Video Article

Saccharomyces cerevisiae Exponential Growth Kinetics in Batch Culture to Analyze Respiratory and Fermentative Metabolism

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Abstract

Saccharomyces cerevisiae cells in the exponential phase sustain their growth by producing ATP through fermentation and/or mitochondrial respiration. The fermentable carbon concentration mainly governs how the yeast cells generate ATP; thus, the variation in fermentable carbohydrate levels drives the energetic metabolism of *S. cerevisiae*. This paper describes a high-throughput method based on exponential yeast growth to estimate the effects of concentration changes and nature of the carbon source on respiratory and fermentative metabolism. The growth of *S. cerevisiae* is measured in a microplate or shaken conical flask by determining the optical density (OD) at 600 nm. Then, a growth curve is built by plotting OD versus time, which allows identification and selection of the exponential phase, and is fitted with the exponential growth equation to obtain kinetic parameters. Low specific growth rates with higher doubling times generally represent a respiratory growth. Conversely, higher specific growth rates with lower doubling times indicate fermentative growth. Threshold values of doubling time and specific growth rate are estimated using well-known respiratory or fermentative conditions, such as non-fermentable carbon sources or higher concentrations of fermentable sugars. This is obtained for each specific strain. Finally, the calculated kinetic parameters are compared with the threshold values to establish whether the yeast shows fermentative and/or respiratory growth. The advantage of this method is its relative simplicity for understanding the effects of a substance/compound on fermentative or respiratory metabolism. It is important to highlight that growth is an intricate and complex biological process; therefore, preliminary data from this method must be corroborated by the quantification of oxygen consumption and accumulation of fermentation byproducts. Thereby, this technique can be used as a preliminary screening of compounds/substances that may disturb or enhance fermentative or respiratory metabolism.

Video Link

The video component of this article can be found at https://www.jove.com/video/58192/

Introduction

Saccharomyces cerevisiae growth has served as a valuable tool to identify dozens of physiological and molecular mechanisms. Growth is measured primarily by three methods: serial dilutions for spot testing, colony-forming unit counting, and growth curves. These techniques can be used alone or in combination with a variety of substrates, environmental conditions, mutants, and chemicals to investigate specific responses or phenotypes.

Mitochondrial respiration is a biological process in which growth kinetics has been successfully applied for discovering unknown mechanisms. In this case, the supplementation of growth media with non-fermentable carbon sources such as glycerol, lactate, or ethanol (which are exclusively metabolized by mitochondrial respiration), as the sole carbon and energy source allows for evaluating the respiratory growth, which is important to detect perturbations in oxidative phosphorylation activity[1]. On the other hand, it is complicated to use growth kinetic models as a method for deciphering the mechanisms behind fermentation.

The study of fermentation and mitochondrial respiration is essential to elucidate the molecular mechanisms behind certain phenotypes such as the Crabtree and Warburg effects[2,3]. The Crabtree effect is characterized by an increase of glycolytic flux, repression of mitochondrial respiration, and establishment of fermentation as the primary pathway to generate ATP in the presence of high concentrations of fermentable carbohydrates (>0.8 mM)[4,5]. The Warburg effect is metabolically analog to the Crabtree effect, with the difference being that in mammalian cells, the main product of fermentation is lactate[6]. Indeed, the Warburg effect is exhibited by a variety of cancer cells, triggering glucose uptake and consumption even in the presence of oxygen[7]. Thereby, studying the molecular basis of the switch from respiration to fermentation in the Crabtree effect has both biotechnological repercussions (for ethanol production) and potential impacts in cancer research.

S. cerevisiae growth may be a suitable tool to study the Crabtree and Warburg effects. This idea is based on the fact that in the yeast exponential phase, the central pathways used to produce ATP are mitochondrial respiration and fermentation, which are essential to

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sustain growth. For instance, the growth of *S. cerevisiae* is intimately related to the function of ATP-generating pathways. In *S. cerevisiae,* the mitochondrial respiration produces approximately 18 ATP molecules per glucose molecule, whereas fermentation only generates 2 ATP molecules, hence it is expected that the growth rate has tight links with the metabolic pathways producing ATP[8]. In this regard, when fermentation is the principal route to generate ATP, the yeast compensates for the low ATP production by increasing the rate of glucose uptake. On the contrary, the glucose consumption by yeast cells that use mitochondrial respiration as the main ATP source is low. This indicates that it is important for the yeast to sense carbohydrate availability before determining how ATP will be generated. Therefore, glucose availability plays an important role in the switch between fermentation and mitochondrial respiration in *S. cerevisiae.* In the presence of high quantities of glucose, the yeast prefers fermentation as the central route to generate ATP. Interestingly, when the yeast is fermenting, the specific growth rate is maintained at its maximum. On the other hand, under low levels of glucose, *S. cerevisiae* produces ATP using mitochondrial respiration, maintaining lower growth rates. Thereby, variation in the concentration of glucose and the use of other carbon sources induce changes in the yeast's preference between fermentative and respiratory growth. By taking into account this fact with the exponential growth equation, one can obtain the biological meaning of kinetic parameters such as doubling time (*Dt*) and specific growth rate (μ). For example, lower μ values were found when the yeast uses mitochondrial respiration as the primary pathway. On the contrary, under conditions that favor fermentation, higher μ values were found. This methodology may be used to measure the probable mechanisms of any chemicals affecting fermentation and mitochondrial respiration in *S. cerevisiae.*

The objective of this paper is to propose a method based on growth kinetics for screening the effects of a given substance/compound on mitochondrial respiration or fermentation.

Protocol

1. Culture Media and Inoculum Preparation

1. Prepare 100 mL of 2% yeast extract-peptone-dextrose (YPD) liquid medium (add 1 g of yeast extract, 2 g of casein peptone, and 2 g of glucose to 100 mL of distilled water). Dispense 3 mL of the media into 15 mL sterilizable conical tubes. Autoclave the media for 15 min at 121 °C and 1.5 psi.
NOTE: The media can be stored for up to one month at 4–8 °C.
2. Inoculate a conical tube filled with 3 mL of cool sterile 2% YPD broth with 250 µL of *S. cerevisiae* cells preserved in glycerol at -20 °C. Incubate overnight in an orbital shaker at 30 °C with agitation at 200 rpm.
3. Streak a Petri dish (60 x 15 mm^2) filled with sterile 2% YPD agar (add 10 g of yeast extract, 20 g of casein peptone, 20 g of glucose, and 20 g of bacteriological agar to 1,000 mL of distilled water) with the cells grown in the conical tube using a sterile loop. Incubate the Petri dish at 30 °C until the isolated colonies show growth.
4. Prepare the pre-inoculum by inoculating a single isolated colony into a conical tube filled with 10 mL of cool sterile 2% YPD broth and incubate it overnight at 30 °C with continuous agitation at 200 rpm.

2. Culture Media and Growth Curves in Microplate

1. Prepare 10 mL of 1% YPD broth (add 0.1 g of yeast extract, 0.2 g of peptone, and 0.1 g of glucose to 10 mL of distilled water), 10 mL of 2% YPD broth (add 0.1 g of yeast extract, 0.2 g of peptone, and 0.2 g of glucose to 10 mL of distilled water), and 10 mL of 10% YPD broth (add 0.1 g of yeast extract, 0.2 g of peptone, and 1 g of glucose to 10 mL of distilled water). Autoclave these growth mediums for 15 min at 121 °C and 1.5 psi.
NOTE: The culture media serves as a control of fermentative growth.
2. Prepare 10 mL of 2% yeast extract-peptone-ethanol (YPE) broth and 10 mL of 2% yeast extract-peptone-glycerol (YPG) broth. For 2% YPE: add 0.1 g of yeast extract, 0.2 g of peptone, and 0.2 mL of ethanol to 10 mL of distilled water. For 2% YPG: add 0.1 g of yeast extract, 0.2 g of peptone, and 0.2 mL of glycerol to 10 mL of distilled water. Autoclave these growth mediums for 15 min at 121 °C and 1.5 psi.
NOTE: Glycerol and ethanol are non-fermentable carbon sources that are exclusively metabolized by mitochondrial respiration. For this reason, they are used as a respiratory growth controls. In this step, the type and concentration of carbon source in the culture media may be changed to test the effects on growth phenotype using yeast extract-peptone (YP) broth (10 g of yeast extract and 20 g of casein peptone in 1,000 mL of distilled water) as a base. To test the effects of other nutrients besides carbon, synthetic complete (SC) medium is supplemented according to the aim of the experiment. The base for SC medium is 1.8 g of yeast nitrogen base without amino acids, 5 g of ammonium sulfate [$(NH_4)_2SO_4$], 1.4 g of monosodium phosphate (NaH_2PO_4), and 2 g of drop-out mix in 1,000 mL of distilled water. Supplement the media with a carbon source and additional nitrogen source in the needed concentrations.
3. Add 145 µL of suitable culture media for the experimental design to each well of a sterile microplate (10 x 10-well) with a lid and inoculate them with 5 µL of the pre-inoculum.
NOTE: If the aim is to screen the influence of substances/compounds on the energetic metabolism of the yeast, that substance/compound should be added during this step. Also, any type of microplate with a lid should be useful.
4. Incubate the multi-well plate in a microplate reader at 30 °C with constant agitation at 200 rpm for 48 h. Measure the optical density (OD) at 600 nm every 30 or 60 min.
NOTE: If the microplate reader does not have the option to incubate the microplate, it may be incubated in an orbital shaker at the same conditions between every measurement of the OD.

3. Growth Curves in Shaken Conical Flasks

1. Add 4.8 mL of the suitable culture media to 20 mL conical flasks and autoclave. Inoculate each flask with 200 µL of the pre-inoculum culture at OD_{600nm} ~2 in cool, sterile 2% YPD broth. Incubate them at 30 °C with constant agitation at 250 rpm for 24 h.

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NOTE: If the incubation period is higher than 24 h, add 12 mL of the suitable culture media to 50 mL conical flasks and autoclave. Inoculate them with 500 µL of the pre-inoculum. It is also important to consider the fermentative growth controls (YP media with 1%, 2%, or 10% glucose) and respiratory growth controls (YP media with 2% glycerol or ethanol).

2. Take 100 µL of the shaken conical flask culture and dilute it in 900 µL of distilled water in a 1 mL spectrophotometer cuvette, and gently mix by pipetting. Measure the OD at 600 nm using a spectrophotometer every 2 h. To obtain the real OD, multiply the result by ten.

4. Data Processing and Kinetic Parameters Calculation

1. **Create a new project file in the statistical package software. In the section of *"New table and graph"*, choose the *"XY"* option.**
    1. Select the option: *"Start with an empty data table"* in the *"Sample data"* section.
    2. Select the *"Points and connecting line"* graph type. Leave the *"X"* section unselected in the segment of "Subcolumns for replicates or error values", and in the *"Y"* section choose the option: *"Enter (10) replicate values in side by side subcolumns and plot Mean and Error SD"*. Click the create button.
    NOTE: The table format has one X column and several Y columns, each with the 10 subcolumns chosen previously.

2. Write the time at which OD measurements were taken in the X column (*e.g.,* 0, 30, 60, 90 min or 0, 1, 1.5, 2 h). In the Y columns, write the OD values obtained from the cultures.
3. Identify the exponential growth phase transforming the Y column data into logarithms. Click the *"Analyze"* button, choose the *"Transform"* analysis, select Y values using Y = Log(Y). Go to the *"Gallery of results"*, select *"Transform"* data table, click the *"Analyze"* button, and choose the *"Linear regression"* analysis. Exclude the OD values that do not follow a linear behavior. To exclude values, select them in the data table and type *"Ctrl + E"*.
NOTE: It is important to exclude values that not follow the exponential phase since the exponential growth equation fits well with the exponential phase.
4. In the *"Data Tables gallery"*, select the data table *"Data 1"*. Click the *"Analyze"* button and choose the *"Nonlinear regression (curve fit)" analysis among the "XY analyses"*.
5. Select the data sets to analyze and click the *"OK"* button. In the *"Fit"* tab choose the *"Exponential equation"* section and select the *"Exponential growth equation"* (, where X is cell concentration, X_0 is cell concentration at zero time, μ is specific growth rate, and t is time). Use the *"Least squares fit"* as a fitting method and leave unselected the interpolate section. Click the *"OK"* button.
NOTE: The tab with the nonlinear fit results is in the *"Gallery of results"*. The software calculates doubling time (*Dt*) and specific growth rate (μ). The software shows μ as K in the results tab.
6. Open a new project file, and in the *"New table and graph"* section, select the column choice. Choose the option *"Start with an empty data table"* and select the desired graph. Choose to plot the mean with the SD. Select the *"Create"* button.
7. Copy the μ or *Dt* values from the nonlinear fit results tab that is in the *"Gallery of results"* and paste them in a column. Finally, select the *"Analyze"* button and choose the desired analysis in the *"Column analyses"* section to compare the kinetic parameters of the different nutrimental conditions.
NOTE: The kinetic parameters obtained from the fermentative growth controls (YP media with 1%, 2%, or 10% glucose) and respiratory growth controls (YP media with 2% glycerol or ethanol) helps establish the threshold of fermentative and mitochondrial respiration, respectively. Comparing the kinetic parameters from the nutritional condition and substance/compound assayed with the respiratory and fermentative growth help identify possible effect on respiratory or fermentative metabolism.

Representative Results

Growth curves can be used to preliminarily discriminate between respiratory and fermentative phenotypes in the *S. cerevisiae* yeast. Therefore, we performed batch cultures of *S. cerevisiae* (BY4742) with different glucose concentrations that have been reported to induce fermentative growth: 1%, 2%, and 10% (w/v)[9]. Cultures showing a fermentative phenotype have a small lag phase and an exponential phase with a high growth rate (**Figure 1**). Ethanol, glycerol, and lactate are the carbon sources that can be metabolized only through respiration; thus, we performed cultures of the yeast using those carbon sources. Cultures that obtain energy mainly by oxidative phosphorylation showed a longer lag phase and slow rate of growth during the exponential phase (**Figure 2**).

Analysis of the growth curves provides only qualitative information; hence, it is important to calculate the kinetic parameters to obtain quantitative information. We calculated the *Dt* and μ using the exponential growth equation (**Figure 3**). We set a threshold for respiratory growth values at *Dt* ≥11.5 h and μ ≤0.059/h. The threshold for fermentative growth was set at *Dt* ≤6.5 h and μ ≥0.149/h (**Figure 3**).

To prove the usefulness of this screening tool we designed three experiments, with the first for evaluating the effects of different resveratrol (RSV) concentrations on *S. cerevisiae* BY4742 cells with different energetic status (**Figure 4**) in shaken flasks. The second experiment aimed to detect the effects of different ammonium sulfate (NH_4^+) concentrations on the energy metabolism of *S. cerevisiae* BY4742 (**Figure 5**) in a microplate. Finally, the third aimed to illustrate how changes in carbon source affect fermentative growth in two different *S. cerevisiae* strains (W303 and the industrial WLP530 used for beer fermentation) (**Figure 6**). In the experiment using RSV, the cell energetic status was modified by varying glucose concentration. At 10% glucose, all RSV concentrations tested did not change the respiro-fermentative phase of the yeast but did decrease the respiratory phase (during the diauxic shift, *S. cerevisiae* metabolized the ethanol produced during the exponential phase by oxidative phosphorylation). The μ values confirmed that under all conditions tested, *S. cerevisiae* showed fermentative behavior, contrary to its phenotype when grown in 2% glycerol (condition used as a respiratory control) (**Figure 4a**). When cells were energized with 1% glucose only, the μ values confirmed that at 0.1, 1, 10, and 100 µM RSV, fermentative behavior in the respiro-fermentative phase was not affected. However, a decrease in the respiratory phase during the diauxic shift was observed. Moreover, at a concentration of 1,000 µM, RSV completely inhibited cell growth.

In the second experiment, cells were supplemented with 10% glucose, a concentration considered sufficient to induce the Crabtree effect in *S. cerevisiae.* Hence, we could observe the effect promoted by the supplementation of different concentrations of NH_4^+ in fermentative metabolism. D_t values suggested that 0.13, 0.66, and 1.99% NH_4^+ favor fermentative metabolism, and it can be observed in the growth curves that these concentrations extended the culture's exponential phase. Nonetheless, at 3.31% mM NH_4^+, an increment in the D_t value showed that this concentration induces a respiro-fermentative metabolism. This phenotype showed an extended lag phase in the growth curves and slower growth rate in the exponential phase (**Figure 5**).

In the third experiment, cells of two different *S. cerevisiae* strains (W303 and WLP530) were grown in different concentrations of sucrose or galactose to test if the effects of the carbon source on D_t values were strain-dependent. As a fermentative control, both strains were grown in 2% glucose, and D_t values were calculated to set a threshold for fermentative growth. The fermentative D_t values for the strains were different, with ≤3.25 h for the W303 strain and ≤6.84 h for the WLP530 strain. Therefore, it is important to highlight the necessity for validating the D_t threshold for the different strains used. Moreover, when sucrose was used as the carbon source, a fermentative phenotype was observed at 2% and 10% in both strains. However, when galactose was used, the strain W303 did not show fermentative behavior in any of the concentrations tested, while the WLP530 strain showed a fermentative phenotype at 2% galactose. Interestingly, the W303 strain grew in all concentrations of both carbon sources tested. Though, the WLP530 strain did not show growth at the lowest concentration of carbon used (0.01%). This may be because WLP530 is used in the beer industry and the carbon concentrations to which it is exposed are generally much higher (**Figure 6**).

Altogether, the data from these three experiments prove that growth curves and kinetic parameters are useful for the preliminary discrimination between respiratory and fermentative growth in *S. cerevisiae*. Also, it is important to highlight that this is a versatile tool that may be used in different aspects of energy metabolism research.

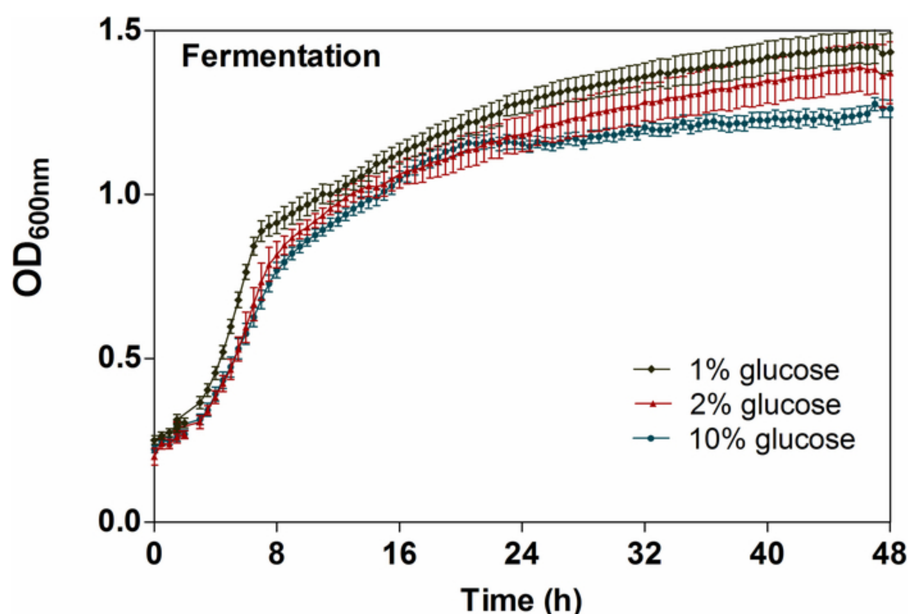

Figure 1: Effect of different glucose concentrations on *S. cerevisiae* growth phenotype. Growth curves were constructed by measuring the optical density at 600 nm every 30 min for 48 h. When *S. cerevisiae* cells ferment, cultures showed a short lag phase and fast growth rate during the exponential phase. The respiro-fermentative phase could be followed by a growth deceleration phase (respiratory phase), where the ethanol produced by fermentation is metabolized using the oxidative phosphorylation pathway, then the stationary phase is reached. Data are presented as the mean ± standard error. Please click here to view a larger version of this figure.

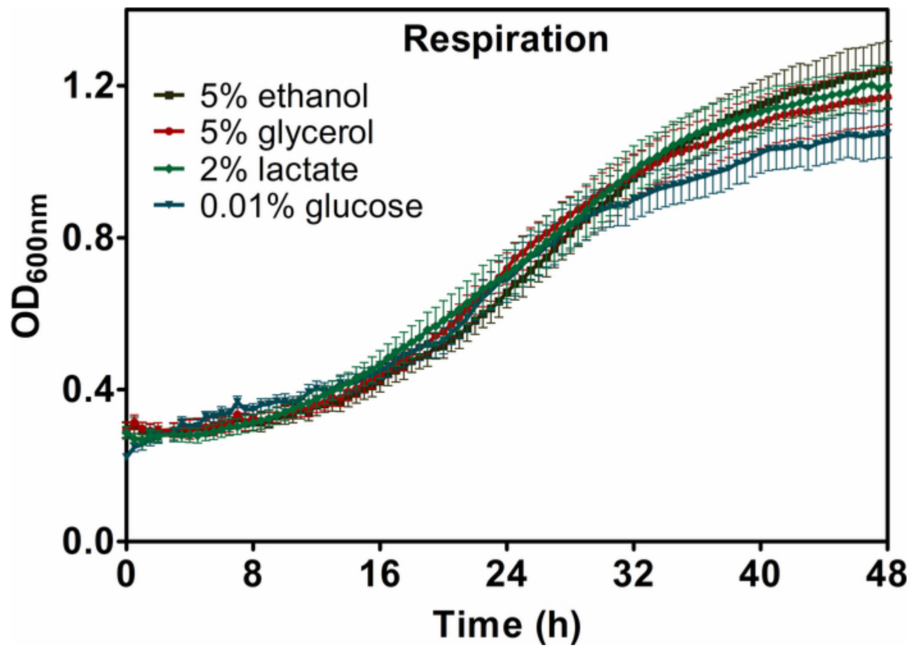

Figure 2: Effect of respirable carbon sources on *S. cerevisiae* growth phenotype. Growth curves were constructed by measuring the optical density at 600 nm every 30 min for 48 h. Cells of *S. cerevisiae* showed a prolonged lag phase and slow growth rate during the exponential phase and generally did not show a diauxic shift. Data are presented as the mean ± standard error. Please click here to view a larger version of this figure.

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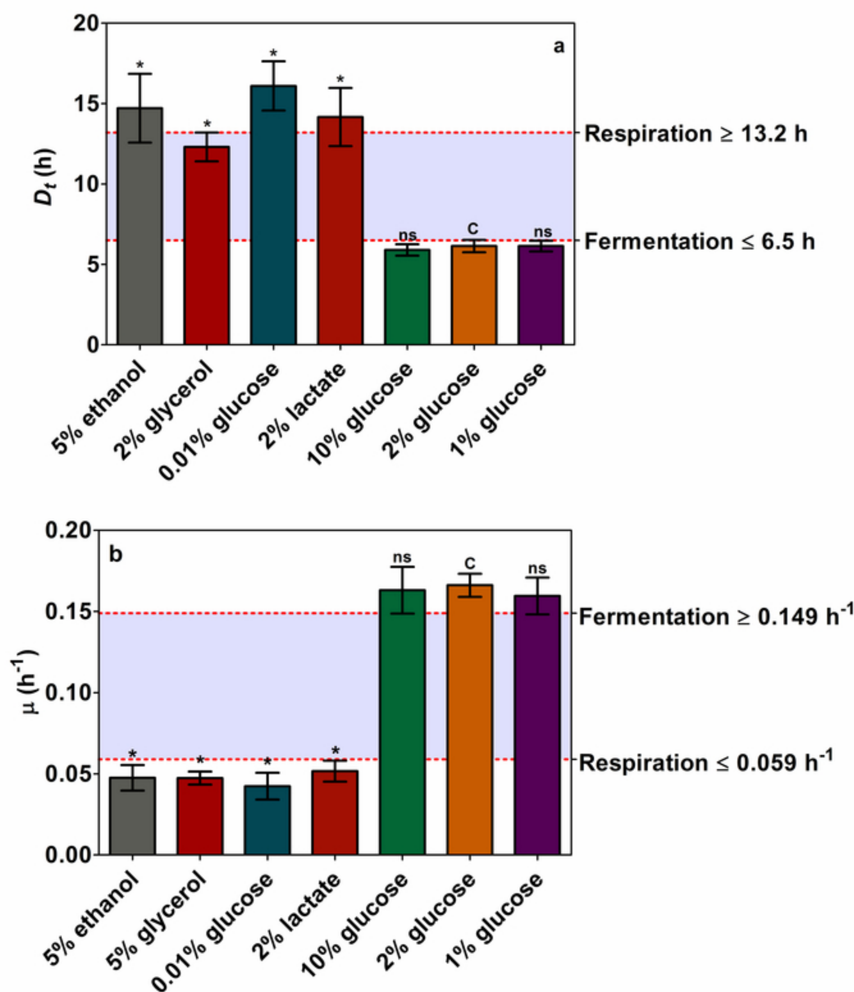

Figure 3: Effect of carbon source and its concentration on *S. cerevisiae* kinetic parameters. (**a**) D_t values of *S. cerevisiae* BY4742 growth under different carbon sources; (**b**) *μ* values of *S. cerevisiae* BY4742 growth under different concentrations of diverse carbon sources. Data are presented as the mean ± standard deviation. Statistical analyses were performed using a one-way ANOVA followed by a Dunnett's test (*p <0.01 vs. 2% glucose). Please click here to view a larger version of this figure.

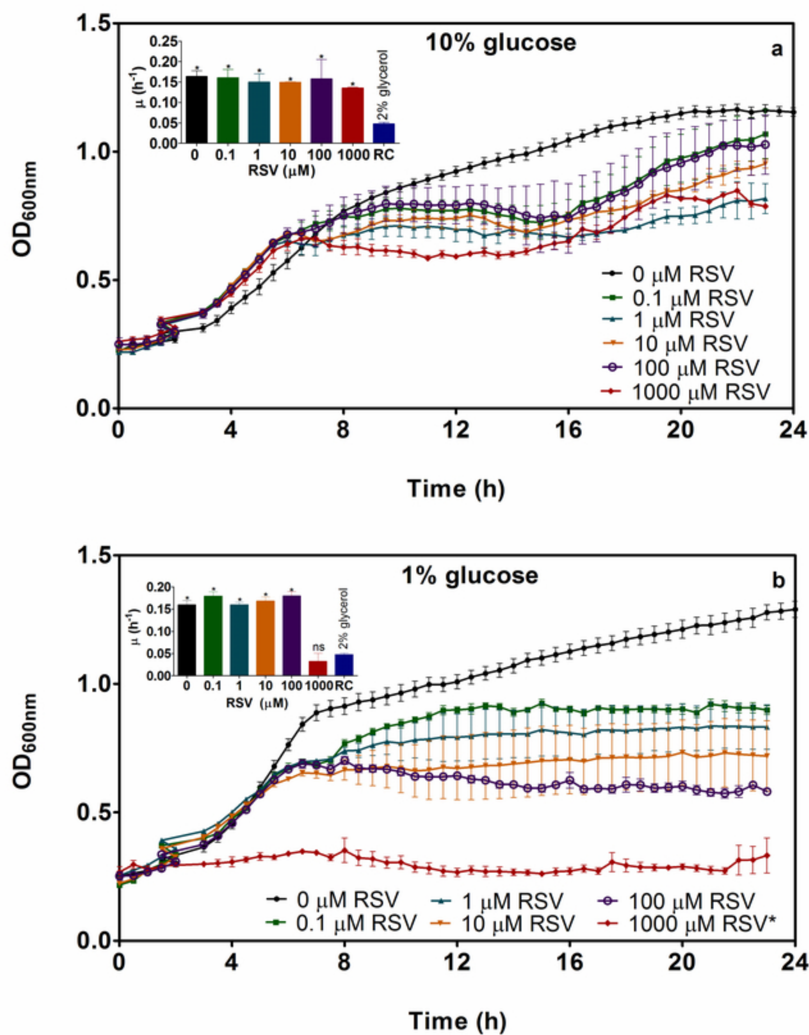

Figure 4: Effect of resveratrol supplementation on different cell energy statuses. (**a**) Growth phenotype and μ values using 10% glucose; (**b**) growth phenotype and μ values using 1% glucose. Data from growth curves are presented as the mean ± standard error, and μ values are presented as the mean ± standard deviation. Statistical analyses for μ values were performed using a one-way ANOVA followed by a Dunnett's test [*p <0.01 vs. respiratory control with 2% glycerol (RC)]. Please click here to view a larger version of this figure.

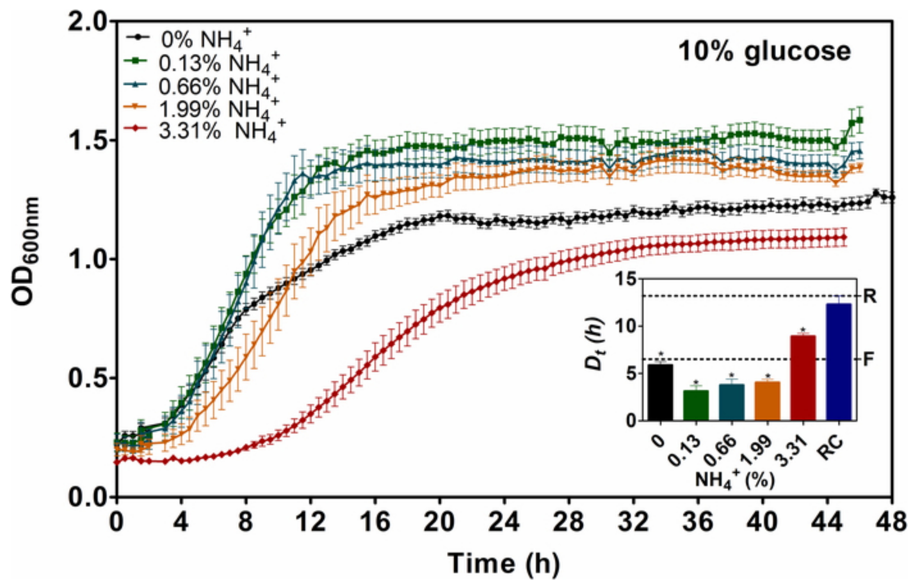

Figure 5: Effect of ammonium sulfate supplementation on *S. cerevisiae* energy metabolism. Growth curves were constructed by measuring the optical density at 600 nm every 30 min for 48 h. Data from growth curves are presented as the mean ± standard error, and μ values are presented as the mean ± standard deviation. Statistical analyses for D_t values were performed using a one-way ANOVA followed by a Dunnett's test [$^*p$ <0.01 vs. respiratory control with 2% glycerol (RC)]. Please click here to view a larger version of this figure.

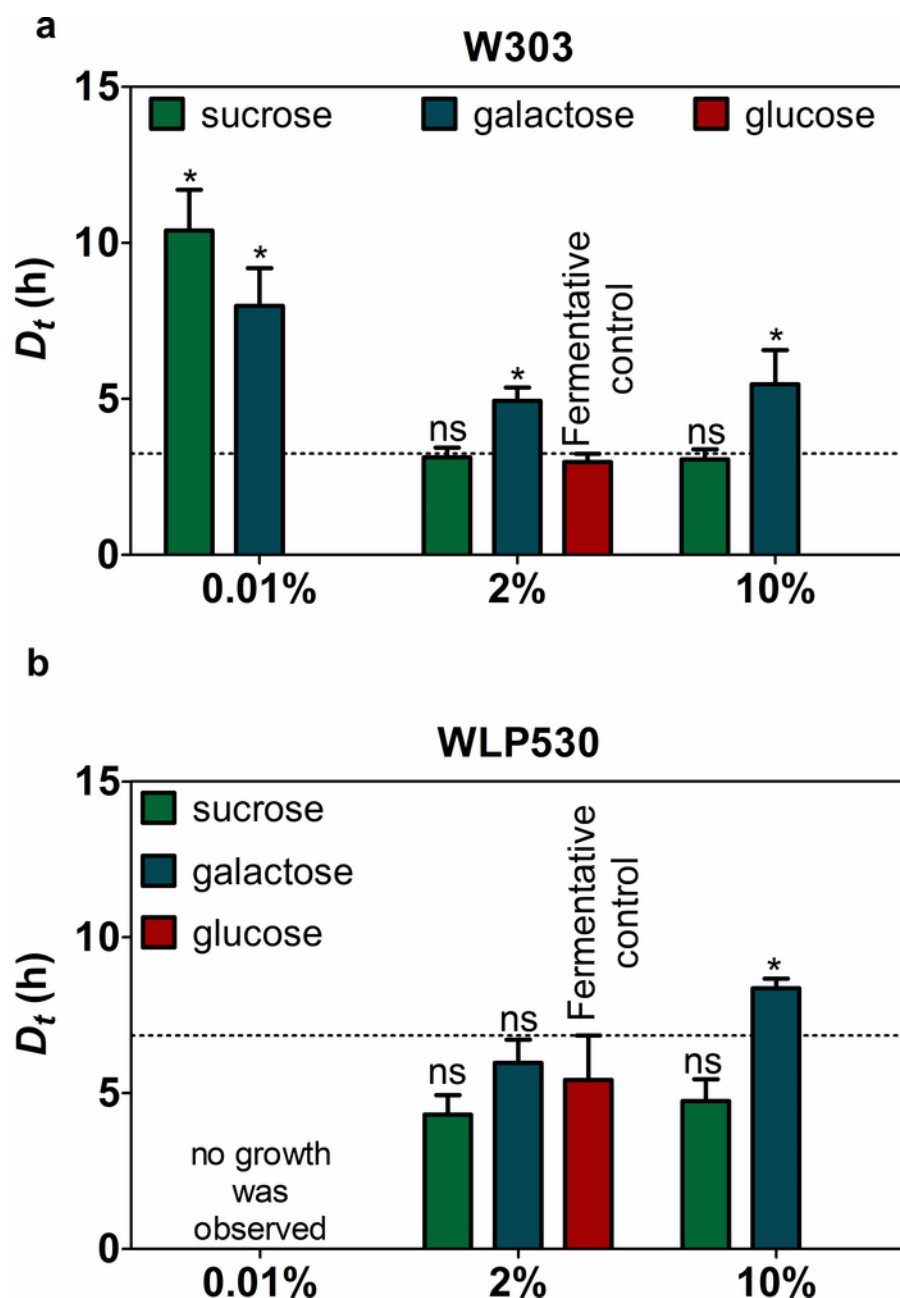

Figure 6: Effect of galactose and sucrose concentration in the fermentative metabolism of *Saccharomyces cerevisiae.* (**a**) D_t values for the W303 lab strain and (**b**) D_t values for the WLP530 industrial strain. Data are presented as the mean ± standard deviation. Statistical analyses for D_t values were performed using a one-way ANOVA followed by a Dunnett's test [*p <0.01 *vs.* 2% glucose (fermentative control)]. Please click here to view a larger version of this figure.

Discussion

A long time has passed since J. Monod[10] expressed that the study of the growth of bacterial cultures is the basic method of microbiology. The advent of the molecular tools delays the usage and study of the growth as a technique. Despite the complexity of growth which involves numerous interrelated processes, its underlying mechanisms can be described by using mathematical models[11]. This is a robust approach that can be used as a complementary tool to elucidate the most intricate molecular mechanisms[12].

To obtain reliable results from this method, it is important to consider the following critical steps. Agitation at 250 rpm is critical to achieving good growth in low carbon sources that exert respiratory growth, since when using lower agitation speeds (150–180 rpm) we observed reduced growth in respiratory conditions. It is important to initiate the protocol employing a fresh stock, and it is also essential to maintain a uniform phenotype in the assays. Growth kinetics threshold values vary among strains and must be validated according to the condition to be used in the assays. The GraphPad Prism software is proposed because the exponential growth equation is already preloaded; however, any other software is suitable,

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provided it allows programming of the exponential growth equation. In the microplate, it is recommended to include three wells with 150 µL of growth media without inoculum — this is a control that indicates if the microplate wells have suffered contamination.

It is important to indicate that plotting the OD data on a linear scale may make it hard to determine the logarithmic growth phase. This problem can be overcome by plotting the OD data in a log scale so that lag phase and logarithmic growth phase are clearly visible.

This protocol is only used to screen a phenotype, so the specific effects on fermentation and mitochondrial respiration must be confirmed. We recommend the quantification of mitochondrial respiration by oxygen consumption assays[13,14], while fermentation can be determined by the accumulation of byproducts such as acetate, succinate, glycerol, and ethanol[9].

S. cerevisiae growth has served as a means for understanding molecular mechanisms and phenotypes, with methods from screening to testing assays. Nonetheless, numerous experimental designs in the literature use yeast growth only as a qualitative parameter (*e.g.,* plate dilutions or growth curves), so the employment of these techniques neglects valuable evidence. For example, the oxygen available in agar plates used for counting microbial population or making serial-dilutions is limited, complicating growth measurement under respiratory conditions. Besides, when two or more cells remain attached to each other, only one colony is observed. Another limitation is the use of growth curves as a visual result to highlight differences in growth, which may leave aside facts about subtle differences. Therefore, the translation of growth information to quantitative parameters permits the achievement of precise data about the exerted phenotype. Nevertheless, the mathematical representation of growth is a complicated scientific enterprise. Most studies examine full growth (lag, log, and stationary phases) of the microorganisms, obtaining high-order polynomial equations that only serve one environmental condition. Despite the relative simplicity when calculating coefficients of these equations by linear regression, it is difficult to assign them to an inherent biological meaning[15]. The analysis of growth by separating growth phases is mathematically reliable and straightforward. An example of this is the exponential growth equation, which is based on first-order kinetics and fits well with a short lag phase and exponential phase.

Finally, studying the log phase using the exponential growth equation is a consistent method to understand the influence of mitochondrial respiration or fermentation on a specific compound or environment. As a proof of concept, quantification of growth in the exponential phase demonstrated that RSV specifically affects respiratory growth[13,14]. Here, growth served as a valuable tool to identify that *RIM15* deletion likely improves fermentative metabolism by partial inhibition of the respiration capacity in *S. cerevisiae*[9]. These data corroborate that observing growth allows for the study of fermentative and respiratory metabolisms and is a simple and reliable method. Hence, this approach allows for screening the effects of a specific substance on mitochondrial respiration or fermentation.

Disclosures

The authors have nothing to disclose.

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