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A high-throughput screening method for general cytotoxicity part I Chemical toxicity

Método rápido para triagem da citotoxicidade geral parte I Toxicidade química

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Abstract

A high-throughput assay intended for screening and ranking the general toxicity of physical or chemical xenobiotics based on a *Saccharomyces cerevisiae* model was developed. In order to standardise the running conditions for the technique the relationship between the absorbance at 525 nm and the number of cells/ml suspended in YPD culture medium (YPD - yeast extract 1%, peptone 0.5% and glucose 2%) was determined and a standard curve, described by the equation $Y=6.8219E^{-08}X + 0.0327$. Cultures of yeast in YPD medium were exposed to different concentrations of nicotine and the growth inhibition evaluated. We could demonstrate by covariance analysis the reproducibility of the results obtained and that method is valid and suitable for its aims.

Key words: *Saccharomyces cerevisiae* model; cytotoxicity screening; growth inhibition by nicotine; high-throughput method.

Resumo

Foi desenvolvido um método destinado a fazer a triagem rápida e o escalonamento da toxicidade geral exercida por xenobióticos tendo como modelo o *Saccharomyces cerevisiae*. Para padronizar as condições de experimentação foi estabelecida a relação entre a absorvência a 525 nm e o número de células em suspensão por mililitro de meio de cultura e calculadas uma curva padrão e respectiva equação definidora ($Y=6,8219E^{-08}X + 0,0327$) Culturas de *Saccharomyces cerevisiae* em meio completo para leveduras (YPD - 1% de glucose 2%, de peptona 0,5% e extracto de levedura 1%) foram expostas a diferentes concentrações de nicotina e a inibição do crescimento avaliada.

Palavras-chave: Modelo *Saccharomyces cerevisiae*; rastreio da citoxicidade, inibição do crescimento pela nicotina; método de alto rendimento.

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Introduction

Cell-based assays are widely used in high-throughput screening to evaluate the effects of toxicants and drugs on their biological targets [1, 2, 3, 4].

Commonly, screening methods used for bioactive xenobiotics such as chemicals or radiation are more or less based on their ability to inhibit the growth rate by interfering with the physiological status of the cells.

Standardized assay conditions enable the use of single values such as the concentration of a biomarker or the inhibition of proliferation to quantify general toxic effects on the cell, as well to study basic biological mechanisms [5].

Cells suspended in a liquid medium scatter the light causing a turbidity that may be measured using a colorimeter. The absorbance increases with the increasing of the number of cells in suspension giving a convenient, rapid and accurate method for measuring cell growth rates. Thus growth monitoring has been made by measuring the absorbance of cellular cultures at different wavelengths, usually between 500 and 650 nm [3, 6, 7].

Saccharomyces cerevisiae is a unicellular eukaryotic, non-pathogenic organism that shares significant genetic and proteic homologies with mammals and has been used as model for a wide range of purposes, from the general toxicity to mechanistic studies [8]. It is, therefore, highly useful in the study of basic biological mechanisms common to fungi, plants, animals, and humans.

Yeast has the advantage of growing rapidly in liquid cultures, the assays are simple, rapid, and cost-effective, require small sample volumes, and can be miniaturized.

The concentration of xenobiotic that causes a growth inhibition of 50% (IC_{50}) is one standard parameter calculated in toxicity studies. The percentages of inhibition on the growth of *Saccharomyces* was chosen in our pilot study to demonstrate the feasibility of this high-throughput assay intended for screening the general toxicity of physical and chemical xenobiotics.

Materials and Methods

Method

The correlation between the cultures absorbance at 525 nm and the number of cells in suspension in YPD was studied and a calibration curve calculated for posterior extrapolations *Saccharomyces cerevisiae* was incubated in absence and presence of different concentrations of xenobiotic at 30º C with 230 cycles agitation maintained up to the time necessary to have a minimum of two hours of *log phase.* Growth curves were generated from the number of cells per/ml YPD evaluated spectrophotometrically.

Reagents

The reagents used were purchased from regular suppliers and nicotine purum =99.0% (GC) from *Fluka Chemika*. A stock solution 100 mM of this compound was used to prepare the solutions tested.

Equipment

Incubators (*LEEC Automatic CO_2 Incubator Model GA3010*) and (*Heidolph Inkubator 1000*), shaker (*Heidolph Unimax 1010*), vortex mixer (*VELP Scientifica ZX*[3]), spectrophotometer (*Biochrom Libra S22*), counting chamber (*Type Neubauer 0.100mm Tiefe Depth Profondeur 0.0025mm*[2]) and microscope (*Novex Microscope K-Range*).

Liquid Cell Culture

The cell cultures to be used in the experiments were obtained from *Saccharomyces cerevisiae*, growing in Petri dishes on complete medium YPD (yeast extract 1%, peptone 0.5% and glucose 2%) containing 1.5% agar, by inoculation into 20ml of YPD in a 50ml Erlenmeyer.. After dispersion, the cells were placed in the LEEC incubator to grown at 30ºC without agitation for a time enough (16 to 20 hours) to obtain approximately $1.0x10^7$ cells per ml.

System Calibration

For each cell concentration the absorbance at 525 nm was measured and the number of cells/ml counted using a optic microscope and a Neubauer type chamber containing 10l of the suspension diluted to 1/50 with YPD medium. The cell suspensions were vortexed just before they were diluted as well before to be transferred into the chamber. The cell counting was performed by two different operators twice and the mean cell concentrations (cells per ml medium) and its standard deviation calculated.

Cytotoxicity

About $0.5x10^6$ cells were transferred into 4mL disposable cuvettes containing YPD medium and aliquots of stock solution of nicotine in order to obtain concentrations 4mM, 6mM, 8mM, 10mM, 12mM and 14mM performing a total volume of 2.2ml. A control of YPD medium was used for each series of tests. The cuvettes were incubated in a Heidolph Inkubator 1000 with shaker at 30º and 230 rpm agitation for five hours. The absorbance of each cell culture was measured at the start of the assay, and after, at regular intervals of 30 minutes except for the two first hours, whereas the measurements interval was 60 minutes. Three series of assays were performed in three different days and the reproducibility of the results studied. Every time the cuvettes were operated, they were vortexed to guarantee homogeneous suspensions.

Math and Statistical Analysis

A covariance analysis was applied to the slopes of the growth curves to test the reproducibility of the method concerning the growth inhibition rate of *Saccharomyces cerevisiae* in presence of test substance.

The statistical significant differences between assays were tested using a significance level of 0.05.

Results

System Calibration

The calibration curve obtained by plotting the absorbance at 525 nm, zeroed with a blank of YPD medium, against the number of cells in each suspension, counted as described above is represented in figure 1.

The cell concentrations (cells/ml) were calculated applying the following formula: nº cells / ml = (X * 1000mm3) / (Y * w^2 * d), being X=nº cells counted; Y=nº of smallest squares counted; d=depth of counting chamber and w=width of 1 square unit.

The cell concentration as a function of the absorbance at 525 nm is described by the equation $Y=6.8219E^{-08}X + 0.0327$, $R^2 = 0.9904$, obtained by linear regression.

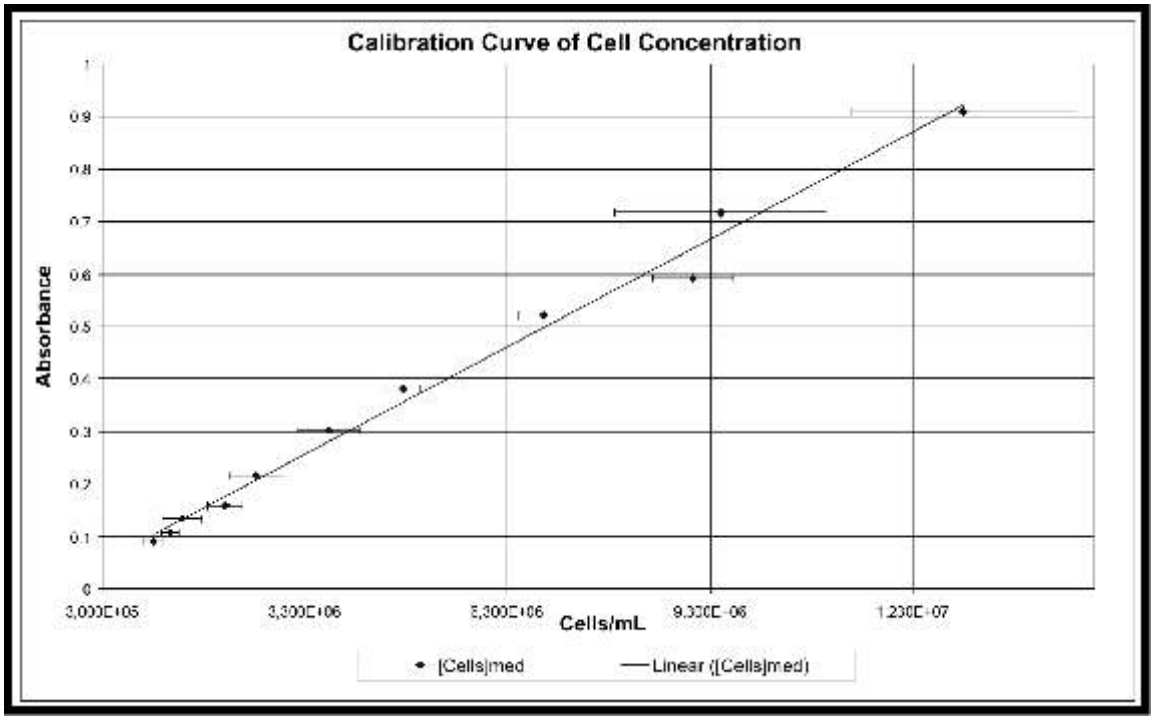

Figure 1 - Calibration Curve of cell concentration in YPD medium as function of absorbance at 525 nm. The errors bars represent the standard deviation of the cell concentration.

Cytotoxicity

The absorbance at 525 nm of the suspensions was measured at intervals as described before and until five hours of incubation were completed. The number of cells/ml in each cuvette was calculated using the equation above. In all the three series of experiments was observed a decreasing of the cellular growth rate when the concentration of xenobiotic (nicotine) increased in the culture medium.

The figure 2 represents the growth curves for each assay during the *log phase*, between 2.5 and 5 hours of incubation, obtained by plotting the logarithm of cell concentrations against the time of incubation.

The specific cell growth rate of the blank was assumed to be 100%, that is, a null percentage of inhibition, and the specific cell growth rates of the cells exposed to different nicotine concentrations were calculated as a percent of the blank rate.

The exposure to concentrations of nicotine 8 mM, 12 mM and 14 mM induced inhibitions approximately 20% (20.89% ± 0.56%)-(IC 20%), 50% (58.68% ± 2.65%)-(IC 50%)-and 99% (IC 99%), respectively.

All percentages of inhibition caused by the different nicotine concentrations assayed are presented in table 1 as well the data of the covariance analysis done to evaluate the method reproducibility.

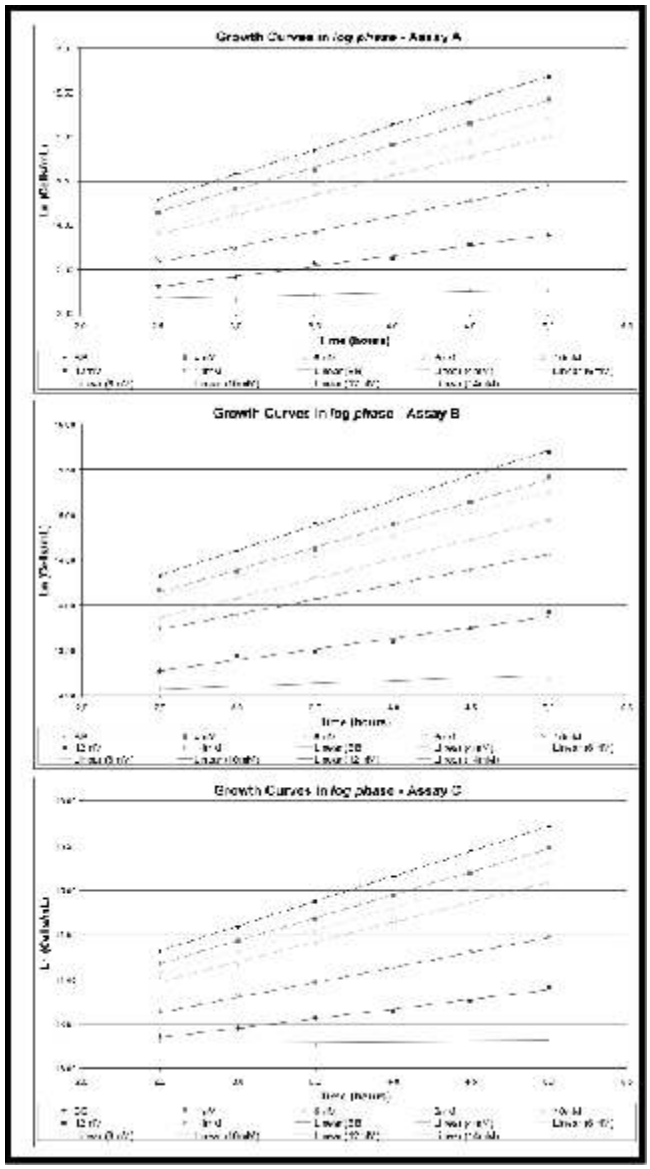

Figure 2 - Growth curves of different cells cultures in *log phase* from the three assays of nicotine cytotoxicity. BB -YPD medium; 4mM-14mM -YPD medium with different nicotine concentrations between 4mM to 14mM

Table 1 - *Log phase* slopes and percentages of inhibition from the three assays of nicotine cytotoxicity and the covariance analysis to three slopes for each nicotine concentration. BB – Blank; SD – Standard Deviation of mean; F0.05(1),2,12 – indicates the significance level, numerator and denominator degrees of freedom, respectively. † - If $F_{calculated} < F_{critical}$ there is no significant statistical difference between the

Nicotine concentration in medium (mM)	Assay A		Assay B		Assay C		Mean Percentage of inhibition (mean ± SD)	Covariance Analysis †	
	Slope	Percentage of inhibition	Slope	Percentage of inhibition	Slope	Percentage of inhibition		$F_{calculated}$	$F_{critical}$ ($F_{0.05(1),2,12}$)
BB	0.5547	0.00%	0.5527	0.00%	0.5591	0.00%	0.00%	0.30	3.89
4	0.5048	9.00%	0.5016	9.25%	0.5173	7.48%	8.57% ± 0.96%	1.07	3.89
6	0.4978	10.26%	0.4673	15.45%	0.4909	12.20%	12.64% ± 2.62%	3.51	3.89
8	0.4409	20.52%	0.4337	21.53%	0.4438	20.62%	20.89% ± 0.56%	0.22	3.89
10	0.3445	37.89%	0.3343	39.52%	0.3355	39.99%	39.13% ± 1.10%	0.54	3.89
12	0.2323	58.12%	0.2413	56.34%	0.2149	61.56%	58.68% ± 2.65%	0.76	3.89
14	0.0426	92.32%	0.0590	89.33%	0.0107	98.09%	93.24% ± 4.45%	2.10	3.89

The mean percentage of inhibition as function of nicotine concentration in culture medium is represented graphically (figure 3), which values were calculated from the equation obtained by linear regression analysis of the raw data. As shown in figure 3, the percentage of inhibition as function of nicotine concentration is best described by an exponential equation.

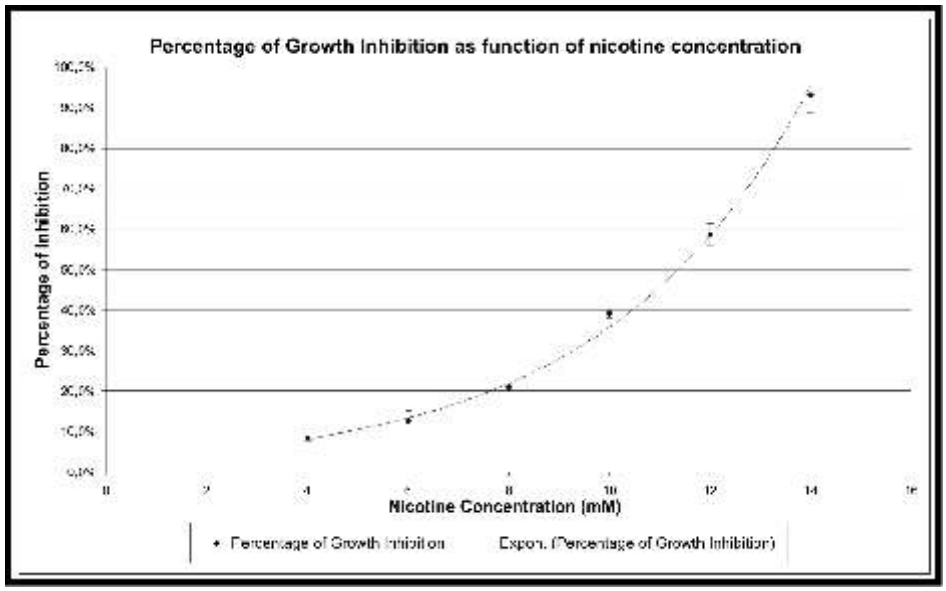

Figure 3 - Linear representation of the percentage of growth inhibition as function of nicotine concentration expressed by the equation $y = 0.0308e^{0.2452x}$

Discussion

The correlation between the absorbance of suspensions of cells and their number has been used as a simple and fast method for counting of *Saccharomyces cerevisiae* and other cells in liquid culture medium [3, 6, 7] The experimental conditions concerning factors as the wave length, the type of the equipment used for the measurements, the concentration of cells in the suspension and its homogeneity are of decisive importance for the results reproducibility and the applicability of the method now developed. The equation generated by regression analysis applied to the pair absorbance/number of cell in suspension is valid until the absorbance does not exceed values near 0.900 corresponding to cell concentrations of approximately $1.3x10^7$cells/mL.

The number of inoculated cells and the incubation time, up to five hours, were adjusted in order to include a linear part of the *log phase*, enough to calculate IC 20% and IC 50%, values usually used to ranking compounds in function of their general cytotoxicity. These experimental conditions maintained the absorbance values under 0.900 and guarantied to avoid time consuming dilutions, an evident drawback for a method developed to be fast and easy to run.

Concerning the growth curves obtained with the described experimental conditions, it was observed that the *log phase* was readily on after 2 hours of incubation at 30º and agitation speed of 230 rpm. Therefore it is possible to collect data after this time to scan the general cytotoxicity of xenobiotics and to rank them for further studies on, for example, mechanisms involved on their biologic effects as well the interaction with other agents that can act as inducers, enhancers or repressors of the xenobiotic effects.

The covariance analysis shown that, there were no significant statistical differences between the values of nicotine cytotoxicity obtained in the three assays, which ensures the reproducibility of the method now developed. Thus *Saccharomyces cerevisiae* as model for *in vitro* studies on general cytotoxicity is valid and suitable to process a large number of samples in relatively short time with high-throughput and reliable results. This method, where up to one hundred samples may be processed in each assay, is a valuable start-point for screening and ranking chemicals cytotoxicity as well for further studies on chemical, photo and photochemical toxicity.

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