Determination of the concentration of microorganisms in real time

Medical–Biological Research & Technologies

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1. Introduction

Quantification of the concentration of microbial cells is an important part of the quality work of every microbiological laboratory.

In this paper, we want to show how the initial cell concentration in the inoculum affects the apparent duration of the cell growth lag phase and how this delay can be applied to calculate the initial concentration of live cells.

2. Materials and methods

Parallel cultivation was carried out on bioreactors of the RTS-1 family.

Under each bioreactor, the initial concentration of cells is indicated (in cells/ml).

$N(o) = 2.8 \times 10^8$

$N(o) = 2.8 \times 10^6$

$N(o) = 2.8 \times 10^4$

$N(o) = 2.8 \times 10^2$

$N(o) = 2.8 \times 10^0$

E.coli BL21 carrying plasmid pET (gfp+ Km+) was used as a model culture. (DNA 2.0, USA). The pET plasmid carries the antibiotic resistance gene Km and the lacZ promoter, which regulates the expression of the fluorescent protein gfp the other is to visually control the purity of the experiment. To set up experiments on the dependence of the lag phase of cell growth on the initial cell concentration, cultivation was carried out under aerobic conditions on a nutrient medium LB (Km+) in an RTS-1 bioreactor up to OD_{600} = 3.0 (OD_{850} =1.5). The volume of the nutrient medium was 10 ml, the cultivation temperature was 37°C.

Previously (see Biosan website, Latvia) we have optimized the cultivation mode of *E.coli* BL21 on the RTS-1 bioreactor and it included the rotation of the tube at 2000 rpm, changing the direction of rotation every second, the measurement of optical density was programmed and in this series of experiments was carried out every 20 minutes. The cultivation mode was not changed in all experiments. The volume of the nutrient medium was 10 ml.

The inoculum was titrated 100-fold to the end point in Luria-Bertani medium and dispensed in 10 ml doses into TPP tubes, the lids of which have "breathing holes" (see TPP website, Switzerland https://midsci.com/ins-lp/tubespin-bioreactors) and placed into three groups of 6 RTS-1 bioreactors. The cultivation process was started simultaneously. Thus, the total number of samples was 18 and the group of 6 reactors of each triplicate contained 100-fold decreasing initial cell concentrations. The titers of starting or initial cell suspensions are given in parentheses. (2.8×10^8, 2.8×10^6, 2.8×10^4, 2.8×10^2 and 2.8×10^0 cells/ml).

To calculate the cell doubling time (doubling time-td), the cell growth rate constant K_{max}(1/hr.) was calculated and the cell doubling time was calculated ($td(hr.)=\ln 2/K_{max}$)

It is known that the suspension of *E.coli* cells with an optical density of 1.0 OD_{600} contains 10^9 cells per milliliter. (see Bionumbers site, *E.coli*) www.weizmann.ac.il/plants/Milo/sites/plants.Milo/files/uploads/keynumberslinks.pdf)

We used this constant to convert OD_{600} to cells per ml.

3. Results

3.1. Growth kinetics vs time of fermentation as function of serial 100-time diluted initial cells concentration in bioreactors, (OD_{600})/ (time of cultivation)/N(o)i.

Optical density data are presented in (OD_{600}). Attention is drawn to the similarity of the obtained dependences of the cell growth curves on the initial concentrations of the inoculum to the PCR kinetics obtained by sequential dilution of the sample. This is understandable, since bacterial cells multiply by doubling and are auto replicators, and nutrient media and growing conditions (critical is the efficiency of aeration of the medium) are identical. The identity of cell growth curves in all bioreactors is confirmed by their presentation in the ln(OD_{600})/ time/N(o)i axes.

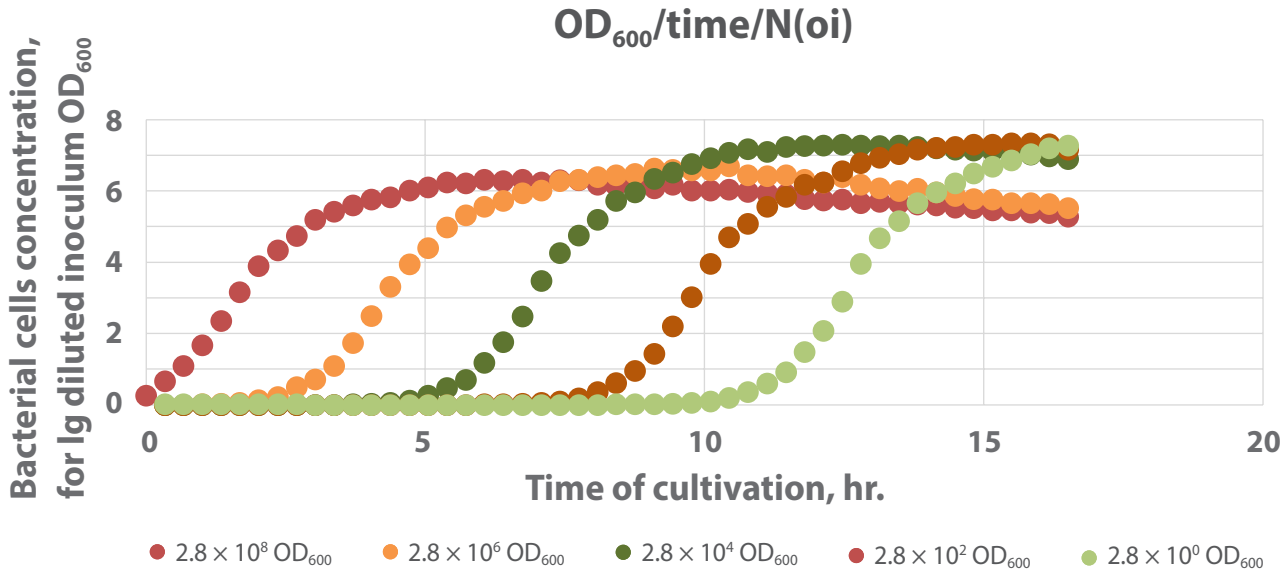
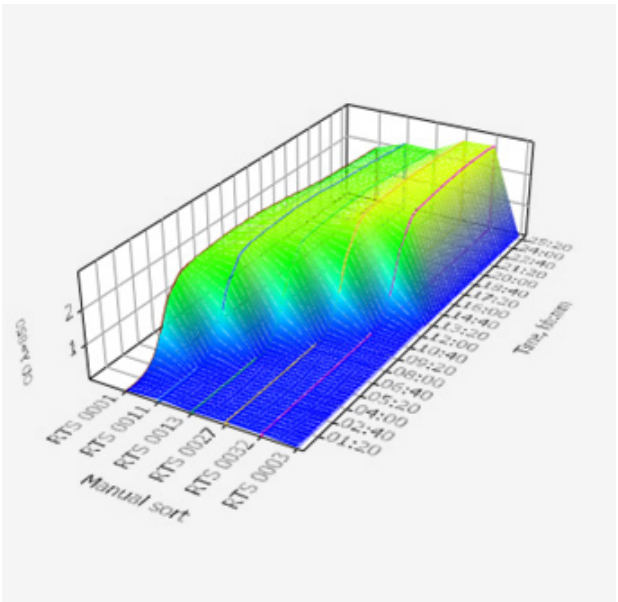


Figure 1. *Shows the data on the dependence of cell population growth on their initial concentration (No)i at a sequential 100 dilution of the inoculum.*



3.2. First derivative of growth kinetics curve, ($\Delta OD_{600}/\Delta t$)/(time of fermentation)/(Lg initial cells concentrations).

The analysis of the obtained growth curves versus time showed a strict sequence of reaching the maximum value of the first derivative ln(OD_{600})/time/N(o)i. (see Fig.2).

It is obvious that the sequential dilution of the inoculum by 100x leads to a shift of the peak of the maximum of the first derivative strictly by 2.7 hours relative to the previous dilution.

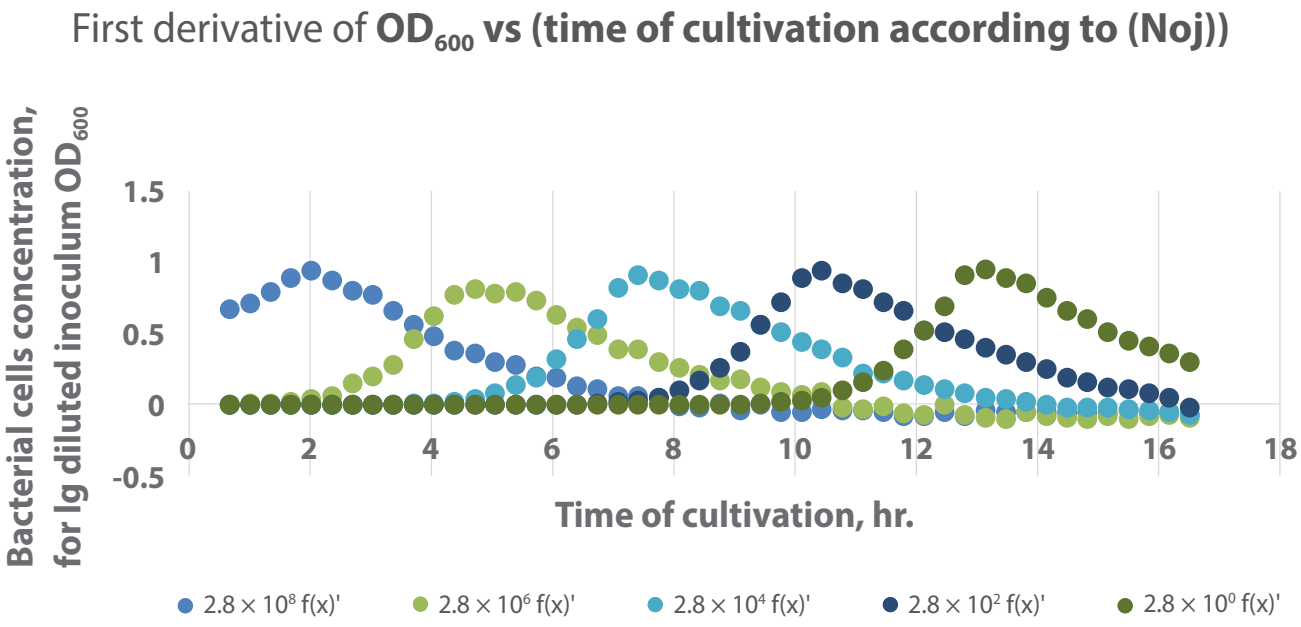


Figure 2. *Dependence of the maximum of the first derivative of cell growth on time, as well as the initial concentration of cells (the values of the initial concentrations in the corresponding bioreactors are given in Fig.1).*

3.3. ln(OD_{600})/time/N(o)

The identity of cell growth curves over time in all bioreactors is confirmed by almost equal slopes of the curves ln(OD_{600})/time/N(o) (see Fig. 3).

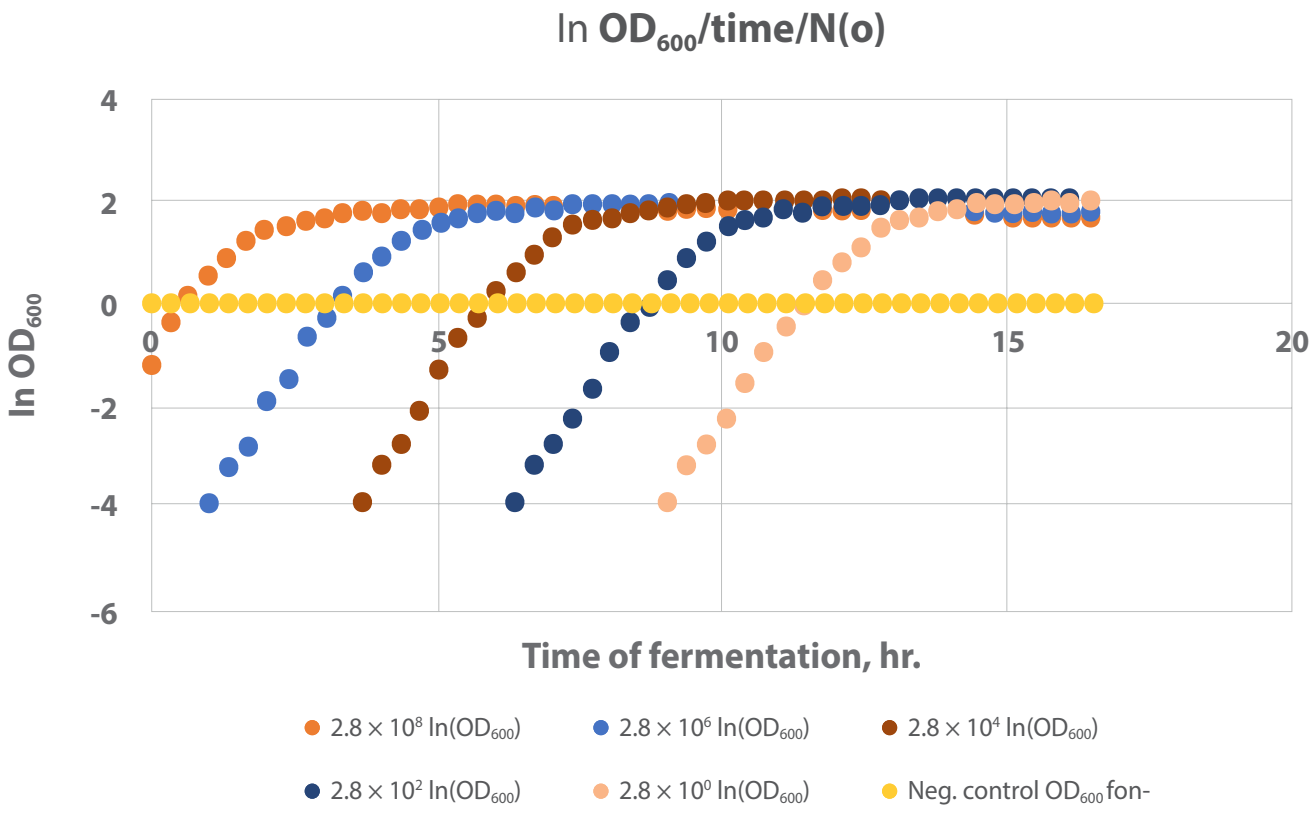


Figure 3. *Representation of cell growth curves versus time (for various initial cell concentrations in bioreactors (OD_{600})/time/N(o) on a logarithmic scale ln(OD_{600})/time/N(o).*

3.4. Optimization of threshold to initial growth rate of *E.coli* BL21.

Properly chosen threshold values are important for further quantitative analysis of unknown initial cell concentrations in bioreactors. For this reason, we took advantage of the developments in the field of quantitative PCR analysis, since both processes are similar and can be mathematically described by the same formulas. It is only necessary to find sections of the initial growth rates in each reactor (V_{max}), and use the obtained data in further calculations. The results of the threshold values obtained in the early, middle and late logarithmic phase of three parallel experiments are shown in **Table 1**.

Table1. *Different Threshold vs initial cells concentrations*

(No)I $2.8\times10^{(x)}$ mg/ml	Time threshold $(\Delta OD_{600})/t)_{max}$	Delta hr.	Threshold OD_{600}=0.8	Delta hr.	Threshold OD_{600}=0.36	Delta hr.
2.8×10^{8}	**2.02**		**1.01**		**0.34**	
2.8×10^{6}	**5.05**	**3.03**	**3.71**	**2.7**	**3.03**	**2.7**
2.8×10^{4}	**7.75**	**2.7**	**6.4**	**2.7**	**5.73**	**2.7**
2.8×10^{2}	**10.44**	**2.69**	**9.1**	**2.7**	**8.42**	**2.7**
2.8×10^{0}	**13.14**	**2.7**	**11.79**	**2.7**	**11.12**	**2.7**

From the results presented in **Table 1**, it follows that 100-fold dilution of the inoculum leads to a shift in the time to reach the set threshold value by 2.7 hours for both the threshold value $((\Delta OD_{600})/\Delta t)_{max}$ and the threshold value OD_{600}=0.8 and OD_{600}=0.36, since the growth curves, as mentioned earlier, are identical. Based on **Table 1**, a graph of the time required to cross the threshold growth curve for 100-fold serial dilutions of the initial cell concentrations in the bioreactors was plotted.

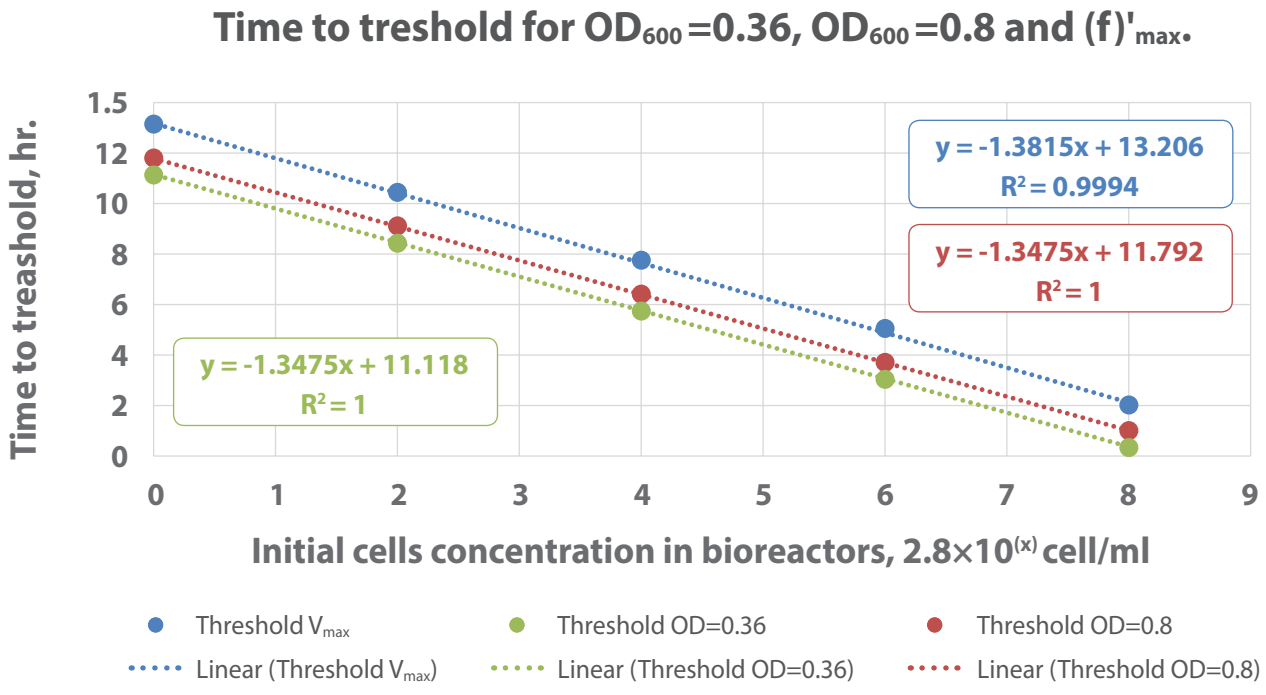


Figure 4. *Calibration plots built on three threshold values.*

Calibration graphs of the intersection of the growth curve (od600)/(time of cultivation) constructed according to the threshold value T(threshold) chosen by us – OD_{600}=0.8 and OD_{600} – 0.36, as well as $(f)'_{max}$ (corresponds to OD_{600}=4) for various initial initial cell concentrations slightly differ from each other by the coefficient (time/(log(cells conc.) from 1.35 to 1.38), but have different values of the free term (from 11.118 hr. to 13.206 hr.).

To figure out which of the above calibration curves is correct, it is necessary to translate the time scale into a scale of the number of cell doublings required to reach a given threshold value. To do this, it is necessary to find the range of initial rates of cell division, when their growth is not limited by the substrate(s) and by-products of fermentation, as well as concomitant physicochemical changes in cultivation conditions (pH, pO_2).

3.4. Maximal growth rate (K_{max}) and dividing time (Td) for *E.coli* on LB media (Km+).

Of greatest interest to quantitative microbiology is the ability to relate T(tr) to cell doubling time t(d). Since the studied strain was "loaded" with a plasmid carrying the Km resistance gene and the fluorescent protein gene, the maximum growth constant ($(K)_{max}$) and cell doubling time (t(d)) were calculated on the LB nutrient medium containing Km (50 µg/ml).

Since Bioreactor **RTS-1** allows you to determine the optical density even with a frequency of 1 time in 2 minutes, it is possible to obtain a sufficient number of necessary points at the earliest phases of growth diapason OD_{600}=0.01 (early log phase) to OD_{600}=4.0 (the maximum value of the first derivative of the growth curve bacteria).

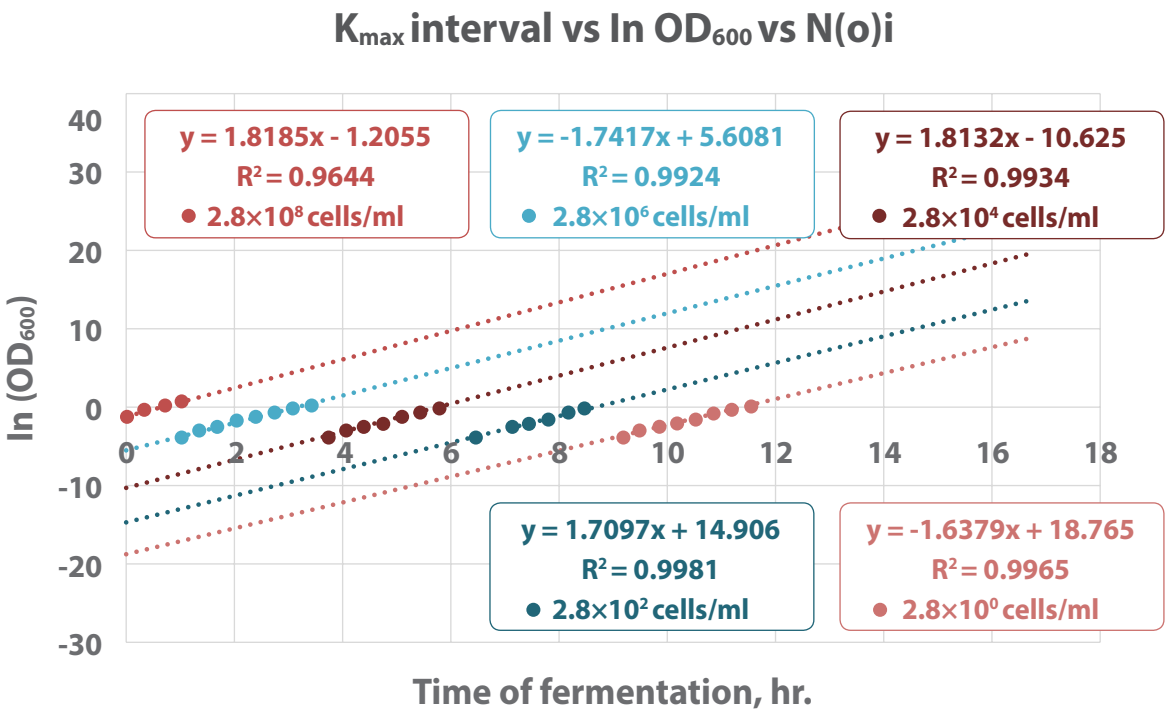


Figure 5. *Linear plots of initial cell growth rates in bioreactors (K_{max}) for various initial inoculum concentrations (N(o)) are presented in the ln(OD_{600})/time/N(o)i axes.*

It is obvious that the slope angles $\ln(OD_{600})/(time)$ for each variant of the initial inoculum concentration are close and the plotted tangents to the points of the initial rates of cell division are practically parallel.

The value of K (1/hr.) in the interval indicated in **Fig. 5** corresponds to the maximum rate of cell division and corresponds to K_{max}, on the basis of which the cell doubling time $t(d)_{max}$ was calculated using the formula $t(d)=\ln(2)/K_{max}$. The data obtained are summarized in **Table 2.**

Table 2.

Bioreactor №	N(o), cells/ml	K(1/hr.)	td(hr.)
RTS23	**2.8×10^8**	**1.82**	**0.38**
RTS24	**2.8×10^6**	**1.74**	**0.40**
RTS26	**2.8×10^4**	**1.81**	**0.38**
RTS29	**2.8×10^2**	**1.71**	**0.40**
RTS31	**2.8×10^0**	**1.67**	**0.41**
	Average	**1.75**	**0.39**
	STDV	**0.06**	**0.01**

3.4. Optimization of threshold to initial growth rate of *E.coli* BL21.

Based on the experimentally obtained value of the maximum cell doubling rate (t(d) equal to 0.39 h), we were able to calculate the number of divisions n(CD) = t/t(d) required to cross the threshold value for each initial cell concentration (N(o)i) **(see Fig. 6).**

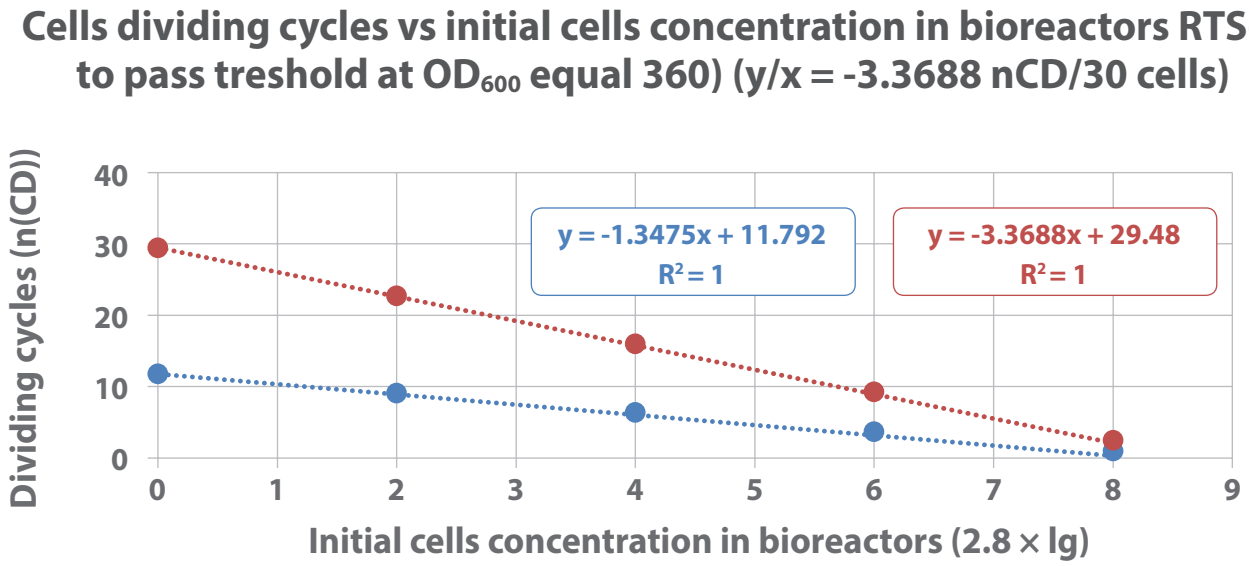


Figure 4. *Graph of the dependence of the time to reach the cell concentration of the threshold value (blue) and the number of necessary doublings of the cell population calculated on its basis (red) from the initial concentration of the inoculum in the RTS-1 bioreactor.*

x = initial concentration of cells in bioreactors in power.

y = t/t(d) number of cells dividing cycles **(n(CD)).**

value -3.3688 = y/x – how many doublings of cells are needed to increase their concentration by 10 times.

value +29.48 = n(CD) at x=0 or initial concentration of cells in bioreactor (2.8 cells /ml). If reactor contain 10 ml liquid total number of cells will be 28 cells/10ml

How can we objectively estimate the coefficient y/x = -3.3688x that we have obtained. This coefficient shows how many cycles of cell division are needed for their concentration to increase by 1 order (ten times). If we express this coefficient in od600, then this is a range from 0.05 to 0.5. – that is, we need 3.37 cell doublings for this. This series, expressed in OD_{600}, is described as follows: 0.05, 0.01, 0.2, 0.04, 0.06 OD_{600}. That is, 3.4 divisions are really needed. And if you make sure that the cells of *E. coli* BL21 double, and not triple, you need to raise 10 to the power of the reciprocal of this coefficient, i.e. in 10^ (1/-3.4). The resulting value, according to our experimental data, will be 1.98 doublings per cell division cycle, which corresponds to an almost ideal replicator selected by Evolution.

The constants thus obtained indicate that if the method is optimized for other microorganisms, then the principles laid down in it can be successfully applied to quantify their ultra-low concentrations on specific nutrient media and conditions of specific cultivation conditions, such as pH, temperature, and specific gaseous medium. Such work was carried out for the homofermentative bacterium *L. plantarum* on the next generation of bioreactors – **RTS-8.**

3. Literature

https://biosan.lv/products/rts-1c-real-time-cell-growth-logger-2

https://midsci.com/ins-lp/tubespin-bioreactors

https://www.weizmann.ac.il/plants/Milo/sites/plants.Milo/files/uploads/keynumberslinks.pdf