μMAX of Saccharomyces Cerevisiae: So Often Used, So Seldom Put into Perspective

Carla Inês Soares Rodrigues
University of Campinas
Bianca Eli Della-Bianca
Nordika do Brasil Consultoria Ltda
Andreas K. Gombert (gombert@unicamp.br)
University of Campinas https://orcid.org/0000-0001-9557-3773

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TITLE:

μ_{MAX} of *Saccharomyces cerevisiae*: so often used, so seldom put into perspective

AUTHORS:

Carla Inês Soares Rodrigues[1,2§], Bianca Eli Della-Bianca[3§], Andreas K. Gombert[1,*]

[1]University of Campinas, School of Food Engineering, Rua Monteiro Lobato 80, 13083-862, Campinas, SP, Brazil

[2]Delft University of Technology, Department of Biotechnology, Van der Maasweg 9, 2629HZ Delft, The Netherlands

[3]Nordika do Brasil Consultoria Ltda, Av. Queiroz Filho 1700, Torre A cj 807, 05319-000, São Paulo, SP, Brazil

[§]Joint first authorship

*corresponding author: gombert@unicamp.br, ORCID: 0000-0001-9557-3773

Carla Rodrigues' ORCID: 0000-0001-5134-0839

ABSTRACT:

The maximum specific growth rate of a microbe in a given growth condition is of primary relevance for biological research and bioprocess development. In the case of the unicellular yeast *Saccharomyces cerevisiae*, this physiological parameter is routinely calculated in (almost) every laboratory, but this procedure conceals several challenges that are often neglected in scientific works, which might lead to misinterpretation of the reported data and of phenomena. We present here several pitfalls involved in μ_{MAX} calculation and interpretation, which was achieved through comparative analyses of: 1) the use of different methodologies for determining cell concentration, 2) different calibration procedures to

correlate indirect (e.g. absorbance) to direct (e.g. dry cell mass) cell concentration measurements, 3) different statistical methods for determining the significance of μ_{MAX} differences, 4) the influence of culture media composition, and 5) the influence of the cultivation system (e.g. microplate, shake-flask or bioreactor). It becomes clear that each of these factors has a great influence on μ_{MAX} calculation and interpretation. We also present a case study involving three yeast strains and three different carbon sources, illustrating that opposite conclusions can be drawn in a screening procedure, if proper caution is not taken during data generation and analysis. Last but not least, we conclude this work with a series of recommendations that we believe could make experimental planning, data generation, μ_{MAX} calculation and interpretation more meaningful and scientifically sound, contributing to the improvement of yeast research and of microbiology in general.

KEYWORDS: cell concentration, microbial growth, *Saccharomyces cerevisiae*, specific growth rate, yeast physiology, μ_{MAX}

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Authors' contributions

Carla Inês Soares Rodrigues: Data collection and analysis, original draft preparation. **Bianca Eli Della-Bianca:** Data analysis, original draft preparation. **Andreas K. Gombert:** Study conception and design, data analysis, draft review and editing, supervision.

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Introduction

Growth of a microbial population is not the increase in size of the individual cells, but rather the increase in total cell number, total cell mass or even total cell volume in the population (Wheals and Lord 1992). Determining the rate at which a microbial population grows is one of the main interests of the fundamental microbiologist, as well as presumably the most important piece of information in an industrial bioprocess. This aspect is captured in a parameter referred to as the specific growth rate, most commonly represented by the Greek letter μ (Doran 2012; Clarke 2013; Liu 2016; Stanbury et al. 2017). Cell growth is an autocatalytic reaction, meaning that the catalyst itself is a product of the reaction (Doran 2012). Hence, the cell (or biomass) specific growth rate, rather than a simple growth rate, is the most appropriate parameter to describe microbial growth. Mathematically,

$$\mu = \frac{1}{X} * \frac{dX}{dt} \qquad \mathbf{1}$$

where X = cell concentration (e.g. in cells/volume or dry cell mass/volume) and t is the reaction time (e.g. in hours).

From equation **1**, it can be observed that μ is similar to the kinetic constant of a 1st-order chemical reaction and has dimensions of time^{-1}. Other formulations for rates, such as total or volumetric rates, are scale-dependent and do not directly reflect catalyst performance.

The exponential growth phase (EGP) occurs very often both in research and in applied cases, and is typically the longest phase of a conventional batch cultivation. During the EGP, cells encounter neither any nutrient limitation nor any inhibition. The population then grows at the maximum possible rate (the maximum specific growth rate, μ_{MAX}) under the applied conditions, until one nutrient becomes growth-limiting or some compound achieves inhibitory concentrations. The term “balanced growth” is often used to describe the physiology of cells during the EGP, since the cell composition typically does not change, although the composition of the nutrient medium is constantly changing (Campbell 1957).

Instead of μ, some professionals prefer to use the doubling (or generation) time (t_G) to quantify the rate of microbial growth. t_G is the time required for the microbial population to double its size (e.g. in terms of cell number or dry cell mass). The two parameters are intrinsically related by the following equation:

$$\mu = \frac{\ln 2}{t_G} \qquad \mathbf{2}$$

We will here only use μ for all our analyses and discussions.

μ cannot be directly measured. Nevertheless, measurements of cell concentration at a minimum of two time points allow for the estimation/calculation of this parameter.

There are several methods to determine cell concentration, including direct cell count, dry cell mass, particle count and colony forming units, among other direct off-line methods (Sonnleitner et al. 1992). Moreover, cell concentration is usually assessed by light-scattering measurements, such as those performed with the use of a spectrophotometer, a ubiquitous laboratory piece of equipment. Other terms used to designate this type of measurement are optical density (OD), turbidity, and absorbance. However, the results of such an indirect analysis need to be calibrated against a direct method, and this requires some caution. Calibration should be performed under a particular condition and applied to this circumstance only. Otherwise, the correlation could be compromised. Even analyses performed with cells from a single cultivation but collected at different growth phases represent a source of error due to inadequate calibration. The possibly different cell morphologies in each growth phase affect deviation of light and compromise the translation of the indirectly assessed cell concentrations into real cell concentrations.

Further options for indirect determination of cell concentration rely on the measurement of a cell component, for instance protein or DNA. In this case, calibration is also necessary and, as discussed above, care should be taken in the sense that cell composition during growth might differ from the one employed during the calibration procedure.

Furthermore, although online methods centered on turbidity, permittivity (Harris et al. 1987), or fluorescence can as well be used to assess cell concentration, as yet they have not substituted the above mentioned off-line methods, which require sampling.

Another relevant aspect for the analysis of microbial growth is the cell cycle, which for yeast comprises the phases G1, S, G2, and M (Juanes 2017). Individual cells in a population are in different phases of the cell cycle, meaning they are not synchronised (Cooper 2019). Thus, for a sample withdrawn from a cultivation, be it a microtiter plate or a million-liter-scale bioreactor, the measured cell concentration involves billions of cells at different stages of the cell cycle.

Fermentation Technology and/or Bioprocess Engineering textbooks usually do not provide a discussion on how cell concentration measurements affect the calculation of the specific growth rate. In one case, it is even stated that "During balanced growth, the net specific growth rate determined from either cell number or cell mass would be the same" (Shuler and Kargi 2002; Liu 2016). Stanbury et al. (2017) present the specific growth rate without any connection to cell concentration determination methods. In one exception, Clarke (Clarke 2013) points out that "μ_{MAX} can vary significantly depending on the method used to measure the cell concentration". This author also mentions that, in the case of the budding yeast *S. cerevisiae,* there might be a difference in μ_{MAX} calculated from cell mass and cell number. This is because, in the beginning of the EGP, yeast cells tend to present many buds (small cells) of lower mass than fully grown cells, which are counted by direct cell counting methods. The opposite is observed towards the end of the EGP, when the budding rate decreases.

There are basically two different approaches to calculate μ_{MAX} from cell concentration measurements. One of them is based on a first adjustment of a growth model to data from an entire batch cultivation, including all growth phases (lag, log, de-acceleration and stationary). Frequently used models include the logistic model, the Gompertz and the Richards models, among others (Pylvänäinen 2005). The second method consists of the integration of equation

77 **1** under the assumption that in the EGP μ is constant and equal to μ_{MAX}. While early
78 researchers used a $\log_2$ or $\log_{10}$ transformation to linearize this equation (Clarke 2013),
79 nowadays, the use of the natural logarithm is common practice:

$$lnX = lnX_0 + \mu_{MAX} * t \quad \textbf{3}$$

80 where X_0 = cell concentration at the beginning of the EGP, corresponding to t = 0.

81 This transformation allows us to calculate μ_{MAX} by plotting ln(X) values along time
82 and taking the slope of the linear region as μ_{MAX}. This procedure also results in the
83 identification of the duration of the EGP. Due to the use of the natural logarithm, μ_{MAX}
84 represents the number of "*e*-fold" generations in a given time point t, or the exponential
85 increase of biomass by a factor of *e* (Manhart and Shakhnovich 2018). We will restrict our
86 analysis and discussion here to this approach, because it is by far the most frequently
87 employed in the context of yeast research.

88 μ_{MAX} is also a key parameter in kinetic models used in biological research and in
89 bioprocess development. In its simplest form, it appears in the Monod equation that relates
90 μ_{MAX} to the limiting substrate concentration S:

$$\mu = \mu_{MAX} * \frac{S}{S + K_S} \quad \textbf{4}$$

91 μ_{MAX} has also been termed the Malthusian parameter and used as a proxy for fitness
92 by part of the scientific community, mainly those involved in population genetics or
93 experimental evolution studies (Lenski et al. 1991).

94 For the sake of completeness, it should be mentioned that there are methods to
95 calculate μ_{MAX} using continuous cultivation data (Jannasch and Egli 1993) and methods that
96 take substrate and product concentrations into account (Oner et al. 1986). We will not discuss
97 them here.

98 Finally, it is important to mention that not only the analytical method used to
99 determine cell concentration influences μ_{MAX} calculations, but also other factors such as the

100 cultivation system. Potvin et al (Potvin et al. 1997) compared μ_{MAX} values obtained for
101 *Lactobacillus plantarum* cells grown in an automated plate reader, in shake-flasks and in a
102 bioreactor, otherwise under similar conditions. Bioreactor cultivations led to higher μ_{MAX}
103 values as compared to shake-flask cultivations, which the authors attributed to external pH
104 control in bioreactors. These authors also showed that the μ_{MAX} calculated from direct
105 absorbance measurements in an automated plate reader, without sample dilution, differed
106 from the values obtained with samples from shake-flask cultivations that were diluted prior to
107 the absorbance measurements. Although these observations seem obvious, this matter has
108 only been given proper attention in few published works.

109 In the only report we identified involving yeast, Stevenson et al. (2016) evaluated the
110 relationship between optical density and cell counts both in *Escherichia coli* and
111 *Saccharomyces cerevisiae* cultures with respect to particle size and shape, refractive index,
112 cultivation volume, spectrophotometer model, cell growth phase, among others. The authors
113 concluded that the cell size effect on the calibration between OD and cell counts was stronger
114 in bacteria than in yeast. This is because the size of the bacterial cells is closer to the
115 wavelength of light (600 nm) used in the OD measurements. In this sense, the bigger size of
116 yeast cells makes them more suitable than bacteria for the application of light scattering
117 techniques at 600 nm or similar wavelengths. Moreover, they demonstrated that the difference
118 between the refractive index of the medium and that of the cells influences the calibration
119 curve. This has implications for yeast research, since sugars commonly used in yeast media,
120 such as sucrose, change the refractive index of the medium significantly.

121 This context motivated us to investigate how different cell concentration
122 determination methods, statistical analyses, cultivation systems, and also culture media
123 influence μ_{MAX} calculations during yeast cultivations performed with different strains,
124 including wild isolates, laboratory and industrial ones.

Material and Methods

Yeast strains and preservation

Eight *S. cerevisiae* strains from indigenous, industrial or laboratory origin were used in this work (**Table 1**). Stock cultures were prepared by growing cells until stationary phase in 500-ml Erlenmeyer flasks containing 100 ml YPD (1% yeast extract, 2% peptone, and 2% glucose) medium. 20% (v/v, final concentration) sterile glycerol was added and 1-ml aliquots were stored in 2-ml cryogenic vials in an ultra-freezer (ColdLab, Piracicaba, Brazil) at -80 °C until further use.

Cultivation media

Yeast cultivations were carried out using either a defined medium (Verduyn et al. 1992), the composition of which altered depending on the cultivation system (**Table 2**), or a complex medium (YPD). Microplate cultivations were performed using both media, whilst shake-flask and bioreactor cultivations were restricted to the defined medium. When needed, urea was used as the sole nitrogen source in replacement for ammonium sulphate, to avoid drastic changes in the broth's pH caused by proton release during ammonium consumption. Glucose was added as carbon and energy source to all cultivation media, unless otherwise stated. Each medium was sterilised either by autoclaving some of its components at 121 °C for 20 min or by filtration through 0.22-µm pore membranes. Glucose, vitamin and trace element solutions were always filter-sterilised to avoid Maillard reactions or thermal decomposition of the components.

Cultivations

Microplate cultivations

All eight strains were cultivated in 96-well microplates (CELLSTAR® flat bottom, mfr. No. 655151 - Greiner bio-one, Kremsmunster, Austria) using the plate reader Tecan Infinite M200 Pro. Initially, cells from the -80 °C stock were streaked onto solid YPD medium (with 2%

agar) and incubated at 30 °C (502 Incubator, FANEM, São Paulo, Brazil) for 48 h. Cells from a single colony were then transferred to a 50-ml centrifuge tube filled with 3 ml of either a defined medium or a complex medium, constituting the inoculum. The inoculum was placed in a shaker incubator (Innova 4430, New Brunswick Scientific, Edison, USA) operating at 200 rpm and 30 °C for 24 h. An aliquot of each tube's content, enough to make 1 ml of a cell suspension with absorbance at 600 nm equal to 1, was then collected. The aliquot was centrifuged at 974 *g* for 5 min (MIKRO200 centrifuge, Hettich, Tuttlingen, Germany), the supernatant discarded and the pellet washed with 1 ml of fresh culture medium. This washing procedure was performed twice. Next, 10 µl of the cell suspension was transferred to one well of a microplate that had already been filled with 90 µl of the same culture medium used for inoculum growth. Once all the desired wells were filled with both medium and cell suspension, the microplate was sealed with PCR sealing film (AMPLlSeal™ - Greiner bio-one, Kremsmunster, Austria). The cultivation was carried out in quintuplicate (5 wells on the same plate) at 30 °C with an orbital agitation amplitude of 3.5 mm and frequency of 198.4 rpm. Absorbance at 600 nm wavelength and 9 nm bandwidth was measured every 15 min during 24 h.

Table 1

Table 2

Shake-Flask cultivations

Shake-flask cultivations were performed with strains CEN.PK113-7D, PE-2, JP1, UFMG-CM-Y257, and UFMG-CM-Y259. First, each inoculum was prepared by transferring cells from one colony of each of the five strains into 500-ml baffled Erlenmeyer flasks containing 100 ml synthetic medium. The inoculum was incubated in a shaker (Innova 4430, New Brunswick Scientific, Edison, USA) at 30 °C and 200 rpm for 24 h. Then, sufficient cell suspension to begin the cultivation with an absorbance at 600 nm of 0.2 was centrifuged at 2153 *g* for 5 min (NT810 centrifuge, Nova Técnica, Piracicaba, Brazil). The supernatant was discarded, cells were washed twice and the cell pellet was resuspended in 1 ml synthetic

177 medium. This cell suspension was transferred to another Erlenmeyer flask containing fresh
178 synthetic medium.

179 Samples of the cultivation broth were collected hourly and their absorbance at 600
180 nm measured in a spectrophotometer (Genesys 20, Thermo Fisher Scientific, Massachussets,
181 USA). Sample pH was read using a pHmeter (DM21, Digimed, São Paulo, Brazil). The
182 cultivations were stopped when the cells reached the stationary phase of growth.

183 ***Bioreactor batch cultivations***

184 To prepare the inoculum for bioreactor cultivation, the content of one cryogenic vial
185 was transferred to a 500-ml Erlenmeyer flask containing 100 ml of medium, which was
186 prepared as described for shake-flask cultivations. The pH of this pre-inoculum medium was
187 adjusted to 6.0 by addition of 2 mol l^{-1} KOH. Cells were propagated at 30 °C in a shaker
188 (Certomat BS-1, Braun Biotech International, Berlin, Germany) under a stirring speed of 200
189 rpm. After 24 h, 1 ml of the pre-inoculum was directly transferred to another shake-flask
190 filled with fresh inoculum medium. Following a second round of growth in a shaker, an
191 aliquot sufficient to start the batch cultivation with an absorbance of 0.2 at 600 nm was
192 collected, centrifuged at 3500 *g* for 3 min, and the pellet resuspended in fresh cultivation
193 medium. Afterwards, the cell suspension was transferred to a 2-l bioreactor (Applikon
194 Biotechnology B.V., Delft, The Netherlands), making up an initial working volume of 1.2 l.

195 Cells were cultivated at 30 °C and 800 rpm until a decrease in the CO_2 molar fraction
196 in the off-gas was observed. Aeration in the bioreactor occurred with compressed air at 0.5 l
197 min^{-1} flow rate injected through a mass flow controller (Model 58505, Brooks Instrument
198 B.V., Hatfield, USA). The pH of the medium was adjusted to 5.0 and kept constant by
199 automatic addition of 0.5 mol L^{-1} KOH solution. Whenever needed, a 10% (v/v) antifoam C
200 emulsion (Sigma-Aldrich, Missouri, USA) was added manually to the broth. Samples of the
201 broth were withdrawn approximately every hour to have their dry mass and absorbance
202 measured. Dry cell mass was determined according to (Olsson and Nielsen 1997), except that
203 the membranes were dried in an oven at 70 °C for 48 h. The result was expressed in g_{DM} l^{-1}.

Absorbance was measured at 600 nm in a spectrophotometer (LibraS11, Biochrom, Cambridge, United Kingdom).

Calculation of the maximum specific growth rates and statistical comparisons

All calculations for statistical comparisons were performed with either GraphPad Prism 8 (San Diego, USA) or Microsoft Excel 365 (Redmond, USA). The maximum specific growth rate (μ_{MAX}) was obtained by plotting the natural logarithm of Abs_{600} (or dry cell mass) values against time and then fitting a linear regression model to the data within the exponential growth phase, the slope of which corresponds to the μ_{MAX}.

Using Microsoft Excel, data from independent replicates were analyzed separately, each one yielding a μ_{MAX} value of its own fitted by the least-squares regression method. The average and the standard deviation of these μ_{MAX} values were then calculated **(Fig. 1, Method A**). Significant changes in μ_{MAX} were evaluated using t-tests with 95% and 99% confidence levels. On the other hand, using GraphPad Prism, data from independent replicates of each experiment were analyzed together, generating one single μ_{MAX} value from one regression line also fitted by the least-squares method. This procedure also generated the standard error of the slope (**Fig. 1, Method B**). Significant changes in μ_{MAX} were evaluated using F-tests with 95% and 99% confidence levels.

Fig. 1

Results & Discussion

Calculated μ_{MAX} values depend on the cell concentration determination and on the calibration with a direct method

In spite of being an indirect method for the determination of cell concentration, Absorbance (Abs) measurements are commonly used during yeast cultivations. Researchers frequently use these measurements to directly calculate μ_{MAX}, by plotting ln(Abs) values against time, identifying the EGP as the linear region, performing a linear regression with the

corresponding data and taking the slope as μ_{MAX}. In other cases, authors report the calibration equation used to convert the Abs data into real cell concentrations, without mentioning how (or under which conditions) it was obtained. Calibration can be performed in different ways and these might influence the calculation of μ_{MAX}. To illustrate this, let us consider the cell concentration data points X_1 and X_2 obtained at two time points during the EGP (t_1 and t_2); from these data, μ_{MAX} can be calculated as:

$$\mu_{MAX} = \frac{\ln X_2 - ln X_1}{t_2 - t_1} \quad \textbf{5}$$

Taking a linear relation (calibration) between Abs measurements and a direct cell concentration (X) method, as follows:

$$X = a * Abs + b \quad \textbf{6}$$

and substituting equation **6** into **5**, results in:

$$\mu_{MAX} = \frac{\ln(a * X_2 + b) - \ln(a * X_1 + b)}{t_2 - t_1} \quad \textbf{7}$$

It is clear from equation **7** that only if the linear coefficient (intercept) b = 0, μ_{MAX} calculated from Abs and true cell concentration measurements will be the same.

In our experience at least, b is usually different from zero. We demonstrate this here with μ_{MAX} calculations from data obtained during bioreactor cultivations of three different yeast strains on glucose, namely CEN.PK113-7D, UFMG-CM-Y259, JP1 (**Table 3**). Samples taken throughout the cultivation had their absorbances measured after proper dilution and their cell concentration determined by a direct method (dry cell mass). μ_{MAX} was calculated using four different approaches: 1) directly from Abs data; 2) directly from dry cell mass data; 3) from calculated dry cell mass values obtained using a calibration equation established between the Abs and the dry cell mass data, including all data points in the cultivation; 4) from calculated dry cell mass values obtained using a calibration equation established

249 between the Abs and the dry cell mass data, including only data points in the EGP (as
250 identified from the dry cell mass data used for calibration).

251 **Table 3**

252 Remarkably, μ_{MAX} values calculated based on approach 1 were in the range of 25 to
253 50% higher than those calculated from dry cell mass data (approach 2). Because the latter
254 approach is based on a direct assessment of cell concentration, widely considered as an
255 accurate analytical method (as long as the appropriate amount of biomass is weighed on the
256 filtration membrane or in the centrifuge tube, (Olsson and Nielsen 1997)), we took this μ_{MAX}
257 value as the reference.

258 On the other hand, μ_{MAX} values calculated using approaches 3 or 4 were much closer
259 to the reference μ_{MAX} value. In the case of the 3rd approach, which includes data points from
260 the lag, EGP and de-acceleration growth phases in the calibration procedure, the calculated
261 μ_{MAX} values differed at most 10% from the reference μ_{MAX} value, even when the calibration
262 had been established with data from a different strain (see supplementary material).
263 Nevertheless, it should be noted that other approaches, such as a modified version of approach
264 3 to force the linear regression to an intercept of zero, or the establishment of a calibration
265 curve between Abs and dry cell mass using the final data point in the cultivation only, lead to
266 the same results as those obtained using approach 1. This latter option would have a very
267 practical implication, since it could allow for the use of shake-flask cultivations monitored by
268 absorbance measurements (which require small sample volumes) along the whole cultivation,
269 accompanied by dry cell mass determination (which requires larger sample volumes) in the
270 final sample only.

271 **Errors associated to μ_{MAX} values depend on the regression method and may alter**
272 **statistical outcomes**

273 Experiments in scientific research are often carried out in replicates, so that statistical
274 comparisons can be performed. It is of interest, for instance, to verify how the μ_{MAX} of a given
275 strain compares to that of another strain under the same conditions, or to the μ_{MAX} of the same

276 strain under different conditions. The error associated to the calculated μ_{MAX} value is therefore
277 critical, since it is the basis for statistical comparisons. One approach to determine the
278 absolute error that affects μ_{MAX} was proposed by Borzani (Borzani 1980, 1994), and it
279 depends on both the relative error of the cell concentration measurements and the duration of
280 the experiment. This methodology was not used here since often researchers do not know the
281 relative error of the cell concentration measurement itself, given that cell concentration is
282 usually measured only once at each time point.

283 Also, we would like to stress that time-series data are not independent, meaning that
284 the value of one data point depends on the value of previous data points. And, strictly
285 speaking, linear regression could not be used when data are not independent (McDonald
286 2014). However, data from microbial growth curves have historically been treated as being
287 independent. This is due to the assumption that "Whether one point is above or below the line
288 is a matter of chance, and does not influence whether another point is above or below the
289 line" (Motulsky 2020). Hence, we also proceeded this way in this work.

290 Using Abs values from exponential growth of strain CAT-1 in microplates, two
291 methods for statistical comparison of μ_{MAX} on defined and complex media were evaluated
292 (**Table 4, Supplementary Material**). Although the final μ_{MAX} values obtained from both
293 methods were the same, each was linked to distinct deviation/error values representing the
294 scattering of the same data.

295 Another analysis we carried out was the removal of outliers, since this is a common
296 procedure adopted by scientists in research. After visual inspection, some data appeared much
297 more distant to the regression lines than the others, with no apparent reason. The removal of
298 outliers based on an informal, visual approach is not recommended; thus the ROUT (Robust
299 regression followed by Outlier identification) method was used. This is an automatic routine,
300 based only on the distance of the point from the robust best-fit curve (Motulsky and Brown
301 2006). We evaluated all data points again in GraphPad Prism software using the ROUT
302 method, set up to eliminate outliers with a coefficient Q = 1% (Motulsky and Brown 2006).

We then calculated μ_{MAX} with the remaining data points by Method B (**Table 4, Supplementary Material**). As expected, different μ_{MAX} values were calculated and their standard errors were lower than the ones obtained by Method B without removal of outliers.

Table 4

Next, we performed statistical comparisons of the data from **Table 4** to check if the methods would yield the same results. Method A required a t-test to compare the averages from different treatments (in this case, the two cultivation media) and define whether their difference was statistically significant or not. A two-tailed, pooled t-test was chosen because we assumed that both populations were independent and normally distributed, their variances were unknown but equal, and the sample sizes were small (n = 5 for each data set) (Montgomery and Runger 2011). Method B, on the other hand, relied on an F-test, which is equivalent to an Analysis of Covariance (ANCOVA). The F-value is based on the residual sum-of-squares of both the common and the pooled regressions, the number of regressions tested, and the degrees of freedom of the pooled regression (details in (Zar 2010) and **Supplementary Material**). For both methods, the null hypothesis was H_0: $\mu_{MAX,1} = \mu_{MAX,2}$, and the alternative hypothesis was H_1: $\mu_{MAX,1} \neq \mu_{MAX,2}$. If the calculated p-value was less than the significance level α (0.05 or 0.01), we would reject the null hypothesis and the μ_{max} from the two cultivation media could be considered different at the significance level used (**Table 5**).

Table 5

Depending on the method and the significance level applied, the outcomes of the comparison diverged, as shown by the resulting p-values. At $\alpha = 0.01$, both methods A and B (with the complete data set) agreed in that the μ_{MAX} values of strain CAT-1 in defined or complex media are not statistically different from each other. However, at $\alpha = 0.05$ the methods disagreed. A different result was observed for strain *S. cerevisiae* UFMG-CM-Y259. At $\alpha = 0.05$ both methods resulted in a significant difference between defined and complex media, whereas that was not the case at $\alpha = 0.01$. Other strains were also tested, but the same

conclusions were achieved from both methods and significance levels (**Supplementary Material**). After the removal of outliers, Method B resulted in completely different conclusions at both α for strain CAT-1, when compared to the same method using all data points.

Even though Method A is widely used due to its simplicity and straightforwardness, it may not be the best way to calculate the error associated to μ_{MAX} values. Each replicate μ_{MAX}, once calculated independently, already has its own error associated to the fitness of the regression line itself. But these errors are not taken into account by Method A as they are simply not calculated, differently from Method B. Additionally, we showed that the removal of outliers was decisive for the results. One can easily see that the comparison between μ_{MAX} values calculated using distinct methods is extremely discouraged. First, because results from statistical comparisons are always to be taken with caution. Second, poorly described statistics in microbial physiology papers makes it difficult to understand how data were obtained and even more difficult to know whether interlaboratory comparisons can be performed.

Influence of the type of medium on μ_{MAX} calculations

Researchers often report μ_{MAX} values of a yeast strain on a given carbon and energy source, such as glucose. However, whether this carbon source is provided in a synthetic defined medium or in a complex undefined medium will influence the growth rate of a microbial population. In principle, μ_{MAX} values should be higher in the latter environment, because cells benefit from compounds that can be taken up directly from the medium, instead of having to synthesize them from metabolic intermediates at the expense of energy. To verify to which extent μ_{MAX} values vary between these two types of media, we evaluated this physiological parameter for eight different *S. cerevisiae* strains cultivated in microplates (**Fig. 2**).

Fig. 2

355 Overall, the μ_{MAX} values were higher for a given strain in YPD medium than in
356 defined Verduyn medium, as expected. Nevertheless, the level to which this occurs varies
357 among strains (**Table 6**), and, for a few cases, the difference between the pair of μ_{MAX} values
358 was not significant at 95% or higher confidence level. The complex/defined μ_{MAX} ratio ranged
359 from 1.12 to 2.33, which is quite remarkable, considering that all strains belong to the same
360 species and that both media employed here are commonly used in experimental research. We
361 were not able to identify any trend in these data, e.g. whether the haploid CEN.PK113-7D
362 strain would present a different behavior than the diploid ones, or whether industrial strains
363 (CAT-1, JP-1, PE-2) would behave differently than the laboratory, the baker's or the wild
364 isolates. This indicates that these results are probably related to cell morphology, which
365 strongly influence Abs measurements (Stevenson et al. 2016), rather than to cells' metabolism
366 or physiology, once again highlighting the importance of taking great care when calculating
367 and/or interpreting μ_{MAX} values from such indirect, light-scattering-based methods.
368 Although complex and defined media must contain all the essential nutrients for cell
369 growth, Abelovska and colleagues (Abelovska et al. 2007) demonstrated that the amount of
370 some compounds can vary up to 20 fold from one sort to another. These authors compared the
371 elemental composition of complex (2% peptone, 1% yeast extract) and minimal media (yeast
372 nitrogen base), and detected lower levels of important enzyme cofactors such as magnesium
373 and manganese in the complex medium. However, for the cofactors iron and zinc, as well as
374 for sodium and potassium ions, which are crucial elements in the generation of
375 electrochemical potential across the cell membrane (Madigan et al. 2012), the results turned
376 out to be the opposite.
377 **Table 6**

378 **Influence of the cultivation system on μ_{MAX} calculations**
379 We assessed how the cultivation system affects the calculation of μ_{MAX} by comparing
380 the calculated values obtained from microplate, shake flask, and bioreactor cultivations of
381 three *S. cerevisiae* strains (**Fig. 3**). The calculations were performed considering the Abs

382 values of distinct samples from the EGP as described in the Material and Methods section
383 (**Fig. 1, Method B**). For any particular strain, the three systems led to different μ_{MAX} values,
384 with the lowest values always being achieved using microplate cultivations. This is consistent
385 with our expectations, and has been observed before with bacteria (Potvin et al. 1997). Cells
386 are exposed to different growth conditions in the three systems, leading to varying oxygen
387 availabilities and pH values. This per se should lead to different physiologies.

388 However, the measuring peculiarities of each system also contribute to the observed
389 differences in μ_{MAX}. While in microplates the absorbance is usually measured without prior
390 dilution of the cell broth, in the other two setups, dilution is performed to assure the measured
391 Abs values fall within the limits of proportionality with cell number or dry cell mass
392 (Madigan et al. 2012). The real Abs is then calculated by multiplying the measured value by
393 the dilution factor. Begot and co-workers (Begot et al. 1996) evaluated the growth of several
394 *Listeria monocytogeneses* strains in both microplate and bioreactor systems, and showed that
395 the range of proportionality between Abs and bacterial population (CFU/mL) depended on the
396 apparatus used to measure Abs, which adds even more complexity and demands prior
397 knowledge on the particular piece of equipment used.

398 In the case of the results shown here, the spectrophotometer used for measuring the
399 absorbance during shake-flask cultivations was different from the one used for the bioreactor
400 cultivations (see Material and Methods section for specifications), as these experiments were
401 performed in different laboratories. Thus, one should also take the contribution of changing
402 the equipment into account, when interpreting these data. As an example of how different
403 spectrophotometers can affect the measurements, Koch (Koch 1970) demonstrated that the
404 standard curves of apparent absorbance versus bacterial dry mass concentrations vary among
405 different instruments under a selected range of wavelengths and aperture widths. By apparent
406 absorbance the author refers to the absorbance measured in non-ideal turbidimeters. Because
407 the absorbance represents the logarithmic difference between the light transmitted by the light

source and the light received by the detector, the slit width plays an important role in quantifying this parameter, and so does the wavelength (Stevenson et al. 2016).

Fig. 3

A practical example on how to misinterpret μ_{MAX} values

To further illustrate the importance of taking proper care while reporting or interpreting μ_{MAX} data, we calculated this parameter for some *S. cerevisiae* strains during cultivations in a defined medium containing a carbon and energy source other than glucose, namely sucrose or fructose. These μ_{MAX} values were then compared to the glucose data, both for microplate and shake-flask cultivations. As an example, a researcher could be interested in verifying on which of the three sugars yeast would grow with the highest μ_{MAX}, or one could be interested in screening several yeast strains for fructophilic behavior, which is a desirable feature in the wine industry, for instance, to overcome challenges with stuck fermentations (Bauer and Pretorius; Berthels et al. 2004; Tronchoni et al. 2009).

The results obtained in microplates do not necessarily corroborate those obtained in shake-flask cultivations (**Fig. 4**). For instance, the UFMG-CM-Y259 strain displayed faster growth on sucrose in the microscale system, compared to its growth on either of the hexoses. In shake-flask cultivations, however it grew with a smaller μ_{max} on sucrose, again compared to growth on glucose or fructose. The CEN.PK113-7D strain also displayed a higher μ_{MAX} on sucrose in microplate cultivations, but no significant difference was observed in the μ_{MAX} values on the three substrates during shake-flask cultivations.

When considering growth on fructose, in comparison to glucose only, the UFMG-CM-Y257 strain showed higher μ_{MAX} on glucose for cultivations using microplates, whereas equivalent growth rates on both substrates were observed during shake-flask cultivations. The opposite was observed for the JP1 strain. Resolving the mechanisms underlying such different behaviors is beyond the scope of this work. Here, the importance relies on the fact that one could easily miss the cultivation system-dependency of μ_{MAX} in *S. cerevisiae*, if a careful evaluation of the reported methodologies was not performed. In fact, in a typical scientific

study, more than one cultivation system is seldom employed. In spite of this, comparisons with literature data are often reported, without properly highlighting the differences in the experimental setup between the evaluated studies, which frequently leads to misinterpretation.

Fig. 4

Final remarks

Determining the maximum specific growth rate is routine in any microbiology laboratory, be it in industry or academia. The several different methods available for this purpose, however, add up to challenge this task. Most frequently, researchers report the μ_{MAX} values they calculate in a comparative manner, either with external publications or with those within their research group. The challenge of these comparative analyses is to assure that the evaluated cultivations and analytical procedures have been executed in the exact same way, and with proper caution. We demonstrated here, through a series of examples, the implications on μ_{MAX} calculations when distinct cultivations setups or analytical methodologies are employed. We, therefore, would like to draw the attention of our fellow microbiologists to the following:

1) Avoid calculations of μ_{MAX} directly from Abs measurements. First convert the Abs data to real cell concentration values using a pre-established calibration equation, obtained under identical cultivation conditions, and only then calculate μ_{MAX}. This calibration equation can be established using data from an entire batch cultivation, but ideally only data points in the EGP should be used to avoid any eventual artifacts introduced by cell morphology changes.

2) When methodologies other than obtaining μ_{MAX} directly from Abs measurements are not an option, one should never think of the calculated values as absolute. Comparisons with data reported in different works should thus be avoided.

3) Always make comparisons of your own calculated μ_{MAX} values with caution and explicitly report the conditions used by other authors or under which other experiments in the same lab were carried out.

4) Do not overstate findings related to μ_{MAX}, since its value can vary with any cultivation detail that is different, such as the geometry of the cultivation vessel, contaminants present in chemicals used to formulate media, rotation radius of the shaker incubator, method used to determine the cell concentration, etc.

5) Decide on a statistical method to use for comparisons between your own μ_{MAX} data and explicitly describe it. Report p-values rather than simply stating the statistical conclusion (Valentin Amrhein 2019).

6) Describe all calculations in detail, even if they are quite obvious to some. Supplementary material in research articles or data repositories could be used for this purpose. This will make comparisons easier, more meaningful and scientifically more sound.

Supplementary material

Fig. S1 Illustration of μ_{Max} calculation, using the eight different methods described in Table S1, for *S. cerevisiae* strains CEN.PK113-7D, JP1, and UFMG-CM-Y259 cultivated in aerobic batch bioreactors with glucose as sole carbon and energy source.

Table S1 Absorbance and cell concentration data for *S. cerevisiae* CEN.PK113-7D, JP1, and UFMG-CM-Y259 grown on glucose in aerobic batch bioreactors. Experiments were carried out in duplicate. Experimental data is highlighted in green.

Table S2 Maximum specific growth rate, calculated using different calibration approaches, of *S. cerevisiae* CEN.PK113-7D, JP1, and UFMG-CM-Y259 during growth on glucose in aerobic batch bioreactors.

Table S3 Maximum specific growth rate (μ_{MAX}) for eight different *S. cerevisiae* strains grown on either defined or complex medium supplemented with glucose as sole carbon and energy source, using microplate as cultivation system. Experiments were carried out in five

replicates, and for each replicate one μ_{MAX} was calculated from Abs600 data within the exponential growth phase (EGP).

Table S4 Comparative statistical analysis, based on method A[a], of the maximum specific growth rates showed in Table S3.

Table S5 Comparative statistical analysis, based on method B[1], of the maximum specific growth rates (μ_{MAX}) of different S. cerevisiae strains grown on either defined or complex medium supplemented with glucose as sole carbon and energy source, using microplate as cultivation system. Experiments were performed in five replicates. One single μ_{MAX} was calculated from Abs600 data from all replicates.

Table S8 Raw Abs600 data from the exponential phase of growth of S. cerevisiae CAT-1 cultivated on either defined or complex medium supplemented with glucose as sole carbon and energy source, using microplate as cultivation system.

Table S7 Calculations for testing for significant differences among slopes for the S. cerevisiae strain CAT-1.

Table S8 Summary of the statistical outcome of the F-test for the S. cerevisiae strain CAT-1.

Table S9 Raw Abs600 data from the exponential phase of growth of S. cerevisiae CAT-1 cultivated on either defined or complex medium supplemented with glucose as sole carbon and energy source, using microplate as cultivation system. The crossed out data represent the outliers identified using ROUT option on GraphPad Prism software with Q = 1%.

Table S10 Calculations for testing for significant differences among slopes for the S. cerevisiae strain CAT-1 after removal of outliers.

Table S11 Summary of the statistical outcome of the F-test for the S. cerevisiae strain CAT-1 after removal of outliers.

Table S12 Comparative statistical analysis, based on method B[1], of the maximum specific growth rates displayed by S. cerevisiae CEN.PK113-7D, JP1,and UFMG-CM-Y259 during growth on synthetic medium supplemented with glucose as sole carbon and energy source, using either microplate, shake-flask, or bioreactor as cultivation system.

513 **Table S13** Comparative statistical analysis, based on method B[1], of the maximum specific
514 growth rates displayed by S. cerevisiae CEN.PK113-7D, JP1, PE-2, UFMG-CM-Y257, and
515 UFMG-CM-Y259 during growth on synthetic medium supplemented with glucose, fructose,
516 or sucrose as sole carbon and energy source, using either microplate or shake-flask as
517 cultivation system.

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Table 1 Yeast strains used in this work.

Strain designation	Group	Ploidy	Precedence	References
CEN.PK113-7D	Laboratory	n	Dr. Peter Kötter (University of Frankfurt, Germany)	(van Dijken et al. 2000)
Fleischmann	Industrial (baking)	2n	Dr. L. C. Basso (USP, Brazil)	(Della-bianca and Gombert 2013)
PE-2	Industrial (fuel ethanol)	2n	Dr. L. C. Basso (USP, Brazil)	(Basso et al. 2008)
CAT-1	Industrial (fuel ethanol)	2n	Dr. L. C. Basso (USP, Brazil)	(Basso et al. 2008)
JP1	Industrial (fuel ethanol)	2n	Dr. M. A. de Morais Jr (UFPE, Brazil)	(Da Silva Filho et al. 2005)
UFMG-CM-Y257	Indigenous[a]	2n	Dr. C. A. Rosa (UFMG, Brazil)	(Beato et al. 2016)
UFMG-CM-Y259	Indigenous[a]	2n	Dr. C. A. Rosa (UFMG, Brazil)	(Beato et al. 2016)
UFMG-CM-Y267	Indigenous[b]	2n	Dr. C. A. Rosa (UFMG, Brazil)	(Beato et al. 2016)

[a]Originally from barks of *Quercus rubra*, located within the Brazilian Atlantic Forest biome.

[b]Originally from barks of *Tapira guaianenses*, located within the Brazilian Cerrado biome.

Table 1 Composition of the cultivation media used in this work.

Cultivation Medium	Components	Composition (g l^{-1})	Cultivation System
Complex (YPD)	Yeast Extract	10.0	Microplate
	Peptone	20.0	
	Glucose	10.0	
Defined Adapted from (Verduyn et al. 1992)	K_2SO_4	6.6	Microplate
	CH_4N_2O	2.3	
	KH_2PO_4	3.3	
	$MgSO_4.7H_2O$	0.5	
	Trace Elements solution	1.0	Shake-flask
	Vitamins solution	1.0	
	Glucose	10.0	
Defined (Verduyn et al. 1992)	$(NH_4)_2SO_4$	5.0	Bioreactor
	KH_2PO_4	3.0	
	$MgSO_4.7H_2O$	0.5	
	Trace Elements solution	1.0	
	Vitamins solution	1.0	
	Glucose	20.0	

Table 3 μ_{MAX} values calculated using four different approaches for three *S. cerevisiae* strains cultivated in aerobic bioreactors with glucose as sole carbon and energy source.

Approach	CEN.PK113-7D		JP1		UFMG-CM-Y259	
	Replicate 1	Replicate 2	Replicate 1	Replicate 2	Replicate 1	Replicate 2
1	0.440	0.415	0.423	0.398	0.413	0.452
2	**0.315**	**0.336**	**0.293**	**0.260**	**0.296**	**0.289**
3	0.327	0.327	0.281	0.286	0.315	0.322
4	0.325	0.331	0.330	0.270	0.316	0.304

Approaches: 1) Directly from Abs data; 2) directly from dry cell mass data; 3) from calculated dry cell mass values obtained using a calibration equation established between the Abs and the dry cell mass data, including all data points in the cultivation; 4) from calculated dry cell mass values obtained using a calibration equation established between the Abs and the dry cell mass data, including only data points in the EGP.

Table 4 Maximum specific growth rates (μ_{MAX}) for strains *S. cerevisiae* CAT-1 and UFMG-CM-Y259 grown in microplates in two cultivation media, calculated using two different regression methods*.

Medium	Method A			Method B (all data)			Method B (without outliers)		
	μ_{MAX}	SD	n	μ_{MAX}	SE	n	μ_{MAX}	SE	n
	CAT-1								
Defined	0.2588	0.0171	5	0.2588	0.0131	40	0.2516	0.0039	32
Complex	0.3221	0.0525	5	0.3221	0.0900	20	0.3436	0.0460	16
	UFMG-CM-Y259								
Defined	0.2500	0.0068	5	0.2500	0.0069	40		N.A.	
Complex	0.2808	0.0253	5	0.2808	0.0081	30		N.A.	

*described in the Methods section. SD is the standard deviation; SE is the standard error of the slope; n is the number of observations.

N.A. = not available. For this case, outliers were not identified.

Table 5 Statistical comparison of μ_{MAX} values for strains *S. cerevisiae* CAT-1 and UFMG-CM-Y259 grown on defined or complex media, using data from Table 4.

	Test statistic	p-value	Conclusion ($\alpha = 0.05$)	Conclusion ($\alpha = 0.01$)
	CAT-1			
Method A	2.5632[a]	0.0335	different μ_{MAX}	same μ_{MAX}
Method B (all data)	1.1016[b]	0.3178	same μ_{MAX}	same μ_{MAX}
Method B (without outliers)	9.9324[b]	0.0029	different μ_{MAX}	different μ_{MAX}
	UFMG-CM-Y259			
Method A	2.6294[a]	0.0302	different μ_{MAX}	same μ_{MAX}
Method B (all data)	7.4850[b]	0.008	different μ_{MAX}	different μ_{MAX}

[a] t-test; [b] F-test

Table 6 Ratio between μ_{MAX} of different *S. cerevisiae* strains in a complex medium (YPD) and in a defined medium with glucose as sole carbon and energy source.

STRAIN →	**CAT-1**	**CEN.PK113-7D**	**Fleischmann**	**JP1**	**PE-2**	**UFMG-CM-Y257**	**UFMG-CM-Y259**	**UFMG-CM-Y267**
Ratio	1.24	1.73	1.41	2.33	1.69	1.16	1.12	1.91

Fig. 1 Methods used for calculating and comparing the slope of regression lines (μ_{max}). Method A yields an average μ_{max} and a standard deviation while Method B yields a unique μ_{max} and a standard error.

Fig. 2 Maximum specific growth rates (μ_{MAX}) of strains grown in microplates in two cultivation media, calculated using two different regression methods (A and B). Depending on the statistical method and the significance level used, distinct conclusions can be drawn. * represent the p-value at which a significant difference between the treatments were observed; ns ($p > 0.05$); * ($p \leq 0.05$); ** ($p \leq 0.01$); *** ($p \leq 0.001$); **** ($p \leq 0.0001$)

Fig. 3 Maximum specific growth rates (μ_{max}) for three *S. cerevisiae* strains grown in a defined medium in three different cultivation systems. Data from different systems were used to calculate and statistically compare μ_{MAX} values using Method B and GraphPad Prism software. This yielded a p-value ≤ 0.0001 (****) for all strains

Fig. 4 Maximum specific growth rates (μ_{MAX}) of *S. cerevisiae* strains grown in microplates or in shake-flasks in a defined medium supplemented with sucrose, glucose or fructose as sole carbon and energy source, calculated by Method B

Figures

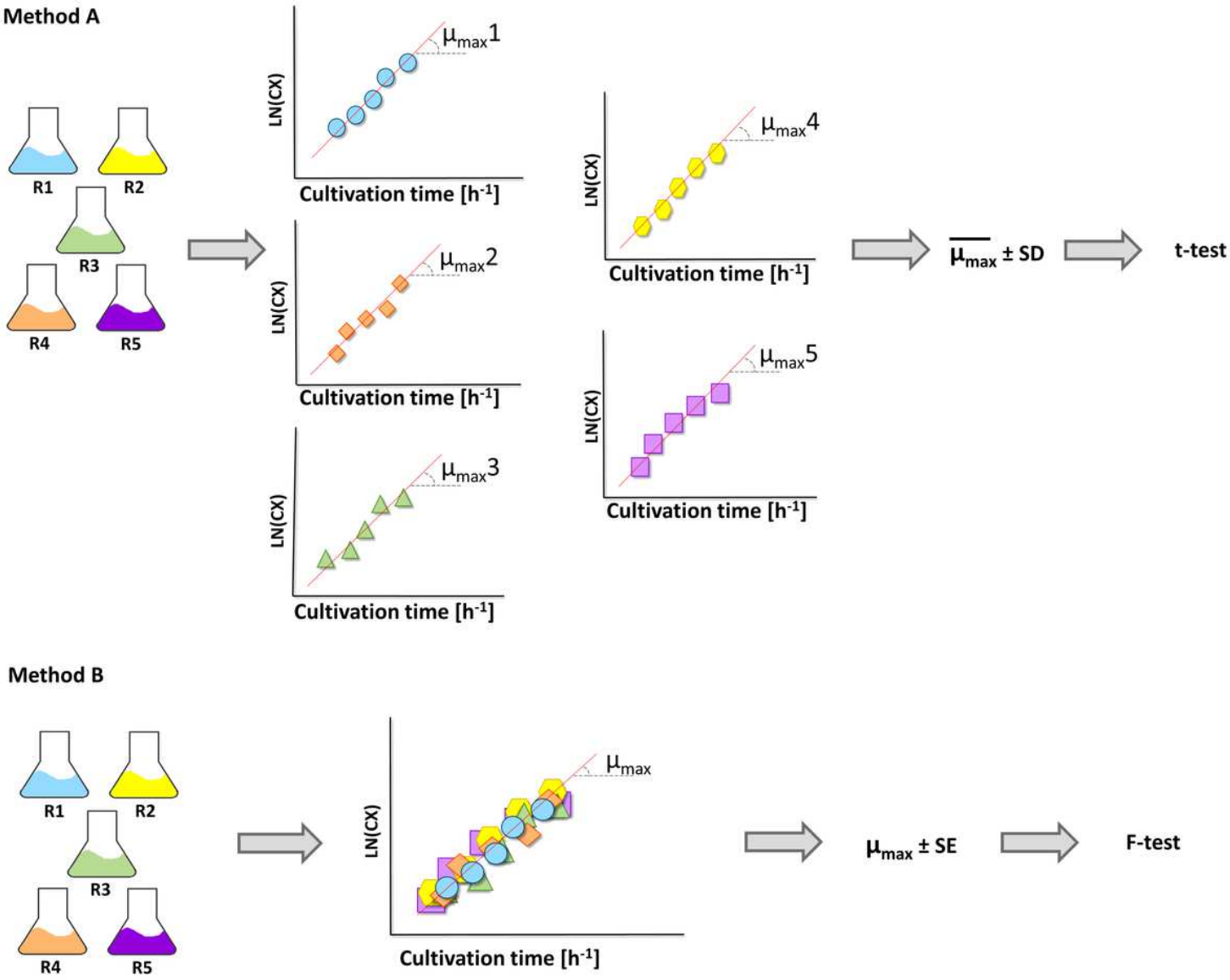

Figure 1

Methods used for calculating and comparing the slope of regression lines (μmax). Method A yields an average μmax and a standard deviation while Method B yields a unique μmax and a standard error.

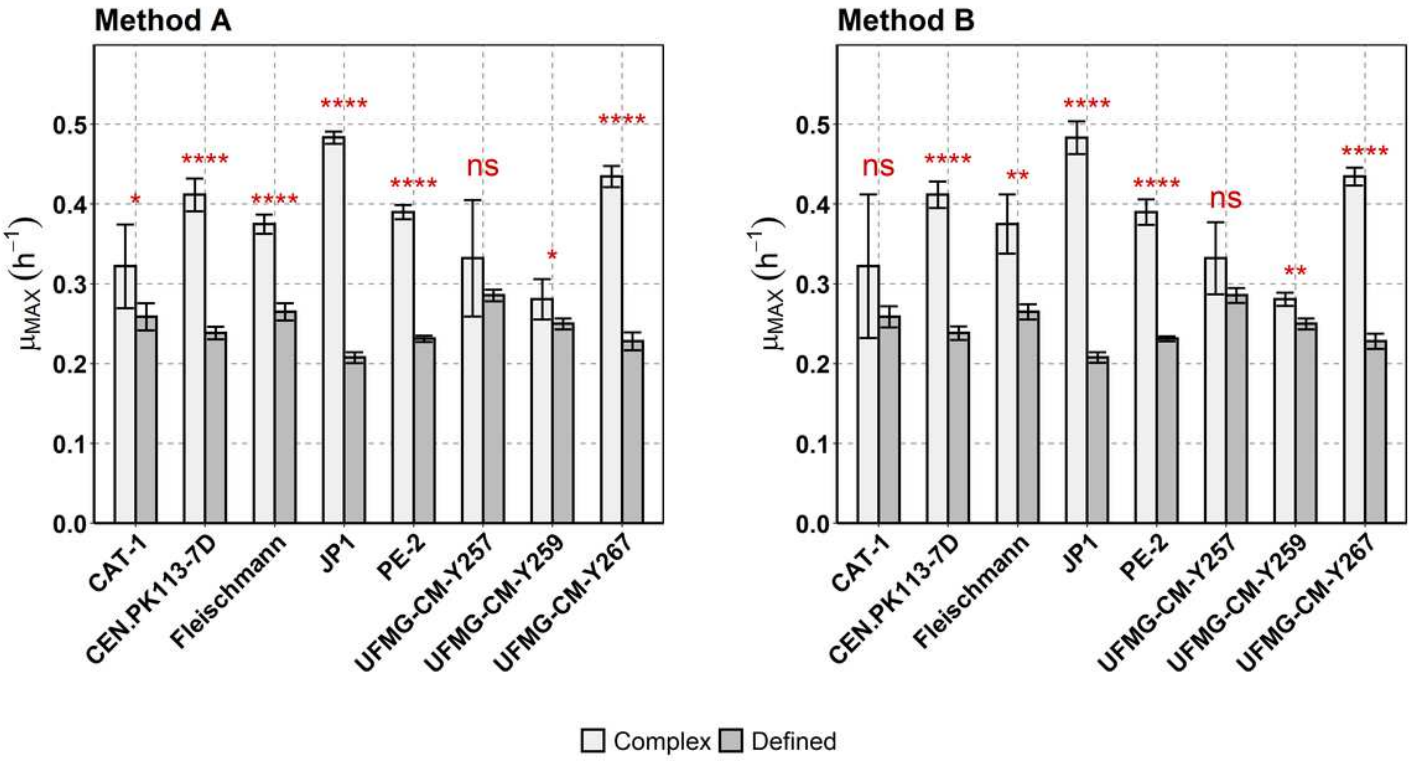

Figure 2

Maximum specific growth rates (μMAX) of strains grown in microplates in two cultivation media, calculated using two different regression methods (A and B). Depending on the statistical method and the significance level used, distinct conclusions can be drawn. * represent the p-value at which a significant difference between the treatments were observed; ns ($p > 0.05$); * ($p \leq 0.05$); ** ($p \leq 0.01$); *** ($p \leq 0.001$); **** ($p \leq 0.0001$)

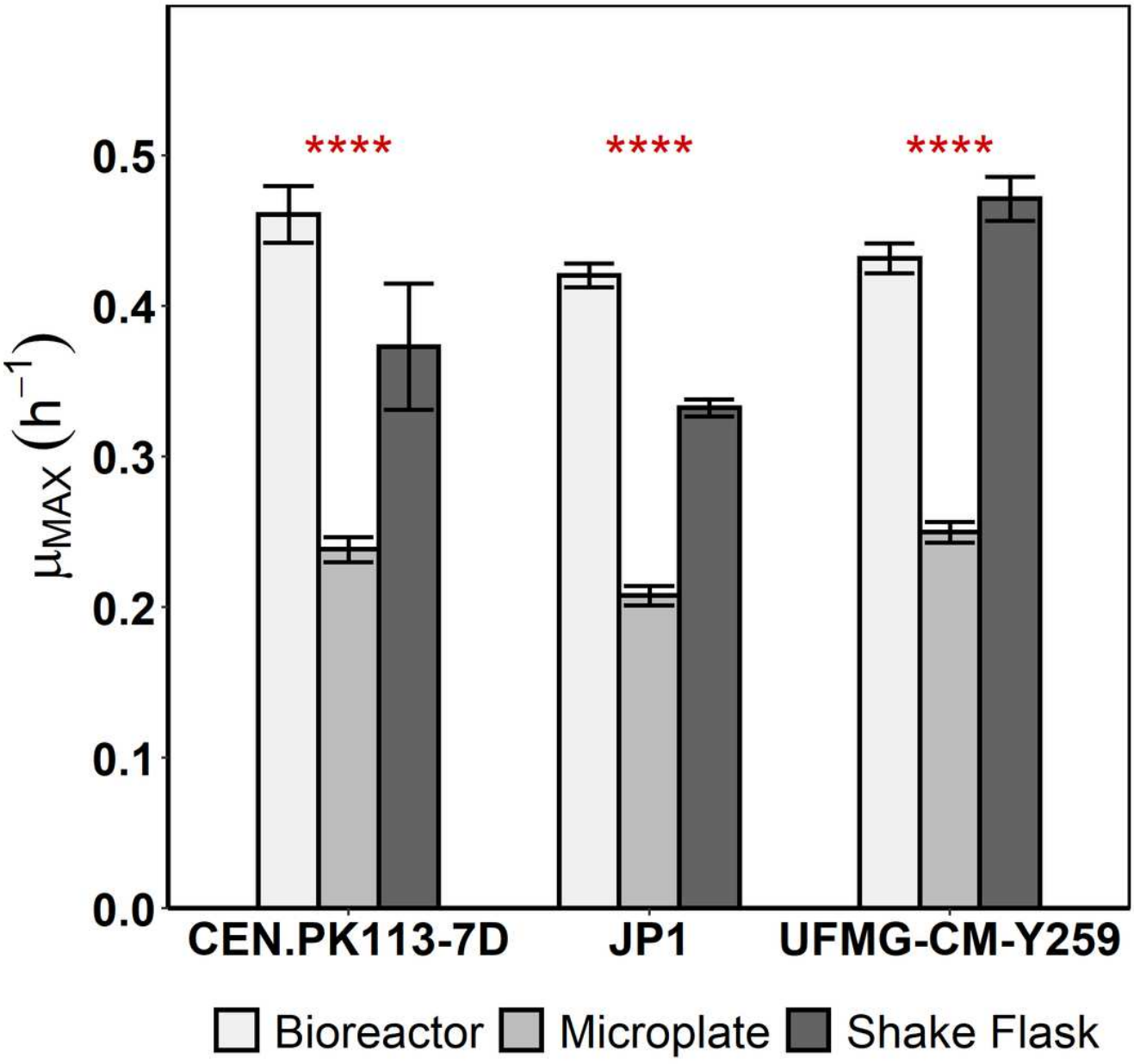

Figure 3

Maximum specific growth rates (μmax) for three S. cerevisiae strains grown in a defined medium in three different cultivation systems. Data from different systems were used to calculate and statistically compare μMAX values using Method B and GraphPad Prism software. This yielded a p-value ≤ 0.0001 (****) for all strains

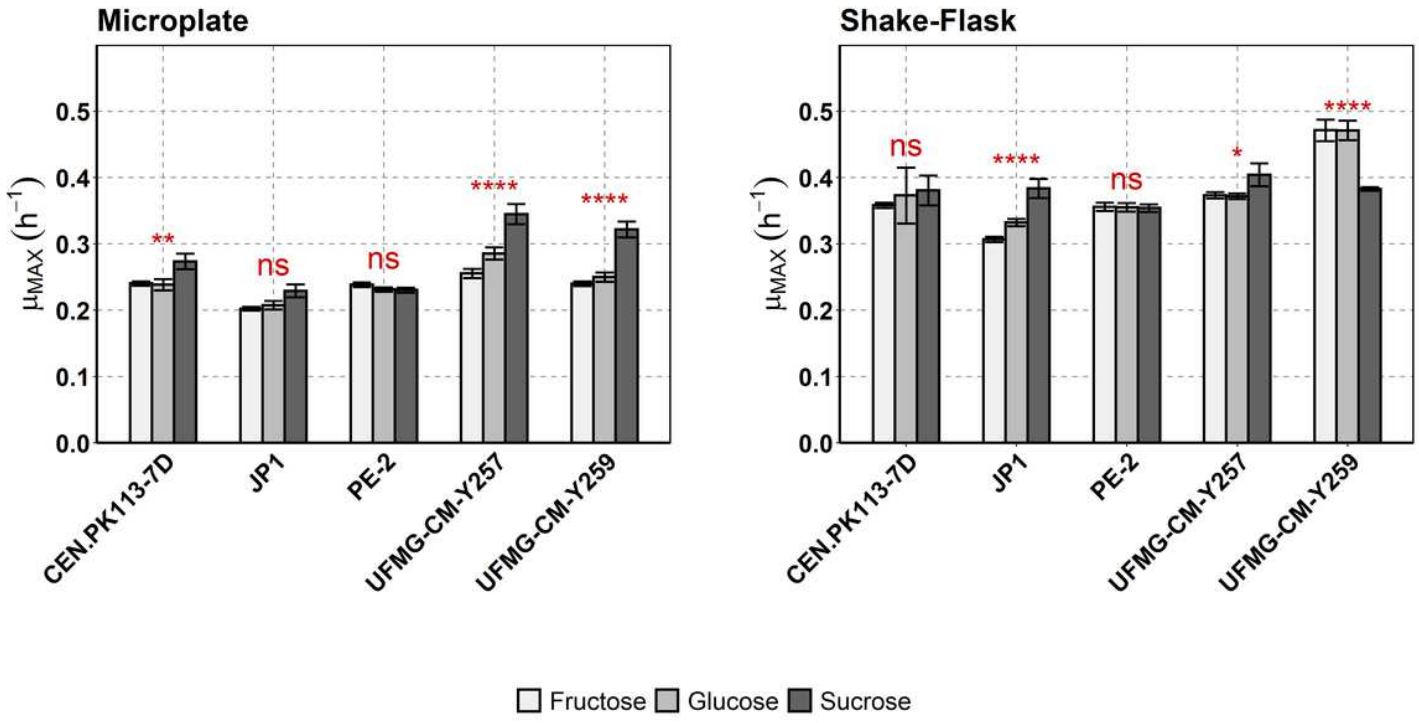

Figure 4

Maximum specific growth rates (μMAX) of S. cerevisiae strains grown in microplates or in shake-flasks in a defined medium supplemented with sucrose, glucose or fructose as sole carbon and energy source, calculated by Method B

Supplementary Files

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- Supplementarymaterial.docx