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Measurement of Yeast Viability/Mortality in the Presence of Chromium(VI)

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Summary

The aim of this work was to develop a mathematical model – a set of equations for studying the effect of Cr(VI) on yeast viability/mortality. The model was built with parameters and a two-stream approach has been developed which includes four parameters that indicate viability like population condition (PC), cell viability in the buffer (V_B), cell viability in the buffer supplemented with the effector (V_{EB}), specific cell viability (V_S), and three parameters that indicate mortality as follows: cell mortality in the buffer (M_B), cell mortality in the buffer supplemented with the effector (M_{EB}) and specific cell mortality (M_S). The parameters were calculated on the basis of primary results [the total cell number per mL (N_T) and the number of viable cells per mL (N_{CFU})]. The total cell number was obtained microscopically with a hemacytometer and yeast viability was determined with the colony count method (spread plate method).

Key words: yeasts, cell viability, mortality, Cr(VI) toxicity

Introduction

The survival of living cells depends on their ability to sense alterations in the environment and to appropriately respond to the newly emerged conditions *(1)*. Knowledge of stress and stress responses is crucial to understand how single-cell and multicellular organisms adapt to changing environmental and physiological conditions *(2)*. The molecular mechanisms induced upon the exposure of cells to such adverse conditions are commonly designated as stress responses. Stress response mechanisms aim to protect cells against the potentially detrimental effects of stress challenges and to repair any molecular damage, and therefore lead to an adjustment of the metabolism and other cellular processes to the new status *(1)*. These stress responses involve aspects of sensing, signal transduction, transcriptional and post-translational control, protein-targeting to organelles, accumulation of protectants, and activity of repair functions *(2)*.

Redox active metals play a major part in the generation of reactive intermediates (free radicals, other reactive intermediates) in the cell *(1)*. Cr(VI) itself is unreactive towards DNA under physiological conditions *in vitro (3)*. However, *in vivo* the reduction of chromium(VI) by cellular reductants (the microsomal cytochrome P–450 system, mitochondrial electron transport chain complexes, aldehyde oxidase, hydrogen peroxide, ascorbate and thiols) to the reactive intermediates, including Cr(VI) thioester, Cr(V), Cr(IV) species and free radicals (hydroxyl and thiol radicals) is thought to be an important step in the mechanism of Cr(VI) induced DNA damage *(3,4)*.

Cells have evolved a number of capabilities to repair macromolecules that are damaged and to inactivate reactive intermediates. Yeast cells contain enzymatic as well as non-enzymatic defense mechanisms against the harmful effects of reactive derivates. The genes encoding components of this defense apparatus, such as those involved in glutathione or thioredoxin biosynthesis, display increased expression after stress challenge. Glutathione and thioredoxin are normally involved in cellular redox-reactions, in particular those involved in

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the establishment and maintenance of the tertiary structure of proteins *(1)*.

Since the measurement of cell viability is a difficult task, there are many methods in microbiology which were developed with the aim of estimating cell viability. A review of available methods shows at least seven different approaches, however, none is comprehensive. The most common methods belong to the group of colony count methods. Among them the pour plate method, spread plate method, thin layer plate method, layered plate method and membrane filter method are available *(5)*. Practically in the same frame the most probable number (MPN) method can be included. After analysing them, it has been determined that all of them suffer from prolonged cultivation time. Rapid methods can be found among solutions dealing with different staining, ATP bioluminescence, microcalorimetry, impedimetry and conductivity and radiometry *(6)*.

The most common is still staining which is mostly performed with 0.01 % solution of methylene blue in a phosphate buffer of pH = 4.6. Staining is sometimes performed with the leuco-base of methylene blue or methylene green. Leuco-bases are prepared by reducing the dye solution with thiosulphate. They can be prepared from various dyes such as toluidine blue, cresol blue, thionin, *etc.* A convenient method based on fluorescence microscopy is shown to be fluorochrome staining (erythrosin B) *(7)*.

Nevertheless, ATP bioluminescence shows very good application. The assay is based on the assumption that living cells of a given type contain a reasonably constant amount of ATP, which is lost rapidly upon cell death. The bioluminescent reaction catalysed by the enzyme luciferase uses the chemical energy contained in the ATP molecule to drive the oxidative decarboxylation of luciferin, with the consequent production of light. For each molecule of ATP, one photon of light is emitted; thus, the amount of light produced is directly related to the concentration of viable cells. Light output, and therefore ATP content, is measured in relative light units (RLUs) *(6,8)*.

Calorimetric data for heat released by cells (microcalorimetry) can be related to microbial growth, and was demonstrated by many publications as a suitable method *(9)*. Cell viability can be also determined on the basis of the measurement of changes in the electrochemical properties (impedance and conductivity) of the growth medium, which is a consequence of metabolic activity of microorganisms *(6)*. However, it has limitations for application in microbiology.

Radiometry has been found specific and accurate. The microorganisms are supplied with radioisotopically labelled substrate. The most common radioisotope is ^{14}C. Labelled $^{14}CO_2$ is produced metabolically, and the amount of radioactivity which is detected using a scintillation counter is representative of microbial activity *(6,9)*.

Since it is generally accepted to analyse microbial status at the beginning *vs.* the end of the process of yeast exposure to a particular environmental stress, some important information about yeast metabolic state might be missed. On this point, one cannot detect all metabolic activity on the basis of total biomass without analysing separately each step within the analytical procedure. As a consequence, the tool which we applied is based on the determination of yeast viability and mortality in each particular step of the analytical procedure. The set of equations based on the calculation of two parameters indicating viability *vs.* mortality in the population has been developed with the intention to establish a rational tool for analysing the impact of the effector.

Material and Methods

Microorganisms and media

The microorganisms *Saccharomyces cerevisiae* – ZIM 321 and *Schwanniomyces occidentalis* – ZIM 763 were obtained from the ZIM culture collection at the Biotechnical Faculty, Department of Biotechnology, Ljubljana.

Media as malt agar (malt extract, 30 g L^{-1}; yeast extract, 4 g L^{-1}; glucose, 4 g L^{-1}; agar, 20 g L^{-1}) and malt broth (malt extract, 30 g L^{-1}; yeast extract, 4 g L^{-1}; glucose, 4 g L^{-1}) were prepared by the standard procedure and yeast growth was conducted on agar plates (28 °C) or on a rotary shaker (28 °C, 200 r.p.m.).

Chromium(VI) stock solution

Cr(VI) stock solution (c Cr(VI) = 10 mM) was obtained using the Cr(VI) compound $K_2Cr_2O_7$.

Experimental procedure

Yeasts were cultivated in malt broth at 28 °C and 200 r.p.m. on a rotary shaker. Biomass was harvested at the early stationary phase by centrifugation (5 min, 4000 r.p.m., at room temperature) and washed twice with MES (2-morpholinoethanesulfonic acid) buffer (pH = 4.0). The yeast suspension with dry mass concentration of 0.05 mg mL^{-1} was prepared in the MES buffer. The Cr(VI) stock solution was added to the yeast suspension to prepare Cr(VI) concentrations of 1, 10, 100 and 1000 μM in the yeast suspension. The samples with a total volume of 10 mL were incubated for 24 hours at 28 °C and stirred.

At the time t = 0 (beginning of the 24 h incubation) the total cell number per mL (N_T) and the number of viable cells per mL (N_{CFU}) were determined for the control sample (only the yeast suspension in the MES buffer). After 24 h incubation the number of viable cells per mL was determined for the control sample and Cr(VI) concentrations (1, 10, 100 and 1000 μM).

The N_T was obtained with a hemacytometer and N_{CFU} as CFU on malt agar.

Results and Discussion

Assessing yeast viability *vs.* death is a difficult task, especially if we take into account non-culturable organisms or organisms which are difficult to cultivate. However, our strains were confirmed previously to be an easily culturable organism, which is quite common among yeasts. The problem of assessing metabolic state at different steps during exposure to environmental stress is not regularly used in stress evaluation procedures. As a consequence, this four-step procedure had been established and standardized with the aim of detecting in which step this effect [in our case Cr(VI)] occurs.

Table 1a. Average values of total cell number per mL (N_T) and number of viable cells per mL (N_{CFU}) and corresponding standard deviation and coefficient of variation from at least three measurements for *Sacch. cerevisiae* – ZIM 321 at the start and end of the experiment without $K_2Cr_2O_7$

	Saccharomyces cerevisiae – ZIM 321					
Time / h	$N_T \cdot 10^5$	s.d. $(N_T) \cdot 10^5$	c.v. (N_T) / %	$N_{CFU} \cdot 10^5$	s.d. $(N_{CFU}) \cdot 10^5$	c.v. (N_{CFU}) / %
0	32.00	1.28	4.0	9.45	0.04	0.4
24	n.d.	n.d.	n.d.	5.74	0.66	11.5

Table 1b. Average values of total cell number per mL (N_T) and number of viable cells per mL (N_{CFU}) and adequate standard deviation and coefficient of variation from at least three measurements for *Schwann. occidentalis* – ZIM 763 at the start and end of the experiment without $K_2Cr_2O_7$

	Schwanniomyces occidentalis – ZIM 763					
Time / h	$N_T \cdot 10^5$	s.d. $(N_T) \cdot 10^5$	c.v. (N_T) / %	$N_{CFU} \cdot 10^5$	s.d. $(N_{CFU}) \cdot 10^5$	c.v. (N_{CFU}) / %
0	91.00	6.12	6.7	30.80	3.55	11.5
24	n.d.	n.d.	n.d.	16.30	1.15	7.1

Legend:

n.d. not determined

s.d.(N_T) and s.d.(N_{CFU}) standard deviations

c.v. $(N_T) = \frac{\text{s.d.}}{N_T} \cdot 100$ and c.v. $(N_{CFU}) = \frac{\text{s.d.}}{N_{CFU}} \cdot 100$ coefficients of variation

Table 2. Average values of number of viable cells per mL (N_{CFU}) and corresponding standard deviation and coefficient of variation from at least three measurements for *Sacch. cerevisiae* – ZIM 321 and *Schwann. occidentalis* – ZIM 763 at the end of the experiment with $K_2Cr_2O_7$

	Saccharomyces cerevisiae – ZIM 321			*Schwanniomyces occidentalis* – ZIM 763		
$c(Cr^{6+})$ / μM	$N_{CFU} \cdot 10^3$	s.d. $(N_{CFU}) \cdot 10^3$	c.v. (N_{CFU}) / %	$N_{CFU} \cdot 10^3$	s.d. $(N_{CFU}) \cdot 10^3$	c.v. (N_{CFU}) / %
1	348.00	22.20	6.4	840.00	101.00	12.0
10	327.00	42.60	13.0	344.00	58.10	16.9
100	113.00	7.08	6.3	1.02	0.11	10.8
1000	68.00	1.50	2.2	0.40	0.04	10.0

Legend:

s.d. (N_{CFU}) standard deviation

c.v. (N_{CFU}) coefficient of variation

On the basis of previous experiments (Tables 1a, b and 2) and experience with the effect of chromium on yeast *(10)* we established a set of parameters which were found to show the yeast physiological status. Regarding this concept a two-stream approach has been developed which includes four parameters that indicate viability and three parameters that indicate mortality, as follows:

Population condition (*PC*) is defined in the early stationary growth phase and represents the ratio between viable cells and total yeast population. Standardized biomass is prepared in the following way: centrifugation, washing twice with the MES buffer and resuspension in MES buffer.

$$PC/\% = \frac{N_{CFU_{t=0}}}{N_{T_{t=0}}} \cdot 100 \qquad /1/$$

$$N_{T_{t=0}} = N_{CFU_{t=0}} + N_{t=0} \qquad /2/$$

$N_{CFU_{t=0}}$ number of viable cells per mL at t = 0

$N_{T_{t=0}}$ total number of cells per mL at t = 0

$N_{t=0}$ dead cells per mL + partly metabolic active cells per mL at t = 0

Since the effect of the buffer is not always predictable and it is known that different buffers affect yeast physiology *(11)*, we considered to evaluate this step of the analytical procedure separately. With regard to this, V_B (cell viability in the buffer) was developed.

Cell viability in the buffer (V_B) is defined as the percentage of viable cells in the buffer after 24 hours of incubation at a temperature of 28 °C. In our case the MES buffer was applied.

$$V_B/\% = \frac{N_{CFU_{t=24h}}}{N_{CFU_{t=0}}} \cdot 100 \qquad /3/$$

$N_{CFU_{t=24h}}$ number of viable cells per mL at t = 24 hours

$N_{CFU_{t=0}}$ number of viable cells per mL at t = 0

Nevertheless, it was found that the effect of the same buffer on various yeast strains can be different (data shown only for two yeasts).

Cell viability in the buffer supplemented with the effector (V_{EB}) is defined as the percentage of viable cells in the presence of a selected effector in the buffer after 24 hours of incubation at a temperature of 28 °C. In our case Cr(VI) in four concentrations (c = 0, 1, 10, 100, 1000 μM) was applied as the effector.

$$V_{EB}/\% = \frac{N_{CFU_{Cr_{c,t=24h}}}}{N_{CFU_{t=0}}} \cdot 100 \qquad /4/$$

$$V_{EB} = V_B \text{ for control}$$

$N_{CFU_{Cr_{c,t=24h}}}$ number of viable cells per mL at a certain concentration at t = 24 h

$N_{CFU_{t=0}}$ number of viable cells per mL at t = 0

Due to the various needs in studying environmental effectors we considered mortality equally important as viability. The following parameters indicate mortality in a set of equations which are the counterpart of equations for viability and essentially design an alternative view of the systematic approach.

Cell mortality in the buffer (M_B) is defined as the percentage of dead cells in the buffer after 24 hours of incubation at a temperature of 28 °C. In our case MES buffer was applied.

$$M_B/\% = 100 - V_B \qquad /5/$$

Cell mortality in the buffer supplemented with the effector (M_{EB}) is defined as the percentage of dead cells in the presence of the selected effector in the buffer after 24 hours of incubation at a temperature of 28 °C. In our case Cr(VI) in five concentrations (c = 0, 1, 10, 100, 1000 μM) was applied as the effector.

$$M_{EB}/\% = 100 - V_{EB} \qquad /6/$$

$$M_{EB} = M_B \text{ for control}$$

Finally, specific cell mortality (M_S) has been established in order to comprehend the negative influences of the selected effector on particular yeasts, which is further followed by specific cell viability (V_S) to complete the systematic approach.

Specific cell mortality (M_S) is defined as the percentage of cell mortality due to a specific effector in a specific period of time, temperature and pH range (24 hours, 28 °C, pH = 4.6) normalized by cell viability in the buffer. In our case Cr(VI) (c = 0, 1, 10, 100, 1000 μM) was applied as the effector.

$$M_S/\% = \frac{M_{EB} - M_B}{V_B} \cdot 100 \qquad /7/$$

$$M_{EB} = M_B \text{ for control}$$

Specific cell viability (V_S) is defined as the percentage of cell viability in the presence of a specific effector in a specific period of time, temperature and pH range (24 hours, 28 °C, pH = 4.6) normalized by cell viability in the buffer. In our case Cr(VI) (c = 0, 1, 10, 100, 1000 μM) was applied as the effector.

$$V_S/\% = 100 - M_S = \frac{V_{EB}}{V_B} \cdot 100 \qquad /8/$$

$$V_{EB} = V_B \text{ for control}$$

The results (Tables 3 and 4) demonstrate the suitability of the proposed model for evaluation of yeast biomass behaviour in the presence of a specific effector, Cr(VI), in four defined concentrations.

Stress generated by transferring yeast biomass from malt broth into the MES buffer (pH = 4.0) reduced yeast cell viability, which was noticed in population condition (*PC*) for a particular yeast. As expected, it was different for various yeasts strains, and lay in the range from 2.78 to 48.58 % for standardized cultivation conditions. All yeast strains were exposed to the Cr(VI) compound in the early stationary phase. The evaluation of MES buffer impact on the yeast cells during 24 hours of incubation revealed its effect in cell death expressed as cell mortal-

Table 3. Parameters [population condition (*PC*), cell viability in the buffer (V_B) and cell mortality in the buffer (M_B)] obtained with a set of equations indicating viability *vs.* mortality for *Sacch. cerevisiae* – ZIM 321 and *Schwann. occidentalis* – ZIM 763 with MES buffer treatment

	Saccharomyces cerevisiae – ZIM 321			*Schwanniomyces occidentalis* – ZIM 763		
c (Cr^{6+}) / μM	*PC*/%	V_B/%	M_B/%	*PC*/%	V_B/%	M_B/%
0	29.53	60.74	39.26	33.85	52.92	47.08

Table 4. Parameters [cell viability in the buffer supplemented with the effector (V_{EB}), cell mortality in the buffer supplemented with the effector (M_{EB}), specific cell mortality (M_S) and specific cell viability (V_S)] obtained with the set of equations indicating viability *vs.* mortality for *Sacch. cerevisiae* – ZIM 321 and *Schwann. occidentalis* – ZIM 763 with $K_2Cr_2O_7$ treatment

	Saccharomyces cerevisiae – ZIM 321				*Schwanniomyces occidentalis* – ZIM 763			
c(Cr^{6+})/μM	V_{EB}/%	M_{EB}/%	M_S/%	V_S/%	V_{EB}/%	M_{EB}/%	M_S/%	V_S/%
0	60.74	39.26	0.0	100.0	52.92	47.08	0.0	100.0
1	36.83	63.17	39.4	60.6	27.27	72.73	48.5	51.5
10	34.60	65.40	43.0	57.0	11.17	88.83	78.9	21.1
100	11.96	88.04	80.3	19.7	0.03	99.97	99.9	0.1
1000	7.20	92.80	88.1	11.9	0.01	99.99	100.0	0.0

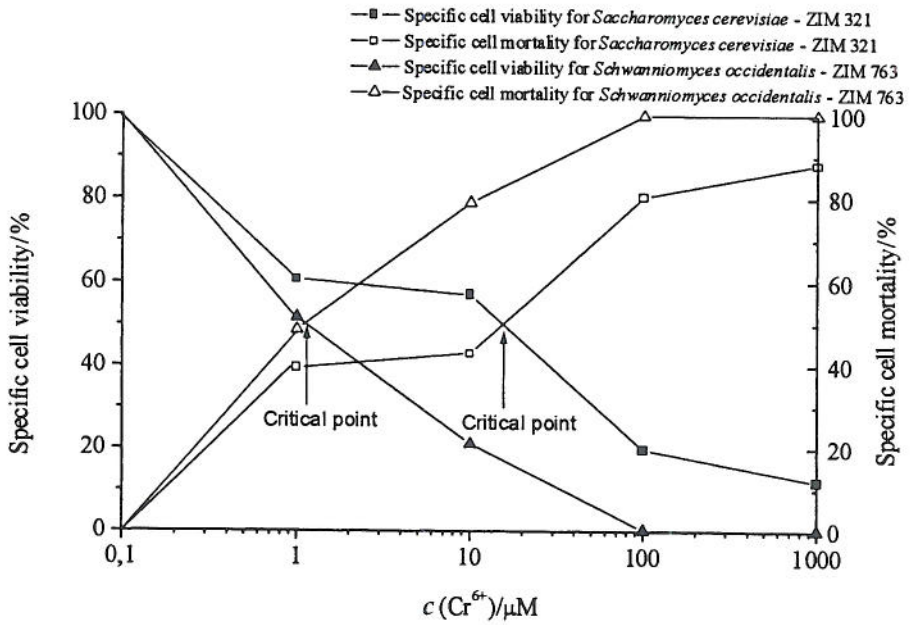

Fig. 1. Specific cell viability and specific cell mortality at different concentration of Cr(VI) for yeasts *Saccharomyces cerevisiae* – ZIM 321 and *Schwanniomyces occidentalis* – ZIM 763

ity in the buffer (M_B). Since the MES buffer environment was shown to be stressful for exposed yeast cells, their reduced viability was taken into account. The mortality reached a value ranging from 16.37 to 67.81 %, which was not expected at the beginning. Further studies of the chromium effect on yeast cells in the MES buffer proved chromium(VI) to be toxic. We expressed that as cell mortality in the buffer supplemented with the effector (M_{EB}). To eliminate the effects of the MES buffer in correlation to the chromium effector we developed Eqs. 7 and 8 to define specific cell mortality (M_S) or specific cell viability (V_S) since these two parameters comprehend solely the Cr(VI) influence on yeast cells. The results of the model evaluation show that the impact of the environment (buffer) cannot be neglected and all the steps should proceed as planned.

The results (Tables 1–4) show parameters for *Saccharomyces cerevisiae* – ZIM 321, a yeast strain which is not sensitive to chromium(VI), and *Schwanniomyces occidentalis* – ZIM 763 which belongs to yeasts that can barely tolerate Cr(VI) in its surroundings. The model has the capacity of recognizing yeast cell status in each experimental step and also in the course of time. We considered this as essential for the purposes of evaluating yeast viability *vs.* mortality in the selected environment. The application of the model through plotting specific cell viability (V_S) *vs.* specific cell mortality (M_S) provided the critical point of Cr(VI) concentration which a particular yeast can tolerate (Fig. 1). This was found particularly useful for screening procedures for randomly or systematically selected microorganisms. The critical point is defined at the intersection of viability and mortality curves. It represents the concentration of an effector where mortality reaches viability. After this crossing point mortality overtakes viability in the particular yeast strain.

Conclusions

Yeast biomass behaviour in the presence of a specific effector was followed in each step of the process and a mathematical model comprising a set of equations was developed. The model was built with parameters and a two-stream approach was developed which includes four parameters that indicate viability like population condition (*PC*), cell viability in the buffer (V_B), cell viability in the buffer supplemented with the effector (V_{EB}) and specific cell viability (V_S), and three parameters that indicate mortality as follows: cell mortality in the buffer (M_B), cell mortality in the buffer supplemented with the effector (M_{EB}) and specific cell mortality (M_S). The proposed model gave the critical point of an effector concentration with the aim to make the model useful for time-consuming screening procedures among microorganisms exposed to Cr(VI) compounds. Two of the parameters profiles show the behaviour of Cr(VI) sensitive organisms (*i.e. Schwanniomyces occidentalis*) and Cr(VI) tolerant organisms (*i.e. Saccharomyces cerevisiae*).

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Mjerenje preživljavanja/smrtnosti kvasaca u prisutnosti kroma (VI)

Sažetak

Namjera je bila razraditi matematički model – niz jednadžbi o utjecaju kroma na preživljavanje/smrtnost kvasaca. Model je izrađen s parametrima, a razrađen je dvostruki pristup koji obuhvaća četiri parametra što označuju preživljavanje, kao što je odnos živih stanica prema ukupnom broju stanica (PC), preživljavanje stanica u puferu (V_B), preživljavanje u puferu kojem je dodan efektor (krom) (V_{EB}), specifično preživljavanje stanica (V_S) i tri parametra što označuju smrtnost: smrtnost stanica u puferu (M_B), smrtnost u puferu kojem je dodan efektor (krom) (M_{EB}) i specifična smrtnost stanica (M_S). Parametri su računani na temelju osnovnih rezultata [ukupni broj stanica u mL (N_T) i broja živih stanica u mL (N_{CFU})]. Ukupni je broj stanica određen mikroskopski s hematocitometrom, a preživljavanje kvasaca određeno postupkom brojenja kolonija.