Journal of Microbiological Methods 161 (2019) 43–46

Contents lists available at ScienceDirect

Journal of Microbiological Methods

journal homepage: www.elsevier.com/locate/jmicmeth

Note

YTOX: a rapid toxicity test based on the dehydrogenase activity of *Saccharomyces cerevisiae* for detection of contaminants in water samples

Luiz Humberto Gomes[a,*], Keila Maria Roncato Duarte[b], Marcos Yassuo Kamogawa[a], Jessica Aparecida Ferrarezi[a], Felipe Gabriel Andrino[a], Ana Claudia LoBuono Tavares[a], Ana Paula Maria da Silva[a], Sergio Escheverrigaray Laguna[c], Simone Possedente de Lira[a]

[a] Escola Superior de Agricultura "Luiz de Queiroz" (ESALQ), Universidade de São Paulo, P.O. Box 9, 13418-900 Piracicaba, SP, Brazil
[b] Unidade de Pesquisa e Desenvolvimento de Tietê - Polo Regional Centro Sul APTA/SAA, Rod 127, km 69, Tietê-SP, Brazil
[c] Universidade de Caxias do Sul- Rua Francisco Getulio Vargas, 1130, Caxias do Sul-, RS, Brazil

ARTICLE INFO

Keywords:
Cadmium
Formazan
Baker's yeast
Gasoline
Fipronil
Methanol
TTC

ABSTRACT

A simple generic toxicity method (test) is proposed using baker's yeast to mediate the reduction of the colourless triphenyltetrazolium chloride (TTC) to red, 1,3,5-triphenyl formazan, which can be extracted by dimethyl sulfoxide (DMSO), enabling the identification of reducible toxic compounds (*e.g.* cadmium, fipronil) in water for consumption.

The rapid detection of toxic reducible compounds and the treatment of contaminants is essential to ensure sanitation, safety and the local population's health (Seth et al., 2016). According to the World Health Organisation (WHO, 2011), only 30% of the world's population has access to good quality drinking water. Therefore, a method that enables the rapid and simple identification of toxic contaminants present in water is both desirable and necessary. Toxicity testing is usually based on exposing a small-sized test organism, (*e.g.* a rodent, worm or microorganism, such as *Daphnia spp., Vibrio fischeri*, *etc.*) to the contaminant of concern and measuring some aspect of its physiological response. Even in natural-source water, compounds such as heavy metals, agrochemicals, antibiotics and petroleum derivatives can be detected and may be toxic even at low concentrations.

The main disadvantage of *Daphnia magna* is the requirement for isogenic or genetically similar organisms in order to guarantee the same physiological conditions and response throughout the test (Rumlova and Dolezalova, 2012). The use of luminescence from the bacteria *V. fischeri* (EN-ISO 11348) avoids this problem, but the technique is costly, as the bacteria have a short lifespan and the test must be performed in saline medium (simulating seawater) specified by the manufacturer. In this context, yeasts such as *Saccharomyces cerevisiae* may offer a more promising model organism as it is a unicellular non-pathogenic eukaryotic microorganism (Ludwig et al., 2009) with very well-defined genetic and physiological properties; it is also relatively easy to obtain, maintain and multiply. Moreover, *S. cerevisiae* is largely used as a model organism for evaluation of cyto- and genotoxicity, as the results obtained with this organism can be extrapolated to humans (Lichtenberg-Fraté et al., 2003; Knight et al., 2004; Välimaa et al., 2008). Several bioassay methodologies have been proposed to evaluate yeast cell metabolic activity and viability, such as growth inhibition tests (Schmitt et al., 2004), assessment of the adenosine-5-triphosphate (ATP) production rate (Estève et al., 2009; Burdock et al., 2011), the determination of acute lethal doses (LD) (Rumlova and Dolezalova, 2012) and minimal inhibitory concentration (MIC) (Oliva-Neto and Yokoya, 2001), as well as colorimetric bioassays to assess cell viability using malachite green and triphenyl tetrazolium chloride (TTC) (Bitton et al., 1984; Hrenovic et al., 2005). Triphenyl tetrazolium chloride acts as a redox indicator and is able to distinguish metabolic activity levels in cells. Several yeast cell dehydrogenases, important for the oxidation of organic compounds in cellular respiration, catalyse the reduction of colourless TTC to red-coloured 1,3,5-triphenyl formazan (Burdock et al., 2011).

As a contaminant of water, cadmium (even at low doses) is extremely toxic, leading to protein denaturation and consequent cell oxidative stress, causing damage to membranes, decreased enzyme activity and a cascade of other metabolic changes (Kurdziel and Prasad,

* Corresponding author.
E-mail address: luhgomes1@usp.br (L.H. Gomes).

https://doi.org/10.1016/j.mimet.2019.04.008
Received 6 February 2019; Received in revised form 10 April 2019; Accepted 11 April 2019
Available online 15 April 2019

2004). Yeasts, however, exhibit tolerance mechanisms in the presence of cadmium. The best known of these involves protein- and peptide-mediated chelation including glutathiones (GSH) and metallothioneins (Ecker et al., 1986). In yeast, GSH forms complexes with cadmium *via* S—H bond formation. Alternatively, metallothioneins can also render the metal biologically unavailable. Once the cadmium has been rendered biologically unavailable by either method, it is stored in the yeast vacuoles, thereby inhibiting its toxic activity and potential effects on yeast metabolism. These mechanisms occur mainly during the lag phase, or when adjusting to the environment. In this phase, the presence of any stressor agent tends to enable yeasts to achieve the lag phase quickly by the activation of the detoxification mechanisms, thereby avoiding major damage to cells. Once the stressor agent is degraded or stored in a biologically non-toxic form, yeast returns to its normal growth cycle (Simonicova et al., 2015).

The YTOX method proposed in this paper is based upon the bioconversion of TTC into triphenyl formazan by yeast dehydrogenase activity, as described by Hrenovic et al. (2005): the more triphenyl formazan produced, the greater the metabolic activity of the cell. The rapid reaction of *S. cerevisiae* to toxic compounds resulting in the inhibition of metabolism confers on yeast the characteristic of an excellent toxicity bioindicator. Since yeasts are eukaryotic, they are more likely to be representative of human cellular responses to contamination than prokaryotic bioindicator species such as *V. fischeri* (Rumlova and Dolezalova, 2012). This characteristic response of yeast cells to toxicity stress can therefore be employed to produce a fast, simple method to identify the presence of toxic agents in aqueous environments. In order to propose a new methodology, the aim of this study was to develop a simple, rapid test, using baker's yeast (*S. cerevisiae*) to determine the presence of toxicants in an aqueous medium (substrate).

Six analytical-grade compounds were used over a range of concentrations: ethanol (analytical grade, 99.5%) 1–24% vv^{-1}, methanol (analytical grade, 99.8%) 1–50% vv^{-1}, cadmium (Specsol, standard solution 100 mg L^{-1}) 0.5–12 mg L^{-1}, fipronil (solution 2.5% vv^{-1}) 0–2.5 mg L^{-1}, and nystatin (Sigma N3503) 1–25 mg L^{-1}. For gasoline (commercial product), 100 mL of commercial gasoline was diluted in 900 mL of water, agitated for 12 h and then the aqueous phase was separated from the free oily phase. The aqueous phase was used at doses of 1–50% vv^{-1}.

A suspension of 200 mg of commercial lyophilised baker's yeast (Fleishman, Mauri and Fermix commercial brands) was agitated in 10 mL of solution A (2% wv^{-1}glucose in distilled water) for 20 min at 25 °C (room temperature) to reactivate yeast cells. Then, 1 mL of this suspension was transferred to each of nine glass tubes (10 mm × 100 mm), centrifuged for 1 min at 3000 x g and the supernatants were discarded. Only reactivated yeast cells remained at the bottom of each tube.

Concomitantly, another dilution on base two was performed. Tubes with 4 mL were analysed for toxic compounds (*i.e.* the water sample) which were vortex agitated for 1 min (Tube 1). Then, 2 mL was transferred from this solution to the next tube (Tube 2) in 1:1 serial dilution with solution A until Tube 8 (Ben-David and Davidson, 2014). Tube 9 was the control tube, with only 2 mL of solution A. The solutions were added to the previously prepared yeast tubes, gently vortex agitated and incubated at 30 °C for 2 h.

After the incubation, tubes were centrifuged for 1 min at 3000 x g and the resulting supernatants discarded. Two millilitres of solution B (2% wv^{-1}glucose and 0.5% wv^{-1}TTC in distilled water) was added to each tube. The tubes were manually agitated, incubated at 30 °C for 15 min and centrifuged for 1 min at 3000 x g. The supernatant was discarded and 2 mL of dimethyl sulfoxide (DMSO) was added. The resulting suspension was agitated at room temperature for 5 min and centrifuged for 1 min at 3000 x g. Aliquots of 200 µL from supernatant of each tube were transferred to 96-well polystyrene microplate with a clear bottom. Based on the colorimetric assay, the absorbance of the resulting supernatant was measured at 485 nm by a microplate reader (Tecan) and data were expressed as percentage values against the non-treated cell samples as a control (100%). Measurements were also performed on a UV–vis spectrophotometer at the same wavelength. Thus, yeast cells were grown in the presence of different dilutions of pollutants, then TTC is added to reduce the dehydrogenases from cell respiration, turning a red colour. So, if cells were mostly dead, no red colour formed.

The Microtox® test was also performed according to the manufacturer's specifications, using an 81.9% screening assay over 15 min (Microtox Model 500, Modern Water). Water samples containing toxic compounds were added at the same concentrations described above.

The EC_{50} (half minimum effective concentration) was calculated according to Esteve et al. (2009) using the Probit method, in which the concentration that affects 50% of the population is used to quantify the toxicity of compounds in aqueous environments.

During the development of the YTOX methodology, the extraction of triphenyl formazan that had formed inside the cells was the first challenge. Gabbita and Huang (1984) used 96% EtOH, but the extraction was inefficient under our experimental conditions, extracting only a fraction of the triphenyl formazan from the cells. Burdock et al. (2011) utilised a modified extraction procedure with DMSO. The concentrations of TTC and glucose was based upon methodology described by Ogur et al. (1957).

In Fig. 1, the toxic effects of cadmium can be observed on the yeast cells and were found to be very similar between each of the baker's yeast commercial brands tested: the data were normalised as the percentage of growth inhibition corresponding to the 0% Cd concentration (negative control). The different brands of baker's yeast also behaved in a similar way in the presence of cadmium. The YTOX methodology showed a confidence limit of 95%, indicated by the letter “f” in Fig. 1. The same confidence limits were observed by Hrenovic et al. (2005) using the commercial Microtox® method, used as the gold standard in this study. In the Microtox® method, the luminescence of bacteria is measured by an enzymatic reaction when toxic agents react with oxygen, aldehydes, luciferases and NADPH, reducing the luminescence produced by the bacteria. A disadvantage of this method is the peculiar conditions required to maintain *Vibrio* in addition to the high cost of the method and the equipment. In this way, the YTOX methodology was found to be more suitable for its purpose. The dilutions in the graph represent the assay methodology, in which the sample water to be analysed was serially diluted.

The comparison of the EC_{50} results for the YTOX and Microtox® methods are detailed in Table 1. The YTOX methodology uses a relatively complex eukaryotic microorganism (*S. cerevisiae*). It requires longer contact time (2 h) to produce effective EC_{50} results compared

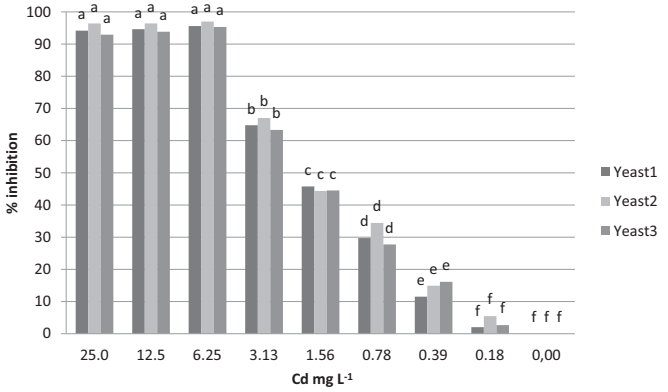

Fig. 1. Evaluation of (cell dehydrogenases) inhibition of three commercial brands of dry active yeast (1- Fleishmann, 2- Mauri and 3- Fermix) in the presence of decreasing doses of cadmium. Use of identical letters indicates that Tukey test ($P < .05$) results show no difference between samples. (Different letters indicate significant differences verified by Tukey test ($p < .05$).)

Table 1
Comparison of EC_{50} results obtained by two methodologies: Microtox® (EC_{50} in 15 min) and YTOX (EC_{50} in 2 h), using a five contaminants (contaminant concentrations expressed in %).

	Microtox	YTOX
	EC_{50} (%)	
Fipronil	0.0000714	0.019
Ethanol	1.62	15.26
Methanol	1.79	25.89
Gasoline	2.64	42.8[a]
Cadmium	0.000121	0.000185

[a] Gasoline (aqueous phase) or else, 10% of total (commercial) gasoline.

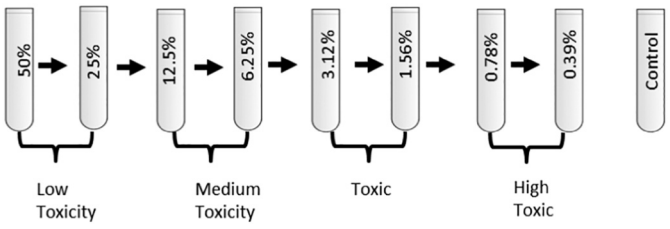

Fig. 2. Design of serial dilutions related to toxicity of the agent in contaminated water samples. The toxicity categories (from "Low" to "High") represent a general classification according to the results obtained by YTOX. Any significant reduction in formazan production by yeast after contact with a toxic agent, compared with the control, is an indication of toxicity. If significant reduction in yeast formazan production is detected after contact with a toxic agent in low concentration (Tube 8, with dilution 0.39%), it indicates that the agent is highly toxic to yeast. On the other hand, if formazan production shows significant reduction only when yeast is exposed to very high concentrations of the analysed agent, it indicates that its toxicity is low.

with the Microtox® method, which uses a simpler prokaryotic marine microorganism (*V. fischeri*) requiring only 15 min to produce the EC_{50} results. The time consuming aspect of the YTOX assay is compensated for by the reliability of the results, since it uses eukaryotic cells (it is based on the metabolic response of eukaryotic cells). Table 1 details the different responses for both tests observed for a variety of toxic agents. For fipronil, the results show a 260.00-fold difference, for ethanol a 9.00-fold difference, for methanol a 14.00-fold difference, for gasoline aqueous phase a 16.21-fold difference and for cadmium a 1.50-fold difference.

Even though the results demonstrate that Microtox® is more sensitive and rapid, a noticeable advantage of YTOX methodology relies on the use of *S. cerevisiae*. Therefore, it is more analogous to human cells in terms of metabolic responses to toxic agents (Ludwig et al., 2009). The cost of the tests are extremely different as the YTOX method can be run 10 times cheaper than Microtox®; the yeast (dried, from commercial brands) does not need a stock system, nor does it depend on a company to supply it, as it can be easily obtained from any local market; moreover, there is no need to order or wait for shipping.

Fig. 2 depicts the YTOX test categorisation of a toxic agent in serial dilutions, from low to high toxicity (from high to low concentration). In this test, any significant reduction in formazan production by the yeast compared with the control is an indication of toxicity.

Yeast has been studied as a toxicity bioindicator by different authors (Rumlova and Dolezalova, 2012; Hrenovic et al., 2005; Bitton et al., 1984), who used the standard analytical approach of observation of dyed yeast cells under a microscope in order to distinguish live from dead cells and to assess cell viability. A significant advantage of the YTOX method is that it results in stained cells by the TTC reaction as a consequence of the procedure. The coloured compound (formazan) can be extracted from the cells using DMSO. This procedure allows for the identification of the metabolic status of yeast cells at the exact moment of contact with stressor substances. The ability to identify cell vitality in this way opens a range of potential commercial uses of YTOX technique; for example, in bakeries, breweries and the alcohol industry. The technique allows for a clear visualisation of the cells status and does not merely distinguish whether cells are alive or dead. These features are additional factors of YTOX representing an excellent methodology for the identification of toxic compounds in water.

The YTOX method can be used to determine the presence of a wide range of water contaminants in a simple procedure, providing linear results at different dilutions (concentrations). Therefore, it is suitable for any laboratory to implement. YTOX is based upon the use of a eukaryotic microorganism, which allows for greater extrapolation of the effects of contaminants to humans and livestock in comparison to tests in which prokaryotic microorganisms are used. An additional advantage is that commercial lyophilised baker's yeast is used for the assay. This allows the method to be replicated without the need for specific yeast strains, even under environmental conditions (avoiding culture contamination issues associated with common lab-only methods), since dried yeast from the local market does not present oscillations in terms of composition or in the strain of *Saccharomyces* used.

Finally, the YTOX method indicates the presence of aqueous contaminants that interfere with the respiratory metabolism of cells. Further studies are necessary in order to validate an appropriate YTOX kit which would be of significant benefit in assuring good quality water for human/livestock consumption to communities worldwide since it is a field-based kit intended for use in poorer, rural communities.

Conflict of interest

The authors declare no conflicts of interest.

Acknowledgements

The authors acknowledge the financial support of FAPESP (Proc. No. 2013/12834-3) for this project. We also Thanks Coordenação de Aperfeiçoamento de Pessoal de Nivel Superior (CAPES) for the Scholarship of Ana C. L. Tavares and Ana P. M. da Silva.

References

Ben-David, A., Davidson, C.E., 2014. Estimation method for serial dilution experiments. J. Microbiol. Methods 107, 214–221.
Bitton, G., Koopman, B., Wang, H., 1984. Baker's yeast assay procedure for testing heavy metal toxicity. Bull. Environ. Contam. Toxicol. 32, 80–84.
Burdock, T.J., Brooks, M.S., Ghaly, E., 2011. A dehydrogenase activity test for monitoring the growth of *Streptomyces venezuelae* in a nutrient rich medium. J. Bioprocess. Biotechn. 1https://doi.org/10.4172/2155–9821.1000101. 101.
Ecker, D.J., Butt, T.R., Sternberg, E.J., Neeper, M.P., Debouck, C., Gorman, J.A., Crooke, S.T., 1986. Yeast metallothionein function in metal detoxification. J. Biol. Chem. 261 (36), 16895–16900.
Estève, K., Poupot, C., Dabert, P., 2009. A *Saccharomyces cerevisiae*-based bioassay for assessing pesticide toxicity. J. Int. Microbiol. Biotechnol. 36, 1529–1534.
Gabbita, K.V., Huang, J.Y.C., 1984. Dehydrogenases activity of activated sludge. Toxical. Environ. Chem. 8, 119–132.
Hrenovic, J., Stilinovic, B., Dvoracek, L., 2005. Use of eukaryotic and prokaryotic biotests to assess toxicity of wastewater from pharmaceutical sources. Acta Chim. Slov. 52, 119–125.
Knight, A.W., Keenan, P.O., Peter, N.J., Fielden, R., Walmsley, R.M., 2004. A yeast-based cytotoxicity and genotoxicity assay for environmental monitoring using novel portable instrumentation. J. Environ. Monit. 6 (1), 71–79.
Kurdziel, B.M., Prasad, M.N.V., 2004. Strazalka K photosynthesis in heavy metal stressed plants. In: Prasad, M.N.V. (Ed.), Heavy Metal Stress in Plants: From Biomolecules to Ecosystems, 2rd ed. Springer, India, pp. 146–181.
Lichtenberg-Fraté, H., Schmitt, M., Gellert, G., Ludwig, J., 2003. A yeast-based method for the detection of cytoand genotoxicity. Toxicol. in Vitro 17 (5–6), 709–716.
Ludwig, J., Schimtt, M., Lichtenberg-Fraté, H., 2009. *Saccharomyces cerevisiae* as biosensor for cyto- and genotoxic activity. In: Kim, Y.J. (Ed.), Atmosphere and Biological Environmental Monitoring, pp. 251–259.
Ogur, M., John St, R., Nagai, S., 1957. Tetrazolium overlay technique for population studies of respiration deficiency in yeast. Science 125, 928–929.
Oliva-Neto, P., Yokoya, F., 2001. Susceptibility of *Saccharomyces cerevisiae* and lactic acid bacteria from the alcohol industry to several antimicrobial compounds. Braz. J.

Microbiol. 32, 10–14.
Rumlova, L., Dolezalova, J.A., 2012. A new biological test utilizing the yeast *Saccharomyces cerevisiae* for the rapid detection of toxic substances in water. Environ. Toxicol. Pharmacol. 33, 459–464.
Schmitt, M., Gellert, G., Ludwig, J., Lichtenberg-Fraté, H., 2004. Phenotypic yeast growth analysis for chronic toxicity testing. Ecotoxicol. Environ. Saf. 59, 142–150.
Seth, A., Klise, K.A., Siirolas, J.D., Haxton, T., Laird, C.D., Asce, A.M., 2016. Testing contamination source identification methods for water distribution networks. J. Water Resour. Plann. Manag. 142 (4), 04016001.
Simonicova, L., Dudekova, H., Ferenc, J., Prochazkova, K., Nebohacova, M., Duzinsky, R., Nosek, J., Tomaska, L., 2015. *Saccharomyces cerevisiae* as a model for the study of extranuclear functions of mammalian telomerase. Curr. Genet. 61, 517–527.
Välimaa, A.L., Virta, M., Karp, M., Kivistö, A., 2008. Real-time monitoring of non-specific toxicity using *Saccharomyces cerevisiae*reporter system. Sensors 8, 6433–6447.
WHO (World Health organization), 2011. Guidelines for Drinking-Water Quality, 4th ed. WHO Press, Malta 564pp.