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ORIGINAL RESEARCH PAPER

NEW FORESIGHT METHODOLOGY FOR TOXICITY ASSESSMENT

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Abstract: We present here an original express method of quantitative assessment based on carbon dioxide intensity production by yeast *Saccharomyces cerevisiae*. Yeast suspension is sucked inside medical syringe, hermetically sealed from one side. During yeast fermentation carbon dioxide intensively releases and the syringe pistol starts to raise. Probes, containing toxic substances show less fermentation efficiency and the height of pistol rising is less compared to the control probe, giving opportunity to assume toxic effects of different substances, foodstuff and medicines Method is independent from highly skilled personnel and hi-tech expensive equipment. By means of a simple technique it allows receiving a comparative assessment of beverages toxicity, such as beer, vodka, energy drinks, etc. and can give information on toxicity and chemical pollution of water, food, and soil. Test for toxic effect of medicines can also become a scope of this approach. According to this concept, it is necessary to replace laboratory animals by other models. Thus, the offered technique is a tool for detection of environment potential danger to humans without experimental testing on animal models. Now days there is worldwide growing tendency: people ignore cosmetic products that were tested on animals. Our method for some extend is able to replace animal models for toxicity assessment.

Keywords: *carbon dioxide, yeast, fermentation, physiological activity, Saccharomyces cerevisiae, toxicity assessment models*

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INTRODUCTION

It is difficult to overestimate toxicity assessment of potentially dangerous substances because this is directly connected with the problem of food, beverages and water safety. The subject of this research was the creation of the adequate model allowing correctly, precisely and quickly estimate potential danger of substances to human organism.

The cornerstone of such models is the possibility of detection of any changes in yeast fermentation intensity. Despite a big arsenal of toxicity assessment methods, modern approaches have a number of restrictions:

1. simple methods are not always informative;
2. highly organized biological objects are expensive;
3. hi-tech equipment demands the presence of qualified technicians.

Creation of experimental models in toxicology is defined by physiology and metabolism. We tried to solve this problem, offering a new express method of toxicity assessment based on the speed of carbohydrates fermentation by yeast *Saccharomyces cerevisiae.*

Some intermediates of Krebs cycle are present in all organisms from bacteria to multicellular eukaryotes [1, 2]. Apparently, early emergency of effective metabolic pathways during evolution defined similarity in living organisms' metabolism [3]. Thus, universal character of biochemistry testifies the unity of all living beings.

The most popular and informative is Ames's test based on prototroph clones of histidine mutants of bacteria *Salmonella typhimurium* growth on selective media under toxic conditions.

Yeast became successful model for biochemical researches, and classical experiments which were carried out on yeast laid the foundation of new science - biochemistry. Louis Pasteur revealed yeast role in alcohol and carbon dioxide formation and utilization of sugars. Eduard Buchner found out that yeast extracts could turn sugars into alcohol. The subsequent investigations in this direction made it possible to Krebs cycle and other metabolic pathways detalization.

The role of yeast in biochemical research, data on their metabolism and universality of metabolic pathways of all living beings gave a chance of using simple eukaryote as convenient model for studying the influence of toxic substances on living organisms. Certainly, various substances differently affect representatives of the living world, because of their individual sensitivity and norms of reactions. Therefore, knowledge of metabolic pathways and mechanisms of toxic substances action on the studied organisms, are important in such research. Experiments on mammals seem obvious in toxicity assessment. However, such experiments could take a lot of time and are very expensive. Detection of statistic results often requires participation of large number of animals. Their norms of reactions vary in wide range, significantly complicating results. Approach of using mammal's cells or bull spermatozoids seems very perspective. Tests on plants, for example, oats and barley speeds germination or *Allium cepa* onions roots growth are also widely used. The fruit fly *Drosophila melanogaster* is well genetically studied object allowing investigations to investigate integrity and quantity of chromosomes, chromosomal reorganizations and frequency of mutations.

Eukaryotic microorganisms, especially *S. cerevisiae* yeast is known as convenient test object for identification of toxic and mutagen substances [4, 5].

The main aim of this work was the creation of an express method of toxicity assessment, using *S. cerevisiae* yeast as test object.

MATERIALS AND METHODS

Strains

The yeast *S. cerevisiae* strain Y-3194 was a test object [6].

Nutrient media

YEPD medium for cultivation of microorganisms was used. Components for cultivation of microorganisms were from Difco. The YEPD medium contains 10 $g \cdot L^{-1}$ of yeast extract, 20 $g \cdot L^{-1}$ of peptone, 20 $g \cdot L^{-1}$ of glucose, 20 $g \cdot L^{-1}$ of agar.

The test for toxic or stimulating action of different substances

We developed a method of yeast activity by their ability of CO_2 formation intensity assessment [7]. We mixed $2.0 \cdot 10^{-4}$ L of the studied substance (lead nitrate at different concentrations: 0, 10, 1, 0.1, 0.05 $g \cdot L^{-1}$; 10 % V/V solution of ethanol, acetone, formalin and chloroform), $2.5 \cdot 10^{-4}$ L of the 8-fold YPD medium, $5.0 \cdot 10^{-4}$ L of glucose solution (400 $g \cdot L^{-1}$ concentration), $5.0 \cdot 10^{-5}$ L of water and $1 \cdot 10^{-3}$ L of yeast suspension $2.0 \cdot 10^{11}$ cells$\cdot L^{-1}$. Distilled water was a positive control, and $2.0 \cdot 10^{-4}$ L of ethyl alcohol (960 $g \cdot L^{-1}$ concentration) was added to negative probe. The suspension was sucked inside a medical syringe (volume 10^{-2} L). In case of air bubbles formation, they were forced out by means of the piston, leaving $2.0 \cdot 10^{-3}$ L of the studied suspension in a syringe. The lower end of a syringe was soldered hermetically. Plastic end of a syringe was melted by flame heating during 10 secs and then lamped by metal tweezers. We placed syringes in thermostat in horizontal for 1 – 4 h at 30 °C. Then we measured piston raising height in each probe. Substance effect on yeast activity was calculated as observed at equation (1) [7]:

$$A = \frac{V_{\exp}}{V_{contr}} \cdot 100\% \qquad (1)$$

where A – physiological activity of yeast;

$V_{\exp}$ – CO_2 volume produced after addition of the studied substance, 10^{-3} L;

V_{contr} – CO_2 volume produced without addition of any substance, 10^{-3} L.

If A < 100 %, the studied substance is toxic;

If A > 100 %, the substance positively influences on physiological activity of yeast.

Determination of oxidation activity by iodonitrotetrazolium chloride (INT)

We centrifuged yeast for 5 min at 3000 rpm, merged supernatant and resuspended in physiological solution to final concentration 10^{10} cells$\cdot L^{-1}$. We mixed $8.0 \cdot 10^{-5}$ L 0.4 % of INT solution (Sigma) and $2.0 \cdot 10^{-4}$ L of yeast suspension in a sterile test tube and incubated at 30 °C for 30 min. In the presence of INT active cells form red granules which one can see at microscope in light field (Axioskop 40 of Zeiss firm, x100 lens, using not fluorescent oil (Zeiss)). Red, insoluble granules of iodonitrotetrazolium formazan, formed as a result of cellular enzymes were observed at microscope. We

calculated cells with high, reduced, and lack of formazan granules. The quantity of granules testifies the activity of oxidizing processes in yeast cell. A large number of such granules give red color to yeast suspension.

Statistical processing of results

Results from at least 3 independent experiments were statistically processed by Excel 2013.

RESULTS AND DISCUSSION

Method adaptation

Yeast activity was measured on CO_2 produced during carbohydrates fermentations in many laboratories. CO_2 formation can be measured by fermenter weighting or by pressure increase in fermentation tank. Earlier we created a method for an express assessment of brewing yeast activity based on intensity of CO_2 production [7]. Here we used similar approach for toxicity assessment. Since we faced a problem of creation of a new express toxicity assessment method to analyze different substances, water samples, drugs, beverages and foodstuff and quickly warn about their potential danger or safety. The more concentrated yeast suspension is used in the experiment, the more CO_2 is produced and the speed and height of the syringe piston raising increase.

Apparently 10 times more concentrated yeast cause 5 times higher the piston raise in identical conditions and during the same time (30 °C, 120 min) (Figure 1).

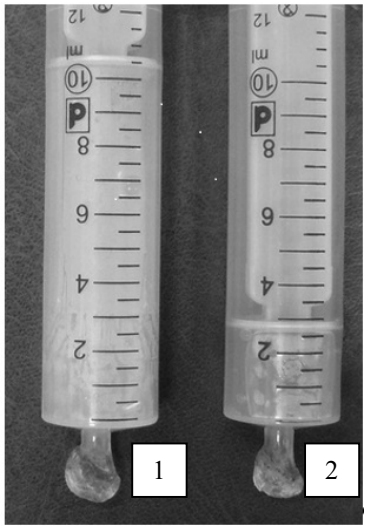
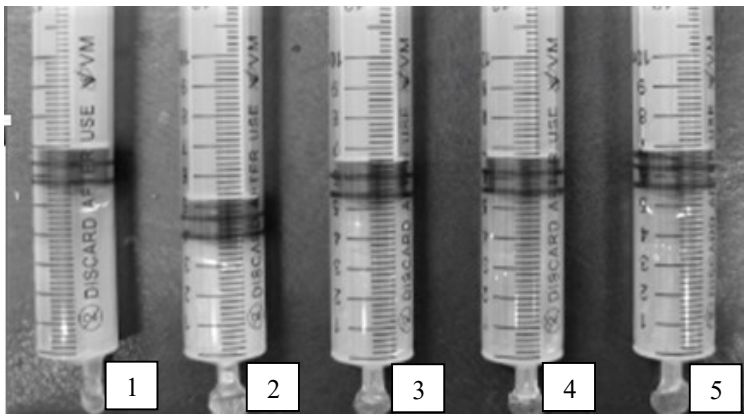


Figure 1. *Height of piston raising depends on yeast concentration used in experiment (1 and 2 – yeast concentration $2.0 \cdot 10^{11}$ cells·L^{-1} and $2.0 \cdot 10^{10}$ cells·L^{-1}, respectively)*

We used obviously toxic substance such as lead nitrate $Pb(NO_3)_2$ for the new method adaptation. Nuclear and absorbing spectroscopies are the most popular analytical methods for lead and other heavy metals detection in biological environments. Despite high sensitivity, accuracy, selectivity, reproducibility, these methods demand expensive equipment and highly skilled technicians.

In our experiments we used 2 yeast metabolism indicators for toxicity assessment: CO_2 production and oxidoreductase activity (INT color reaction). Results of CO_2 production and activity of oxidoreductase at various lead nitrate concentrations are shown on Figure 2.

a



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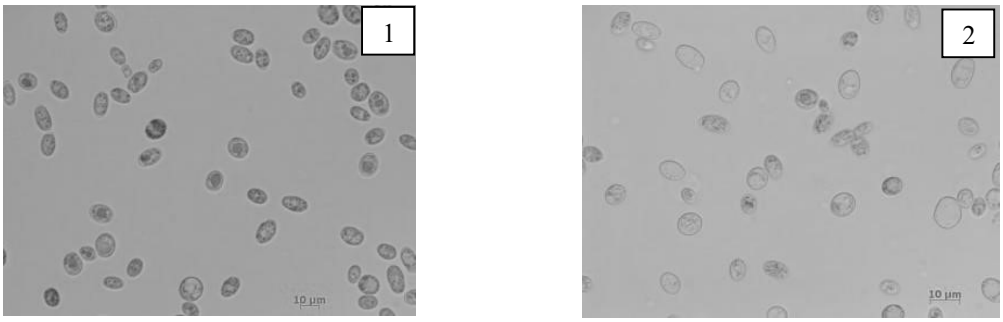

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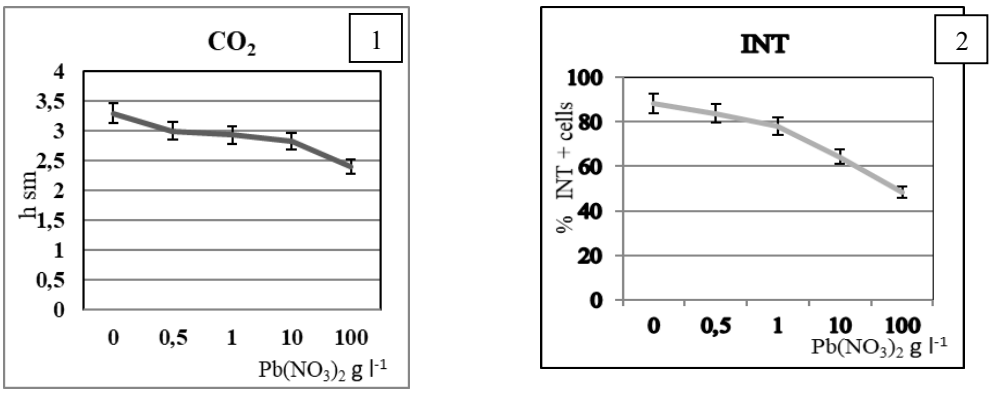


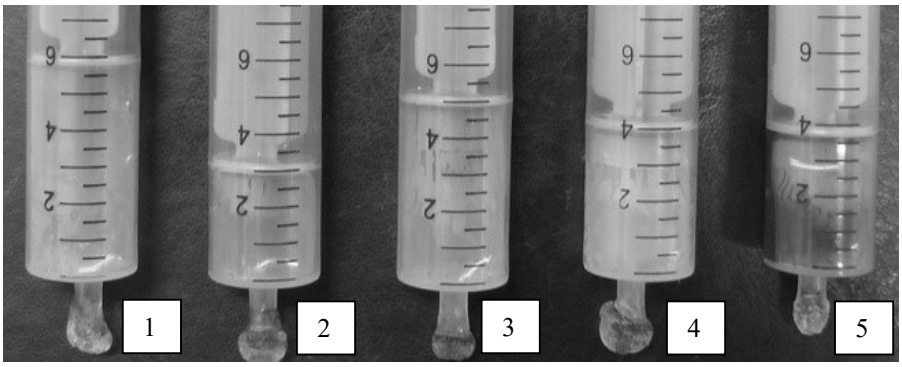
Figure 2. *Influence of various concentration of lead nitrate on intensity of CO_2 production and oxidoreductase inhibition (INT staining): a - intensity of CO_2 production at different $Pb(NO_3)_2$ concentrations: 0, 10, 1, 0.1, 0.05 g·L^{-1}, 1–5, respectively; b - inhibition of oxidoreductase (0 and 100 g·L^{-1} $Pb(NO_3)_2$), reduction of quantity of formazan granules yeast cells 0 and 100 g·L^{-1} $Pb(NO_3)_2$, photo 1 and 2, respectively (x 1000); c – correlation between CO_2 and INT production at various $Pb(NO_3)_2$ concentrations, graph 1-2, respectively*

Oxidoreductase inhibition method correlates with toxic lead salts concentrations (Figure 2b). We found liner reduction of fermentative and oxidoreductase activity by $Pb(NO_3)_2$ concentrations increase (Figure 2c).

Toxicity differentiation

In further experiments, we used our method for the analysis of toxic substances with various cell toxic mechanisms. It is difficult to overestimate relevance of toxicity of different substances comparison. We compared low-toxic (NaCl) and highly toxic $Pb(NO_3)_2$ by our method (Figure 3).

a



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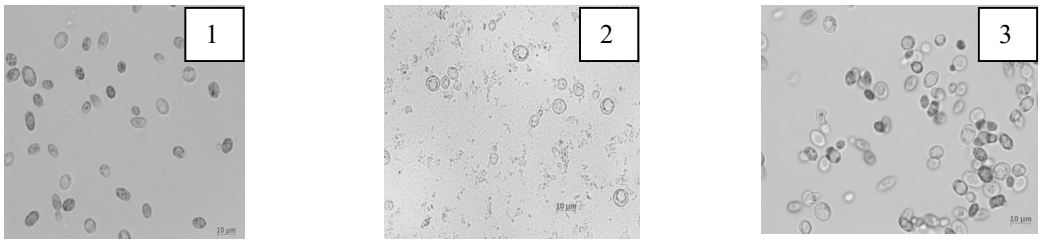

Figure 3. *Influence of various substances on intensity of CO_2 production and oxidoreductase inhibition (INT staining): a - intensity of CO_2 production after addition of water (positive control), ethanol 40 % V/V, NaCl 1 % m/V, $Pb(NO_3)_2$ 1 % m/V, EtBr 0.01 % m/V 1–5, respectively; b – formazan granules reduction water (positive control), $Pb(NO_3)_2$ 1 % m/V, NaCl 1 % m/V, photo 1, 2 and 3, respectively (x 1000, INT staining)*

It is visible that the low-toxic substance NaCl inhibits formation of CO_2 and oxidoreductase less, than highly toxic $Pb(NO_3)_2$ at the same concentration (Figure 3). Addition of $Pb(NO_3)_2$ 1 % m/V leads to dramatic consequences for yeast cells (Figure 3b, 2). It is visible that yeast cells enlarged and busted, and their contents left out from the cells and formed rather large colloidal particles. It is known that lead ions induce formation of colloids in mammals' blood that has similarity to a picture we demonstrated with yeast cells after $Pb(NO_3)_2$ action at high concentration. Besides, 10 $g \cdot L^{-1}$ of $Pb(NO_3)_2$ almost completely suppress yeast oxidoreductase, thus the cells are not able to form INT stained formazan granules. Addition of low-toxic NaCl 1 % m/V % leads to less fermentation and oxidoreductase inhibition (Figure 3b, 3).

The new method can be also used for organic substances toxicity assessment. In the same experiment we compared toxic action of 10 % V/V ethanol, acetone, formalin and chloroform (Figure 4, 1 - 5).

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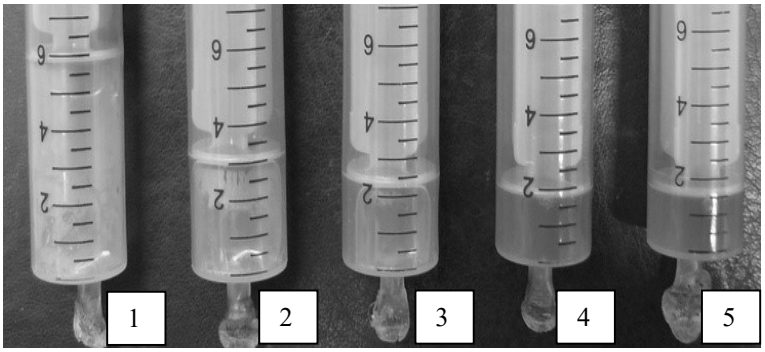

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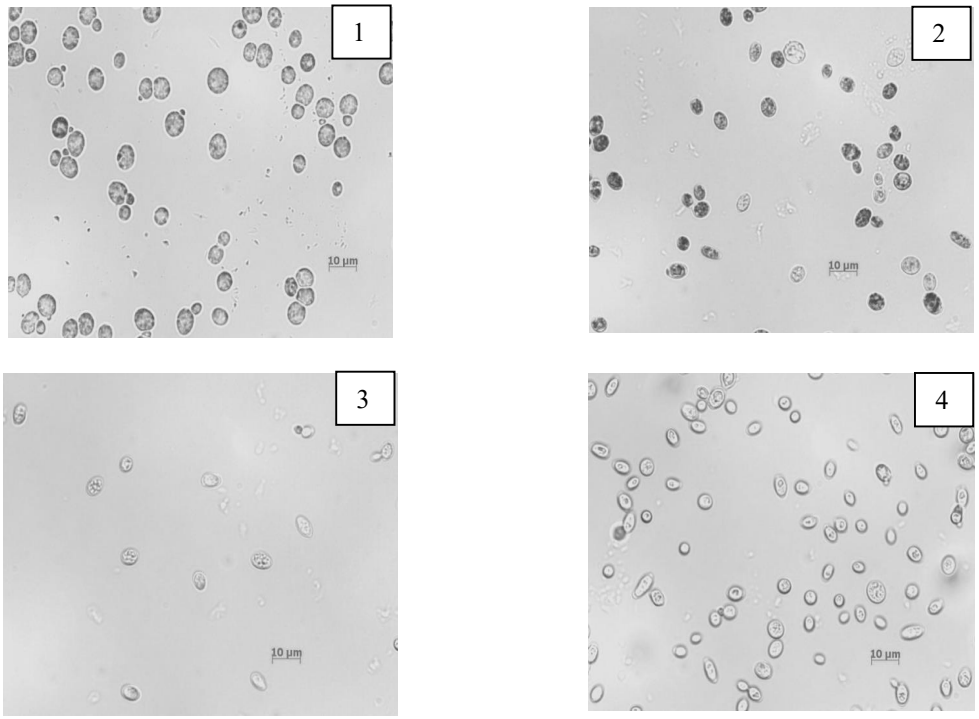


Figure 4. *Influence of various substances on intensity CO_2 production and oxidoreductase inhibition (INT staining): a - Intensity of CO_2 production after addition of water (positive control), ethanol 10 % V/V, acetone 10 % V/V, formalin 10 % V/V chloroform 10 % V/V 1–5, respectively; b - inhibition of oxidoreductase (INT staining) water (positive control), ethanol 10 % V/V, formalin 10 % V/V chloroform 10 % V/V 1–4, respectively*

It is visible that formalin and chloroform are more toxic, than ethanol and acetone (Figure 4) that correlates with known data on toxicity of these substances and with data on classification of substances danger. According German Water Hazard Class (WGK) acetone belongs to the 3rd hazard class, and formalin to more dangerous class 2. Acetone is a natural metabolite and is formed at decarboxylation of corresponding acid. Its concentration increases in blood at glucose deficiency conditions.

Formalin and chloroform almost completely block oxidoreductase unlike ethanol (Figure 4). Yeast cells don't contain granules of a formazan in the presence of those

substances. After ethanol addition they are capable to form formazan but their morphology is obviously changed because of dehydration caused by ethanol.

Reproducibility and sensitivity

We compared reproducibility and sensitivity of our method with well known and widely used methylene blue staining. Same probes were analyzed by both methods (Table 2; Figure 5). We incubated $2.0 \cdot 10^{-3}$ L of yeast suspension $5.0 \cdot 10^{10}$ cells$\cdot L^{-1}$, with a toxic substance in a syringe with hermetically sealed end for 4 hours at 30 °C. We measured the volume of produced CO_2 by estimating the high of syringe pistol. Then we cut off the sealed end of the syringe, transferred the yeast suspension into Eppendorf tubes and stained $5.0 \cdot 10^{-5}$ L of it by methylene blue (MB) to count dead cells in the suspension.

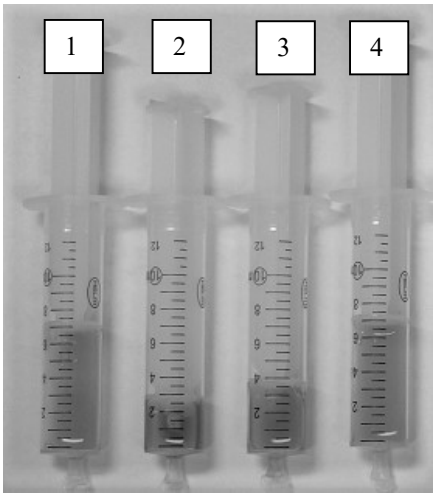


Figure 5. *Influence of various $Pb(NO_3)_2$ concentrations on intensity of CO_2 production in probes with 0, 1, 0.5, 0.05 g$\cdot L^{-1}$ 1–4, respectively*

The reproducibility of these 2 methods is demonstrated at Table 2 and Figure 5.

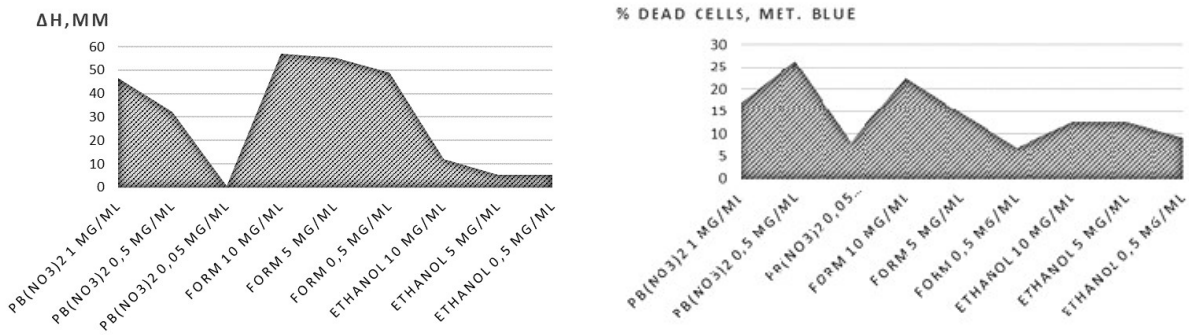
Table 1. *Toxic assessment by CO_2 and MB 2 methods*

Toxic substance	Method CO_2, $\Delta h \cdot 10^{-2}$ [m]	Method met. blue MB [% dead cells]
$Pb(NO_3)_2$ 1.0 [g$\cdot L^{-1}$]	4.67±0.27	16.8±5.12
$Pb(NO_3)_2$ 0.5 [g$\cdot L^{-1}$]	3.23±0.37	26.4±9.3
$Pb(NO_3)_2$ 0.05 [g$\cdot L^{-1}$]	0.067±0.033	8.2±0.9
formalin 10.0 [g$\cdot L^{-1}$]	5.7±0.3	22.5±10.58
formalin 5.0 [g$\cdot L^{-1}$]	4.9±0.2	14.67±7.36
formalin 0.5 [g$\cdot L^{-1}$]	5.53±0.77	7.07±2.76
ethanol 10.0 [g$\cdot L^{-1}$]	1.2±0.52	12.6±2.9
ethanol 5.0 [g$\cdot L^{-1}$]	0.53±0.23	12.7±2.1
ethanol 0.5 [g$\cdot L^{-1}$]	0.4±0.15	9.13±2.43

Reproducibility is higher at higher toxic concentrations. It is reliable for biological methods.

While comparing sensitivity of new method (Figure 6a) and methylene blue staining (Figure 6b) it is easy to see that new method is quite sensitive to $Pb(NO_3)_2$ and formalin. It is possible to detect up to 0.05 g$\cdot L^{-1}$ (0.005 % m/V) of Pb $(NO_3)_2$ and 0.5 g$\cdot L^{-1}$

(0.05 % V/V) of ethanol in probe. So the physiological sensitivity of the method is rather high.
Methylene blue staining is less sensitive for all three methods.



a **b**

Figure 6. *Method sensitivity: a - new method; b - methylene blue staining*

The comparacence of different substances in one experiment, using the same yeast biomass in the same conditions is the advantage of the new method. The possibility to compare the experimental and control probe in each experiment is the key to use different strains and yeast generations because we have the reference – positive control to compare the toxic effect of experimental substance on yeast CO_2 production. Positive effect of experimental substances (glucose or other fermentation stimulators) can be also demonstrated by this method.
The main result of this work is the creation of new express method of toxicity assessment which is based on intensity of carbon dioxide production yeast *S. cerevisiae*.
Various methods of biotesting are used as solutions of such important tasks as toxicological assessment of industrial and city sewage, carrying out environmental assessment of new technological processes, materials, treatment facilities etc. [8 – 10].
Yeast *S. cerevisiae* is one of the most convenient and adequate test objects. Unlike many microorganisms applied in toxicity tests they are not pathogenic. Being the lowest eukaryote, yeast has similar metabolic pathways, mechanisms of membrane potential maintenance, nuclei and other cellular organelles. In spite of the fact that yeast – mono celled microorganisms, they possess mechanisms of cellular death, characteristic the highest eukaryote. All these circumstances testify to possibility of use of yeast for toxicity biotesting [11, 12].
Thus, yeast appeared a successful model and for studying cellular death process. The similarity of highest and the lowest eukaryote cellular mechanisms give the adequate foundation for using yeast as test object for toxicity studying.
Methods of toxicity detection have to be not only sensitive, revealing toxicity of wide range of potentially toxic substances, but also fast and cheap and be able to provide assessment of huge number of chemical compounds before they are widely used in industry, agriculture, food, cosmetics and medicine. It is obvious that tests on laboratory animals don't meet the requirements of speed, low cost and ensuring reliability of results and also demand participation of a large number of animals.

CONCLUSIONS

The problem of toxicity assessment is complicated because of test-object metabolism specific characteristics. However, we managed to solve the problem, using low eukaryotic microorganisms, yeast *S. cerevisiae* as a model, possessing similarity of metabolic pathways caused by the evolution mechanisms of adaptation and cell death.
New method of toxicity detection was successful for various toxic substances. The accurate effect of inhibition of CO_2 production by yeast was detected in the presence of not only lead salts, but also such substances as acetone, chloroform and formaldehyde.
The new method gives the chance to receive fast result and to increase productivity of toxicity assessment laboratories and centers, as it reduces time of analysis from several months to 1 - 4 hours.
Simplicity of method execution doesn't demand difficult and expensive equipment and reagents and makes it possible to detect toxicity in field conditions. The offered technique is simple, effective, reproduced, doesn't demand high cost equipment and has practical interests in the field of toxicity effects of water, chemicals, pesticides, herbicides, fertilizers detection. The method can be also used for toxicity assessment of beverages, food and other products, taking in account carbohydrate content.

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