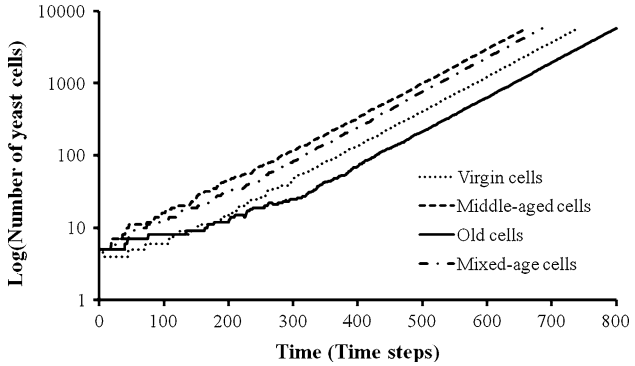

Fig. 4 Time evolutions of the logarithm of the viable yeast cells in the simulated cultures from the four different small inocula corresponding to the four age groups: virgin or daughter cells with no scars, young parents or middle-aged cells with one to five scars, old parent cells with more than five scars, and mixed-age cells ($N_0 = 5$ cells)

exponential phase. Figure 3 shows the initial population growth from four different small inocula ($N_0 = 5$ cells): daughter or virgin cells, young parent cells or middle-aged cells, old parent cells and mixed-age cells. Taking into account that the simulations are stochastic realisations of the implemented model, the four simulations were chosen randomly from the large set of combinations performed. In this case, we can observe the different dynamics in the population increase along the first time steps. We see that the fastest growth initiation corresponds to the young parent cells, followed by the inoculum that has cells of heterogeneous ages, while virgin and old cells have longer lag periods. This is a proof of the ability of INDISIM-YEAST to distinguish differences in the evolution of a population that emerges from small inocula with different age structures. In fact, it is particularly useful in the study of small inocula during the initial steps of growth because of the large influence of the discrete and asymmetrical nature of yeast cellular division [13]. It is here that this model has an edge over top-down continuous models, which are useful when the initial population contains a large number of cells and the interest is the inspection of average behaviours exhibited by those cells.

Figure 4 plots the logarithm of the number of viable yeast cells of the full evolution reported in this study, and reflects the transition from the lag to the exponential phase. Also, this figure shows that all simulated cultures reach similar maximum possible growth rates: the exponential phase maximum growth rate, μ. That is, the characteristics of the inocula do not seem to affect this parameter [13], which depends mainly on the microbial strain and the medium conditions. In a previous study, several simulations were performed to analyse the effect of two factors—the inoculum size and genealogical age of the cells which made it up—on the lag phase and first division time, showing that the shortest lag phase and time to first division were obtained with the largest inocula and with the youngest inoculated parent cells [13].

Thus, seeding a fermentation with yeast consisting primarily of aged cells would result in an extended lag phase in the fermenter due to slow progression through the cell cycle. In pitching yeast consisting primarily of newly budded virgin cells, the time taken to reach the critical size required for the first division would result in a slight delay in the onset of growth. However, middle-aged mothers would rapidly divide, reducing fermentation lag time. Therefore, it has been suggested that mother cells that are young to middle aged represent the most active portion of the pitch population in terms of rapid growth and yeast biomass production [24, 25, 30].

Figure 4 also shows how the maximum number of viable cells is displaced towards a larger number of time steps with old cells, in comparison with virgin and middle-aged cells.

The behaviour shown in Figs. 3 and 4 corresponds to the first stages of the inocula developments until the exponential phase is reached. After that, different phases may emerge naturally from the collective behaviour of the yeast cells as time goes by, from their individual responses to the different environmental conditions found in the medium [29].

Mean biomass evolution of the yeast population

Figure 5 presents the simulation results for the temporal evolution of the mean biomass. Here we actually see the increase or decrease in mean biomass during the first stages of the growth of the culture depending on the group of cells that configure the different inocula: an increase for virgin cells and a decrease for young and old parent cells.

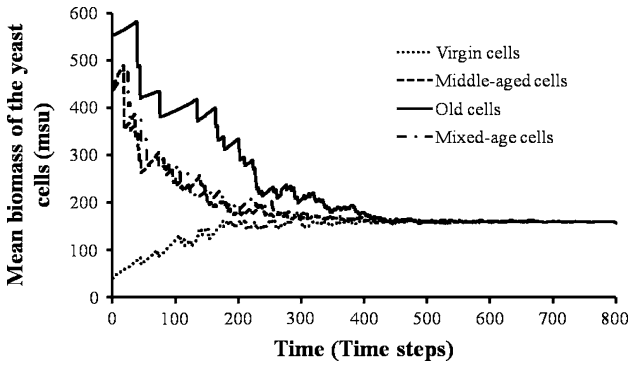

Fig. 5 Time evolutions of the mean biomass in simulated yeast cultures from the four different small inocula corresponding to the four age groups: virgin or daughter cells with no scars, young parents or middle-aged cells with one to five scars, old parent cells with more than five scars, and mixed-age cells ($N_0 = 5$ cells)

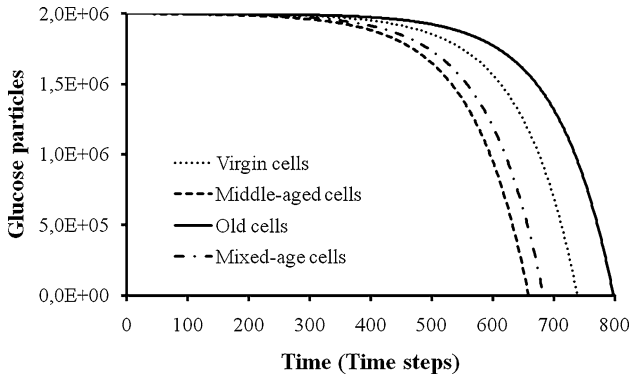

Fig. 6 Time dependence of nutrients in the simulated yeast cultures at each fermentation from the four different small inocula corresponding to the four age groups: virgin or daughter cells with no scars, young parents or middle-aged cells with one to five scars, old parent cells with more than five scars, and mixed-age cells ($N_0 = 5$ cells)

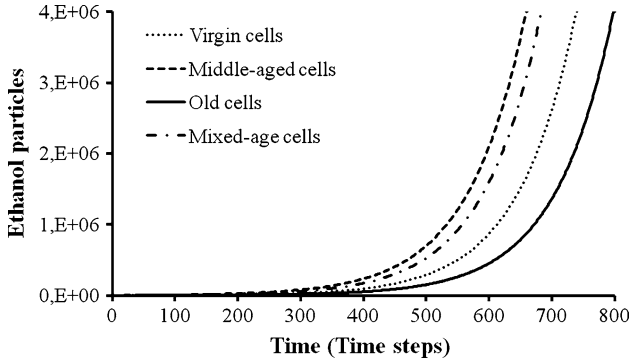

Fig. 7 Time dependence of metabolites in the simulated yeast cultures at each fermentation from the four different small inocula corresponding to the four age groups: virgin or daughter cells with no scars, young parents or middle-aged cells with 1–5 scars, old parent cells with more than five scars, and mixed-age cells ($N_0 = 5$ cells)

The evolution of the mean biomass of the inoculum with the five cells chosen randomly from different genealogical ages has a very close evolution to the group of middle-aged cells. Nevertheless, it is evident that this behaviour is influenced greatly by the final genealogical ages that those few cells possess. All of them converge on approximately the same mean value, which remains stable during the exponential phase growth. It is important to note that this first period of adaptation, i.e. the lag phase and the initial time steps to reach the exponential phase of the culture, takes different times, approximately 200, 300 or 400 time steps for young parent cells, daughter cells and old parent cells, respectively (Fig. 4). The adjustments in the mean biomass during the lag and pre-exponential phases are a consequence mainly of the budding yeast reproduction of the cells and the non-viability of some of these cells. We shall return to this point below when we present the simulation results of the distributions of genealogical age and biomass for the population at certain time steps.

Consumption of glucose and ethanol production

Figures 6 and 7 depict the time evolutions of nutrients (glucose particles) and metabolites (ethanol particles), respectively. These evolutions for each age case have similar shapes but the rates of consumption of glucose and production of ethanol are different. Fermentation that originates in middle-aged cells has a greater ethanol production than in the other cases, but it stops earlier. The population growing from the old inoculated cells has the slowest ethanol production but for the longest time; nevertheless, in the end a similar level of ethanol is achieved in the four cases. Our results show that the initial age of the seed yeast cell influences not only population growth, but also the rate of nutrient uptake and ethanol production. The increased fermentation time observed experimentally for virgin individuals is of particular significance to the brewing industry as an extended fermentation time has a direct impact on plant efficiency, with subsequent financial implications [25]. In other words, the individual features of the inoculated cells influence the profile of the fermentation. To better characterise the initial steps of ethanol production (as a consequence of glucose consumption), as well as to capture the effect of the inoculum on this parameter, sets of 50 independent runs selecting inocula that combined the two factors, size and genealogical age, were controlled. In order to compare the fermentation profiles, we accounted for the time required to reach a predetermined ethanol production (2×10^6 ethanol particles). This condition corresponds approximately to the middle stage of fermentation, when glucose is still present in the medium without limitations as a source of nutrient.

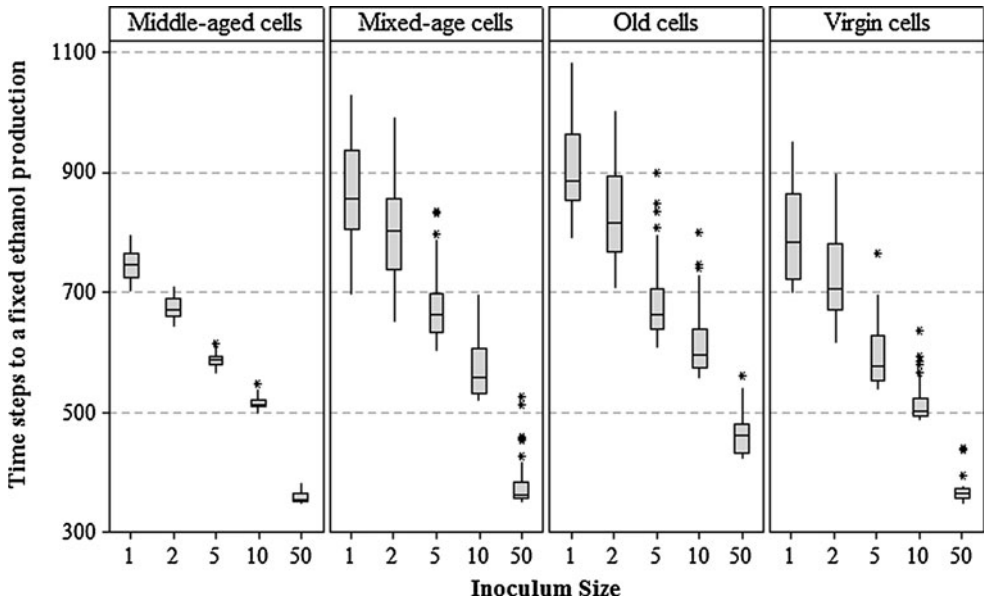

Fig. 8 Time steps to achieve a fixed ethanol production (2×10^6 ethanol particles) versus inoculum size depending on the genealogical age of the set of cells. *Box plots* illustrate the simulation series where inocula combining the two factors, size and genealogical age. Different inoculums sizes (from 1 to 50 cells) with only virgin cells (zero scars), middle-aged cells (one to five scars), old cells (six scars or more), or mixed-age cells were chosen to perform sets of 50 independent runs for each combination. A *middle line* inside the *box* represents the median. Outliers—data that were more than 1.5 times the interquartile range above or below the *box*—are shown as *asterisks*

This information enables the construction of box plots (also called box-and-whisker plots), which are particularly useful for showing the distributional characteristics of data and for comparing the set of data corresponding to these groups (Fig. 8). Outliers, unusually large or small observations corresponding to certain simulations, can be identified in some of these graphs. These are the values beyond the whiskers, which in other cases extend to the highest and lowest data values. The tops of the boxes are the third quartiles and the bottoms are the first quartiles, with the middle lines showing typical values, i.e. the median values for these sets of data. The simulation results shown in Fig. 8 show the expected influence of these initial features of the inocula on the time required to achieve this fixed amount of ethanol. This time has minimum values for the youngest parent yeast cells or middle-aged cells, and as the genealogical age of the inoculum increases this time lengthens. Also, the best group, the young parent cells, always exhibits much less variability in these times than the other groups. Additionally, irrespectively of their ages, the largest inocula reach the smaller values, whereas for small inocula the variability exhibited by the cultures to achieve this amount of ethanol is large, except for the group of middle-aged cells.

A recent experimental study investigated fermentation and metabolism characteristics under five initial cell densities [5], and the results showed that production of ethanol was higher whereas the fermentation time was shorter in higher inocula size fermentations. It has been suggested by some authors that mother cells that are young to middle-aged represent the most active portion of the pitch population [24]. Although quantitative comparisons cannot be made, the tendencies found are in agreement with these simulation results. Enhanced glucose consumption rate and ethanol productivity in specific characteristics of the initial yeast cells implied higher fermentation capability, which was beneficial for industrial ethanol fermentation.

Distributions of biomass and genealogical age throughout inoculum development

In the model implemented in INDISIM-YEAST, we consider that, in a yeast cell, the bud grows steadily in size as the cell cycle progresses, and that after cell separation, the daughter cell continues growing in order to achieve the critical size, or mass, required for replication (Fig. 1). Furthermore, the parent cells increase their size slowly over several generations (Fig. 2). As a result of these processes, the cell population exhibits a distribution of sizes, ages and duration of these phases over several cycles. All these distributions relate microscopic with population parameters. They reflect the state of the yeast population at given times during the fermentation process. Figures 9 and 10 show the evolution of these distributions for the growth of a small old-parent-cell inoculum, although the same analysis was performed with virgin and middle-aged cells (data

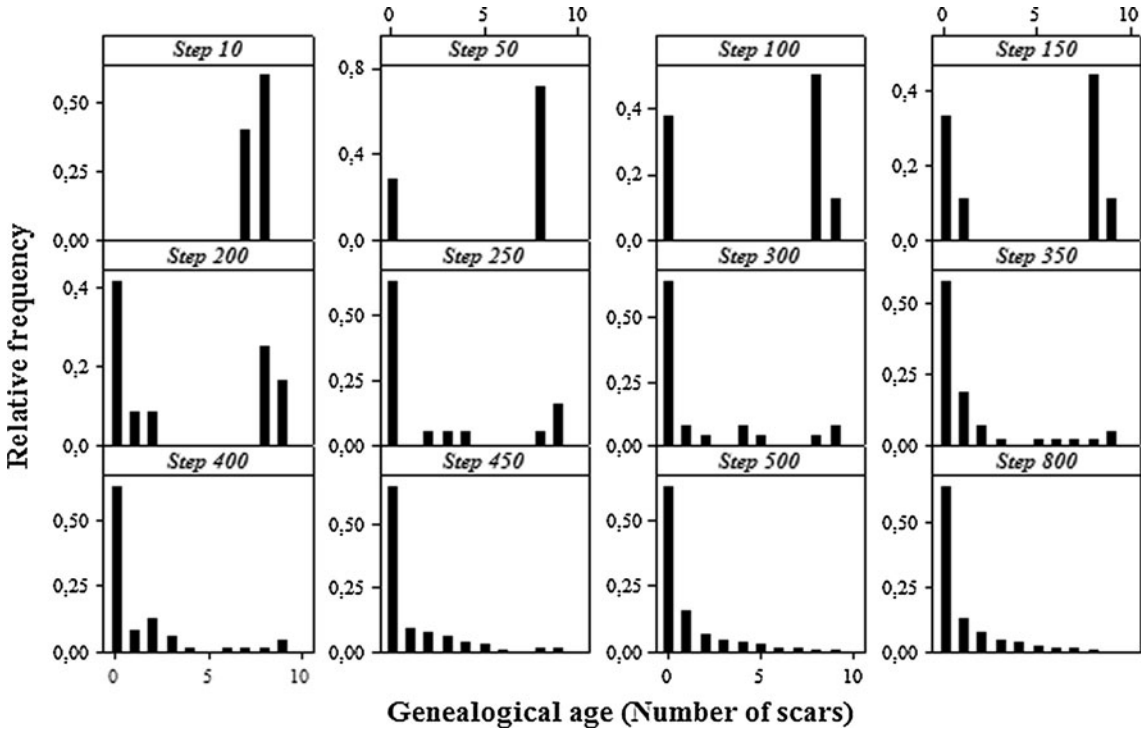

Fig. 9 Bar charts of the distributions of genealogical age of yeast cells in the simulated yeast culture from the small inoculum with the old cells ($N_0 = 5$ cells) at different steps of the simulated evolution

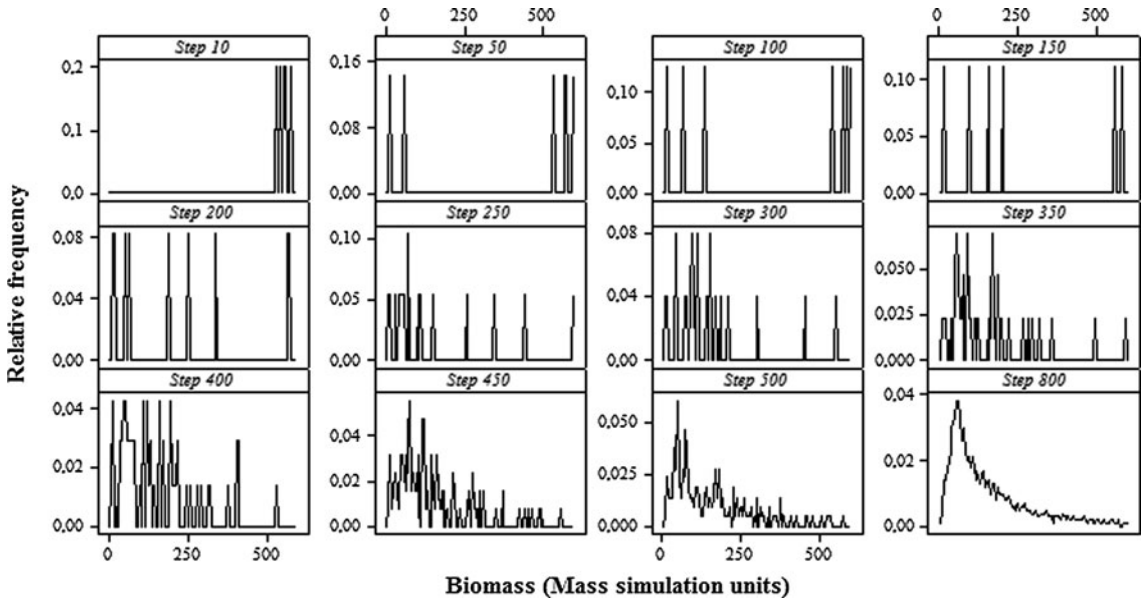

Fig. 10 Histograms of the distributions of biomass of yeast cells in the simulated yeast culture from the small inoculum with the old cells ($N_0 = 5$ cells) at different steps of the simulated evolution

not shown). The INDISIM-YEAST outcomes reveal that the virtual population is asymmetrical, with a more complex distribution than if bipartition or symmetrical division into two equal microbial cells took place [28].

Figure 9 shows the evolution of the distribution of genealogical age during the first steps of the evolution; the fact that the old cells of the inoculum are generating new and smaller daughter cells (virgin cells) is clearly

illustrated. The structure of the population during this first part of its development is evolving and adapting to the distribution shape that is imagined for an optimal and exponential growth (achieved at around time step 400 and maintained during this phase). After this adaptation, the shape of the distribution remains stable and constant until the end of the simulation, with the depletion of the nutrient (approximately at time step 800). Our simulation results let us see the effect of the duration of the cellular cycle for daughter cells (genealogical age 0), which is, on average, longer than that for parent cells (genealogical age greater than 0). Here, the fraction of parent cells may then follow, albeit only approximately, a geometrical series. For brewing yeast, the mean lifespan depends on the strain and is generally found to be ten or more replications [24]. In contrast, a population of yeast may be considered to be immortal because, although individuals will senesce and die, the continual generation of new daughter cells ensures that the population is maintained indefinitely under favourable environmental conditions. Moreover, the distribution of genealogical ages is affected by the intrinsic variation in the duration of the cellular cycle of each cell. The relative frequencies of the ages shown in the last bar charts of Fig. 9 show a percentage of daughter cells higher than 50% and close to 65%. With increasing genealogical age, the frequencies become smaller following an approximately geometrical series with a ratio of 0.5. These distributions correspond to the exponential phase and exhibit a largely asymmetrical, right-skewed or positive skew, distribution of yeast cells. A reproducible flow cytometric determination of the average bud scar number per cell of a yeast population would be useful. In this line, some recent advances connected to beer fermentation technology have been reported [20].

Figure 10 presents histograms of the distribution of masses resulting from the same simulation. These also correspond to different periods of development in the fermentation process. We have chosen the sample of mass distribution histograms with the same time steps as in Fig. 9, in order to compare them with corresponding genealogical age distributions. In both cases, more steps have been chosen at the start of the fermentation to control changes in the population structure, until the stable distribution that corresponds to the exponential phase achieved (the last step drawn). This last biomass histogram matches the typical shape of distribution of masses during the exponential growth phase: the bell-shape with a positive skew, with a long tail for larger biomasses (the right tail is longer; the mass of the distribution is concentrated on the left of the figure). The biomass distributions shown in Fig. 10 complement the information provided in Fig. 5 on the evolution of the mean biomass. The distributions of genealogical age and biomass for the other three simulations (inocula with virgin, middle-aged cells, and mixed-age cells) also show this kind of adaptation to a stable distribution corresponding to the exponential phase. The difference is the number of time steps that they need to reach it; they need less time. In addition, the manner in which these adaptations take place changes from one case to another, basically because the two origins are different (data not shown).

Physiological heterogeneity stems primarily from progression through the cell cycle and describes morphological differences between individual cells. Examples of physiological heterogeneity in yeast include size differences between mother and daughter cells, bud scarring and surface wrinkling. INDISIM-YEAST takes these features into consideration, and the simulation results presented in this study are a consequence of the integration of different parts of the yeast cell model used.

Within the stated limitations, this study mimics the industrial production of beer, which reuses yeast cropped at the end of a fermentation in subsequent fermentations, so that immediate and long-term fermentation performance is conditioned by the characteristics of these reused inocula. Our findings are in broad agreement with experimental research [3, 5, 20, 22, 24, 25, 30], and support the researchers' view that artificial selection of a population enriched with young or aged individuals influences yeast fermentation performance. The possibility of estimating the variation of a population, not only in number or biomass, but also in age, would lend favour to the proposition that the production capacity of each yeast cell depends on its cellular cycle state, its vitality and its ageing.

However, INDISIM-YEAST methodology faces some limitations that are shared by other IbMs, relating mainly to the difficulty in quantifying individual yeast parameters. Although another version of INDISIM was already assessed with data sets showing that parameterisation and calibration could be carried out reasonably well under controlled laboratory conditions [10], further analyses and work are necessary to deal with quantitative simulation results and goodness-of-fit to experimental data in the studied yeast fermentation process.

In view of the fact that the mathematical treatment of these processes to be integrated in kinetic or continuous models is very complex and presents some significant difficulties [17], the use of this IbM, INDISIM-YEAST, offers diverse and attractive possibilities for the continuing exploration of fermentations [13], and specifically protocols related to "repitching" of yeast in the brewing industry. Ongoing modelling advances mean that these modern IbM simulators, like INDISIM-YEAST, may now be used or considered by non-specialists in mathematics to effect a revitalisation in our understanding of yeast heterogeneity. Computational approaches or simulation studies cannot replace direct experimentation, but they represent

an additional resource for testing hypotheses with an economy, speed, and flexibility that cannot be matched by 'hands-on' microbiology.

Conclusions

Our simulation results show that the genealogical age of yeast cells and the small size of daughter cells compared to older cells are two individual characteristics that are significant in determining the evolution of a yeast culture during its first stages. Also, the initial conditions of the seed yeast cells influence not only population growth, but also the rate of nutrient uptake and ethanol production. Moreover, evolution of the biomass and genealogical age distributions of yeast populations during the first stages of growth are dissimilar and depend on the initial configuration of the inocula. In this way, with INDISIM-YEAST, we can compare and contrast both the individual and global properties of yeast cells and populations at chosen time steps during simulated growth, gaining insight into the interrelation between these two types of data. Examination of the internal structures of virtual yeast populations by biomass and genealogical age distributions provides useful knowledge of the macroscopic behaviour observed in experimental research.

The tendencies found in the simulations resemble those seen during 'serial repitching' in beer fermentation, although additional work must be done to improve the simulation model and to integrate new biological features into yeast cellular activity (such as internal reservoirs, or the possibility to use other metabolic pathways by controlling nitrogen, glycerol or other abiotic elements). This study highlights one of the benefits of IbMs, i.e. the fact that the ability to amass volumes of data on individual-based observations of selected yeast brings new challenges in ordering and understanding such information.

In general, the possibilities opened up by IbM approaches are the development of computing tools that allow integration of the knowledge of the systems considered, orientation of experimental design, prediction of temporal evolutions or behaviours, evaluation of experimental results, testing of hypotheses, revealing relations between parameters or variables, and ultimately, understanding of the phenomena or the target system. Indeed, the latter is the main goal of the IbM approach presented here. Apart from providing fresh perspectives on issues of concern to basic science, the tools and technologies of single-cell microbiology, jointly with individual-based simulations, make the problems of interest to researchers in applied science attractive. The convergence of novel measurement techniques and innovative modelling approaches, at scales previously inaccessible to researchers, may greatly alter the way in which we examine beer yeast fermentations.

Acknowledgements We thank Drs. Anna Gras and Rosa Carbó for several comments and suggestions. We gratefully acknowledge the financial support of the Ministry of Education and Science (Plan Nacional I+D+i) through Grant CGL2007-65142/BOS, and the Universitat Politècnica de Catalunya.

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