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Development of a Yeast Inhibitive Assay for Anionic Heavy Metals Using the 1-(4,5-Dimethylthiazol-2-Yl)-3,5-Diphenyltetrazolium Bromide (MTT) Assay

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ABSTRACT

Currently, heavy metals pollution is significantly becoming a global concern as it causes severe toxicity towards human health and the environmental condition. Intensive efforts to develop a highly sensitive, rapid, and simple toxicity assay to assess the toxic effects of heavy metals in aquatic bodies have been done. An emerging tool to solve this matter is by using microorganisms and one of them is yeast. In this study, a rapid, simple, and cost-efficient toxicity assay by using the Baker's yeast (*Saccharomyces cerevisiae*) respiration assay with a tetrazolium dye (MTT) is developed as potential environmental biomonitoring tool. To achieve high sensitivity, optimization using one-factor-at-a-time (OFAT) was first carried out. The best conditions giving optimum response occurred at pH 5.8 and 30 min contact time. Molybdate and chromate exhibited exponential decay type inhibition curves with calculated half maximal inhibitory concentration, IC₅₀ of 1.137 mg/L for molybdate and 1.247 mg/L for chromate. The Limits of detection (LOD) were 0.313 mg/L and 1.247 mg/L for molybdate and chromate, respectively. The newly developed assay can help in monitoring heavy metals pollution in rivers and agricultural areas.

INTRODUCTION

Human activity has resulted in the widespread distribution of heavy metals, which are naturally occurring elements. Various industrial, residential, medicinal, agricultural, and technical processes are the primary sources of these inorganic pollutants. Heavy metals are being deposited in our waterways, soils, and environment due to the rapid growth of the agriculture and metal sectors, poor waste management, fertilisers, and pesticides. Due to their long-term presence in the environment, heavy metals are considered highly hazardous chemical pollutants [1]. Because of their bioaccumulation, nonbiodegradability, and high toxicity to humans and other ecosystems, these pollutants pose a serious threat to human health [2]. Some metal ions have multiple oxidation states, and they can exist as cation or anion. The hazard and toxicity level of many heavy metals are highly influenced by their oxidation state and solubility [3]. Heavy metals and toxic organic compounds have polluted the environment since the industrial revolution and continue to do so today [4]. Heavy metals contamination levels in water must therefore be closely

monitored and their toxicity assessed, as this has become a major environmental concern [1]. Bioassays are required for environmental sample screening due to the high cost of chemical analysis procedures. Traditional ecotoxicity testing can be expensive, time-consuming, and inaccurate, but microorganism-based bioassays can be more cost-effective, time-efficient, and accurate [5]. Many traditional bioassays in effect-directed analysis (EDA) are still prohibitively time-consuming and labour-intensive [6].

Yeasts have been identified as a viable animal-free alternative to other commonly used microorganisms in toxicological bioassays. Yeast-based assays can be used for preliminary screening of environmental samples for high levels of contamination because they can provide a quick preview of the possible toxicity level before further instrumental analysis is carried out. Toxicity tests using *Saccharomyces cerevisiae*-based bioassays, such as testing for inhibition of yeast metabolic activity and growth inhibition assays, have been carried out in particular. It is cheaper, faster, and requires less sample volume

than traditional ecotoxicity tests such as *Daphnia magna* bioassays based on yeast phenotypes. Numerous studies have shown that yeast, *Saccharomyces cerevisiae*, is an effective test organism for heavy metal toxicity assays [7–9]. As a result, more study and research are required to obtain a more accurate and dependable result from the assay.

The amount of heavy metal pollution in Malaysia's environment continues to rise as a result of rapid industrialization, urbanization, agricultural advancement, and other modern-era activities. Because of Malaysia's rapid industrialization, urbanization, agricultural advancement, and other modern-era activities, heavy metal pollution in the environment continues to rise. Water quality is one of the most critical environmental challenges associated to sustainable development, particularly in terms of ensuring national drinking water safety. Adilah *et al.* suggested based on the high levels of heavy metals in the water samples, the researchers concluded that nearby mining activity is the primary cause of pollution in the Class III (water supply requires significant treatment) Jemberau River and Chini River [10]. Nevertheless, another study found that anthropogenic activities such as livestock rearing, and oil palm planting are major contributors of low-level heavy metal contamination in the Linggi River [11]. According to the Environmental Quality Report (2017), the percentage of conformity for chromium was just 62 percent, while arsenic was 54 percent for municipal water supply [12].

MTT assay is initially developed based on the ability of the bacterium *Rhizobium meliloti* to reduce a water-soluble tetrazolium dye, MTT (3-[4,5-Dimethylthiazol-2-yl] 2,5-diphenyl-tetrazolium bromide) that results in a color change from pale yellow to insoluble purple-blue formazan. As toxic compounds inhibit reduction of the dye, lower color intensity indicates less reduction of MTT-formazan and so higher inhibition from a toxic compound. This assay offers a simple, fast, and inexpensive method as it does not require special equipment or training to run, but its sensitivity compares favorably to Microtox™ and Polytox™ microbial assays [13]. A *Bacillus* sp.-based MTT assay was also developed and tested to be sensitive towards toxic response [14].

MATERIALS AND METHODS

Yeast preparation

Yeast preparations were done in sterile condition in the laminar flow. 1 M glucose, distilled water, centrifuge tubes, 500 mL conical flask and pipette tips used were autoclaved at 121 °C for 20 min beforehand. 1.0 g of commercial instant dry Baker's yeast (Mauripan) were weighed and suspended in 25 mL of 1 M glucose (final concentration of 0.05 M) and 475 mL of distilled water in a conical flask. The top of the flask was covered with aluminium foil, and it was incubated in water bath at 30 °C for 10 min. The yeast suspension was centrifuged at 2,500 rpm for 5 min at 25 °C. The supernatant was discarded, and the pellet was weighed to obtain the wet weight. Depending on this weight, the yeast was re-suspended in distilled water, 1:1 (w/v). Yeast stock solution were kept in a styrofoam ice box filled with ice, and it was stored in the chiller for further usage. Yeast suspensions were diluted with 4 times dilution in 50 mM pH 5.8 phosphate buffer before it was used in the assay.

Preparation of Reagents

50 mL pH 7.4 PBS was prepared by the addition of 0.4 g NaCl, 0.01 g KCl, 0.072 g Na₂HPO₄, and 0.012 g KH₂PO₄ in 40 mL distilled water. The buffer solution was stirred using magnetic stirrer until there was no precipitate present. Final pH value of 7.4 was adjusted with the addition of 0.1 M HCl and/or 0.1 M NaOH using a pH meter. Final volume was then made to 50 mL. The buffer was stored in chiller at 4 °C. 4 mg/mL MTT powder (Sigma-Aldrich) was prepared by dissolving 1.0 mg of MTT powder in 25 mL of 10 mM pH 7.4 PBS [15]. The solution was stirred using a magnetic stirrer until there was no precipitate present. The MTT solution was then stored at 4 °C in a 50 mL Falcon tube wrapped with aluminium foil. Preparation was done in dark. Heavy metals which are molybdate (VI) (Na₂MoO₄, Sigma-Aldrich), chromate (VI) (K₂Cr₂O₇, Sigma-Aldrich) and arsenate (V) (Na₂As₃O₄, Sigma-Aldrich) were prepared from analytical grade commercial salts by diluting the metal salts in distilled water. Only molybdate was prepared by diluting the metal salt in distilled water over the heat. 1000 mg/L stock solution were prepared.

MTT assay

The assay mixture contained 100 µL of yeast suspensions, 50 µL of phosphate buffer (pH 5.8, 50 mM) and various volume of tested anionic heavy metals from stock solutions of 1000 mg/L (depends on final concentration in the assay) in 1.5 mL tubes (Eppendorf). Yeast suspension without any toxicant was prepared as a control sample. The mixture was first pre-incubated for 30 min at room temperature (25 °C). The MTT assay was carried out by adding 100 µL of MTT solution into the mixture and final volume was made to 1 mL using phosphate buffer (pH 5.8, 50 mM). The reaction mixture was then incubated in water bath at 30 °C for 20 min before it was centrifuged at 5,000 rpm for 5 min and the supernatant were discarded. 1 mL of 50% DMSO was added to solubilize the formazan. The reaction mixture was vortexed and another incubation in water bath at 37 °C was done for 20 min. The solution was centrifuged again at 5000 rpm for 5 min before color development was measured at 570 nm using UV-Vis spectrophotometer. Distilled water is used to autozero the spectrophotometer. All tests were performed in triplicate.

Determination of IC₅₀ value

Anionic heavy metals that showed significant inhibition towards yeast activity during screening were tested further. The half maximal inhibitory concentration (IC₅₀) was determined using seven different concentrations of anionic heavy metals, which are (1, 5, 10, 25, 50, 75, 100) mg/L.

Data and statistical analysis

The percent inhibition was computed according to the following formula:

$$\% \text{Inhibition} = \frac{\text{Initial control absorbance} - \text{Final sample absorbance}}{\text{Initial control absorbance}} \times 100$$

The values displayed are means and their standard deviations. GraphPad prism version 7.0 was used to analyse the data (Graphpad Software Inc., San Diego, CA). A one-way analysis of variance (ANOVA) with post hoc analysis by Tukey's test or a student's t-test was used to compare groups. P 0.05 was regarded as statistically significant.

Determination of anionic heavy metals that cause 50% inhibition towards yeast activity (IC_{50}) and limit of detection (LOD) values were calculated from regression curve generated using GraphPad prism 7.0 non-linear regression analysis for one-phase exponential decay models software.

RESULTS AND DISCUSSION

Tetrazolium salts can be used to detect dehydrogenase activity or other enzyme systems where redox equivalents are produced. Therefore, MTT assay is beneficial for testing cell proliferation and cell viability and is also used for cytotoxicity tests [16]. Viability can be defined as the ability of cells to grow, reproduce, and interact with the surroundings, or can be understood as the ratio between the number of living cells to dead cells [17]. Formazan is formed from the cleavage of tetrazolium ring by active mitochondria of living cells. Hence the amount of formazan formed is directly proportional to the number of living cells [15]. When cells die, they no longer have the ability to reduce MTT into formazan.

Since MTT is positively charged, they readily penetrate viable eukaryotic cells, thus color formation functions as a convenient marker of only the viable cells [18]. Mitochondrial complex II, succinate dehydrogenase is responsible for the reduction of MTT to formazan. The MTT dye-reduction system is appealing due to its simplicity, economic and relatively rapid [19]. No studies or data have been reported on the application of MTT assay for measuring the inhibition of heavy metals on Baker's yeast activity. Adding the fact that inadequate study was done regarding this, an attempt to develop a yeast inhibitive assay using MTT as indicator should be conducted in the aim of obtaining a highly sensitive toxicity assay.

Effect of pH

pH of the medium plays an important role in the conversion of MTT to formazan by mitochondrial yeast. Yeast needs the optimum pH to grow to produce the maximum response to the MTT assay. Extracellular pH has a vital role in growth and the maintenance of the normal function of yeast cells [20]. Seven different pH in increasing order – 4.0, 4.5, 5.0, 5.75, 5.8, 6.5 and 7.0 were used to study the effect of pH on the absorbance value of formazan produced by yeast cells. The overlapping buffer system used for pH optimization studies were citrate buffer for pH 4.0 to pH 5.75, and phosphate buffer for pH 5.8 to 7.0. The assay was conducted without the presence of toxicants to identify maximum yeast activity that could give the highest absorbance.

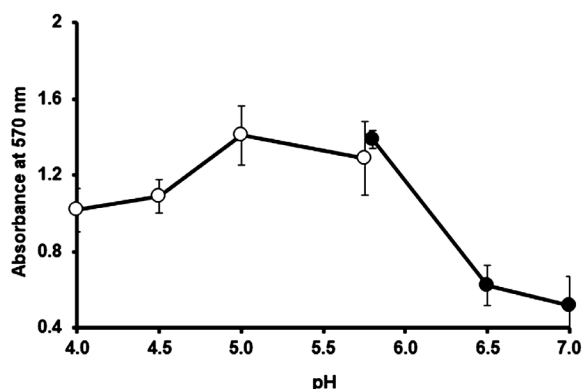


Fig. 1. Effect of different pHs on the absorbance value at 570 nm. ANOVA analysis suggest that the optimum pH was between pH 5.0 and 5.8. Data is mean $\pm$ standard error of the mean (n = 3).

The bell-shaped curve in Fig. 1 indicates that the yeast activity was relatively moderate at pH 4.0 and 4.5, then slowly increased to pH 5.0 in acetate buffer. The activity levelled out at pH 5.0 to pH 5.75 and pH 5.8. However, the activity sharply decreased beyond pH 5.8 to 7.0 in phosphate buffer. ANOVA analysis suggested that the optimum pH was between pH 5.0 and 5.8. The result showed that yeast activity has a narrow pH range as the activity of each mean point only from pH 5.0 to 5.8 showed no significant difference ($p < 0.05$) either in acetate buffer or phosphate buffer. pH 5.8 was selected as optimum pH and deemed to be suitable for measuring the effects of metal ions towards yeast activity to attain the maximum inhibition of metal ions at the experimental conditions. Optimum condition of slightly acidic for *Saccharomyces cerevisiae* has been reported and similar to the results reported by [21].

Effect of contact time

The inhibition of metal ions towards yeast activity was studied using various contact time - 10, 20, 30, 40, 50 and 60 min. The assay was conducted with the same time and concentration of metal ion to identify the optimum time for maximum inhibition of metal ions towards yeast activity. Percent activity of yeast after exposure to metal ions for different number of times were compared to control which indicated 100% activity as there is no inhibition by any toxicant. After incubation of the yeast cells with metal ion for 10, 20, and 30 min, percent activity of yeast decreased, with 30 min showed the lowest yeast activity (Fig. 2).

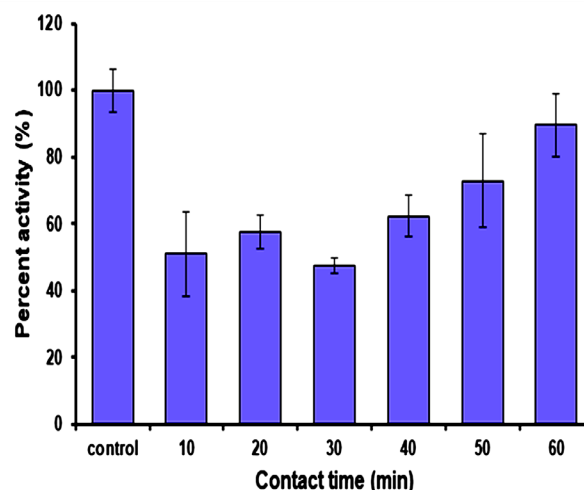


Fig. 2. Effect of different contact time of yeast with anionic heavy metal on the percent activity of yeast. Data is mean $\pm$ standard error of the mean (n = 3).

This was most likely owing to the effect of metal ions on yeast cells, which caused cellular metabolism to halt for a short period of time, resulting in a decrease in mitochondrial function against hazardous species. After 60 min, the % activity of yeast rose, which was due to the mitochondrial stress reaction, which led to an increase in mitochondrial activity in creating reactive oxygen species (ROS) to minimise the influence of metal ions [22]. According to Fig. 2, 30 min corresponded to the optimal time, which means that metal ions inhibit yeast activity at the highest percentage (52.50%) at 30 min. Thus, pre-incubation period of 30 min for exposure of metal ions with yeast cells is sufficient to attain the maximum inhibition of metal ions at the experimental conditions.

Screening of anionic heavy metals

Screening was conducted to study the inhibitory effects of anionic heavy metals on yeast activity. The final concentration of 100 mg/L was used for potassium dichromate, sodium molybdate and sodium arsenate. Inhibitory effects were assessed by measuring the percent activity of yeast after exposure to heavy metals for 30 min, compared to control with 100% activity. Based on the Malaysia Marine Water Quality Criteria and Standards in the Environmental Quality Report (EQR) by Malaysian Department of Environment ([12], maximum value of total suspended solid for Class III (ports, oil & gas fields) and Class E (mangroves estuarine & river-mouth water) is 100 mg/L. Hence, the screening of heavy metals was conducted at maximum concentration range of 100 mg/L to determine whether they significantly inhibit yeast activity or not.

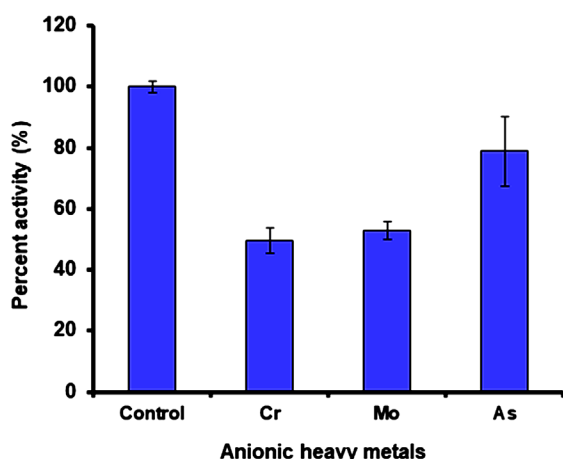


Fig. 3. Percent activity of yeast after inhibition by 100 mg/L of chromate, molybdate and arsenate compared to control. Data is mean $\pm$ standard error of the mean (n = 3).

Out of three metal ions tested, two exhibit inhibition of yeast activity at 100 mg/L (**Fig. 3**). The inhibition shown by the anionic heavy metals on yeast activity is 50.32% for chromate, 47.08% for molybdate, and 21.10% for arsenate. Arsenate at the highest concentration range (100 mg/L) did not cause more than 50% inhibition towards yeast activity. Therefore, it was not tested further and it can be concluded that this assay is not sensitive to arsenate. Chromate at 100 mg/L caused more than 50% inhibition towards yeast activity and hence was further tested using various concentrations. Meanwhile molybdate at 100 mg/L caused inhibition near 50% and was included to be tested further.

Determination of IC_{50} values for anionic heavy metals

Yeast cells were exposed to seven concentration of metal ions in increasing order - 1, 5, 10, 25, 50, 75, and 100 mg/L. The PRISM non-linear regression analysis for one-phase exponential decay model software was used to construct IC_{50} of regression curves for heavy metal concentrations. When utilised to represent inhibitory effects of heavy metals, such as Microtox™, this model can provide relevant comparison values in the form of IC_{50} [5]. In this study, we utilized the one-phase exponential decay model, which gave good fitting to the experimental data, with the coefficient of determination (R^2) values ranging from 0.8468 to 0.9245. Both chromate and molybdate exhibited exponential decay type inhibition curves (**Figs 4 and 5**). The calculated IC_{50} values for anionic heavy metals were observed at 1.137 mg/L and 2.921 mg/L, in the order of $MoO_4^{2-} < Cr_2O_7^{2-}$ respectively.

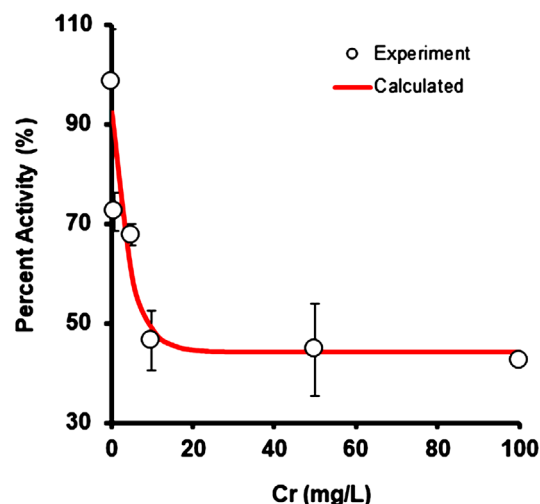


Fig. 4. Percent activity of yeast for different concentration of potassium dichromate. Data is mean $\pm$ standard error of the mean (n = 3). The LOD was 1.247 mg/L, IC_{50} was 2.921 mg/L and R^2 was 0.8468.

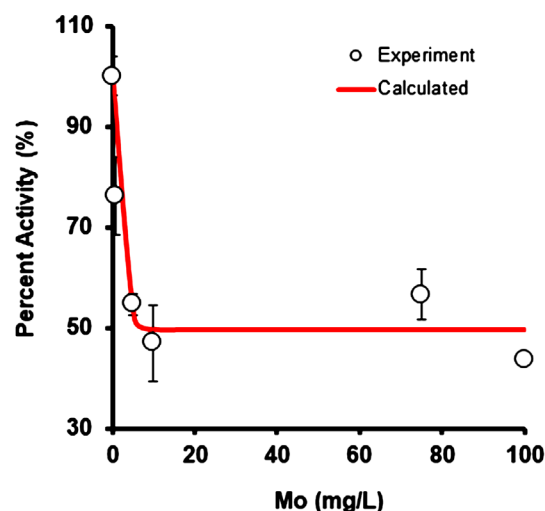


Fig. 5. Percent activity of yeast for different concentration of sodium molybdate. Data is mean $\pm$ standard error of the mean (n = 3). LOD was 0.313 mg/L, IC_{50} was 1.137 mg/L and R^2 was 0.9245.

Sensitivity of yeast inhibitive assay for chromate Comparison to yeast-based assay

The IC_{50} values for 30 min exposure obtained for chromate using the newly developed assay were compared with the results of other toxicity tests that used yeast as test organism. The comparative LC_{50} (lethal concentration that causes 50% toxicity), EC_{50} (effective concentration that causes 50% response) and IC_{50} (concentration that causes 50% inhibition) data from yeast-based assay for chromate in comparison to other bioassay shows that the toxicity value obtained from this study (2.921 mg/L) was highly comparable to a study by [23] and was much lower than [24] (**Table 1**). This means that the developed assay is equivalent in sensitivity to Baker's yeast assay by Gong *et al.* [23] but is far more sensitive than Fai & Grant [24] assay that used yeast strain NCYC 2939 as test organism.

Table 1. Sensitivity of yeast assay to chromate in comparison to IC₅₀, EC₅₀ or LC₅₀ of yeast-based assay.

IC ₅₀ , EC ₅₀ or LC ₅₀ (mg/L)			
	Baker's yeast ^a	Yeast strain NCYC 2939 ^b	Baker's yeast ^c
Chromate	2.5	12.4±1.77	2.921

^a [23] ^b [24] ^c From this study

Comparison to other toxicity assay

The IC₅₀ values for 30 min exposure obtained for chromate using the newly developed assay were compared with the results of acetylcholinesterase *Daphnia magna* and Microtox™ toxicity tests reported in the literature. Comparative IC₅₀, EC₅₀ or LC₅₀ data for chromate in different toxicity tests showed that the IC₅₀ value for chromate in this study is higher than those for acetylcholinesterase and *Daphnia magna*, but much lower than Microtox™ assay (**Table 2**). This means that the developed assay showed lower sensitivity compared to acetylcholinesterase and *Daphnia magna* but has higher sensitivity than Microtox™ assay for chromate.

Table 2. Sensitivity of yeast assay to chromate in comparison to IC₅₀, EC₅₀ or LC₅₀ of acetylcholinesterase, *Daphnia magna* and Microtox™.

IC ₅₀ , EC ₅₀ or LC ₅₀ (mg/L)				
	Acetylcholinesterase ^a	<i>Daphnia magna</i> ^a	Microtox™ ^b	Baker's yeast ^c
Chromate	0.632	0.29	19.35	2.921

^a [25] ^b [26] ^c From this study

Sensitivity of yeast inhibitive assay for molybdate

The IC₅₀ values for 30 min exposure obtained for molybdate using the newly developed assay were compared with the results of acetylcholinesterase and *Daphnia magna* toxicity tests reported in the literature. Comparative IC₅₀, EC₅₀ or LC₅₀ data for molybdate in different toxicity tests showed that the value obtained in this study is much lower compared to both acetylcholinesterase and *Daphnia magna* assay. However, as shown in **Table 3**, the data obtained from this study are in sharp contrast with the results reported by [25] and [27]. Overall, the developed assay has the highest sensitivity for molybdate.

Table 3. Sensitivity of yeast assay to molybdate in comparison to IC₅₀, EC₅₀ or LC₅₀ of acetylcholinesterase and *Daphnia magna*.

IC ₅₀ , EC ₅₀ or LC ₅₀ (mg/L)				
	Acetylcholinesterase ^a	<i>Daphnia magna</i> ^a	<i>Daphnia magna</i> ^b	Baker's yeast ^c
Molybdate	26,492	2847.5	367.8	1.137

^a [25] ^b [27] ^c From this study

Potential of the yeast inhibitive assay as biomonitoring tool

Limit of detection (LOD) is the lowest concentration of a heavy metal revealing a distinguished signal before it turns saturated. Commonly, LOD of a system is also defined as three times the standard deviation of a reference sample [28]. To assess the potential of the developed assay as a biomonitoring tool, the LOD value obtained were compared with the Maximum Permissible Limit (MPL) as reported by Malaysian's Department of Environment (DOE). The targeted sensitivity of the developed assay is the Malaysian Department of Environment standard for the maximum permissible limit (MPL) of heavy metals, specifically Class III (Water Supply III – Extensive treatment required). The level of MPL that exceeded Class III will be classified as polluted and not suitable for irrigation and livestock drinking [29]. Based on the MPL level reported in the Environmental Quality Report (EQR), chromate could only be detected at LOD level of the developed assay. Meanwhile there is no available data on the MPL level of molybdenum (**Table 4**). It is generally accepted that an assay can be judged satisfactory

or acceptable if the IC₅₀ of heavy metals is within the MPL threshold for Class III or within the LOQ level. Despite being within the limit of detection (LOD), the assay is deemed subpar. As a result, heavy metal contamination is frequently multi-elemental instead of single-elemental [6]. Thus, the cumulative toxicity of heavy metals is more important as they will register an inhibition to the assay and deemed fit to be sent for instrumental validation [30]. In general, the values of IC₅₀, EC₅₀ or LC₅₀ are usually used to benchmark bioassays [28].

Table 4. Summary of IC₅₀ studies.

Anionic heavy metals	Equation	R ²	IC ₅₀ -min 30 (mg/L)	LOD (mg/L)	MPL ^a (mg/L)
chromate	One phase decay	0.8468	2.921	1.247	1.4
molybdate	One phase decay	0.9245	1.137	0.313	No available data

^a Maximum Permissible Limit (Class III) [29]

CONCLUSION

In conclusion, a yeast inhibitive assay for anionic heavy metals using MTT assay was developed. The parameters for optimum assay conditions were optimized using OFAT method and the assay gave optimum response at pH 5.8 and contact time of 30 min. The sensitivity of the assay is proven to be good as it can detect chromate pollution in Class III Rivers based on the MPL level. The results in this study revealed that Baker's yeast is suitable to be used as test organism in toxicity assay to detect the pollution of anionic heavy metals. To further increase the sensitivity of the assay, several recommendations were proposed for future studies. Parameters such as temperature and type of solvent to dissolve formazan crystals can be further studied as it may provide more specific results. Further optimization using statistical approach like Response Surface Methodology (RSM) that could overcome the limitations of OFAT method can be carried out. Other than that, environmental sample from polluted rivers can be tested to prove that the assay can be applied for field trying and have potential to be developed as a biomonitoring tool.

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REFERENCES

1. Ranjbar L, Yamini Y, Saleh A, Seidi S, Faraji M. Ionic liquid based dispersive liquid-liquid microextraction combined with ICP-OES for the determination of trace quantities of cobalt, copper, manganese, nickel and zinc in environmental water samples. 2012;
2. Tian M, Fang L, Yan X, Xiao W, Row KH. Determination of Heavy Metal Ions and Organic Pollutants in Water Samples Using Ionic Liquids and Ionic Liquid-Modified Sorbents. J Anal Methods Chem. 2019;2019.
3. Vodyanitskii YN. Determination of the oxidation states of metals and metalloids: An analytical review. Eurasian Soil Sci 2013 4612. 2014 Jan 5;46(12):1139–49.
4. Gidlow DA. Lead toxicity. Occup Med. 2015 Jul 1;65(5):348–56.
5. Jung K, Bitton G, Koopman B. Assessment of urease inhibition assays for measuring toxicity of environmental samples. Water Res. 1995;29(8):1929–33.
6. Shukor Y, Baharom NA, Rahman FA, Abdullah MP, Shamaan NA, Syed MA. Development of a heavy metals enzymatic-based assay using papain. Anal Chim Acta. 2006;566(2):283–9.
7. Malara A, Oleszczuk P. Application of a battery of biotests for the determination of leachate toxicity to bacteria and invertebrates from

- sewage sludge-amended soil. Environ Sci Pollut Res. 2013;20(5):3435–46.
8. Monarca S, Zani C, Richardson SD, Thruston Jr. AD, Moretti M, Feretti D, et al. A new approach to evaluating the toxicity and genotoxicity of disinfected drinking water. Water Res. 2004;38(17):3809–19.
9. Sirisattha S, Momose Y, Kitagawa E, Iwahashi H. Toxicity of anionic detergents determined by *Saccharomyces cerevisiae* microarray analysis. Water Res. 2004;38(1):61–70.
10. Adilah AN, Marhidayu MSSN. Study on characteristics of sediment and bed load discharge in Sungai Jemberau at Tasik Chini. IOP Conf Ser Earth Environ Sci. 2019 Mar;244:012046.
11. Elias M, Ibrahim S, Samudin K, Kantasamy N, Rahman S, Hashim A. Rare earth elements (REEs) as pollution indicator in sediment of Linggi River, Malaysia. Appl Radiat Isot. 2019 May 1;151.
12. DOE. Environmental Quality Report [Internet]. 2017 [cited 2022 Jan 22]. Available from: <https://enviro2.doe.gov.my/ekmc/wp-content/uploads/2018/10/EQR-2017.pdf>
13. Botsford JL. A simple, rapid, inexpensive assay for toxic chemicals using a bacterial indicator. Stud Environ Sci. 1997 Jan 1;66(C):429–43.
14. Isa HWM, Mustafa M, Wan Johari WL, Syahir A, Shukor MY, Nor Azwady AA, et al. Development of a Bacterial-based Tetrazolium Dye (MTT) Assay for Monitoring of Heavy Metals. Artic Int J Agric Biol. 2014;16:1123–8.
15. Sánchez NS, Königsberg M. Using yeast to easily determine mitochondrial functionality with 1-(4,5-dimethylthiazol-2-yl)-3,5-diphenyltetrazolium bromide (MTT) assay. Biochem Mol Biol Educ. 2006 May;34(3):209–12.
16. Hayon T, Dvilansky A, Shpilberg O, Nathan I. Appraisal of the MTT-based assay as a useful tool for predicting drug chemosensitivity in leukemia. Leuk Lymphoma. 2003 Nov;44(11):1957–62.
17. Rumlova L, Dolezalova J. A new biological test utilising the yeast *Saccharomyces cerevisiae* for the rapid detection of toxic substances in water. Environ Toxicol Pharmacol. 2012 May 1;33(3):459–64.
18. Riss TL, Moravec RA, Niles AL, Duellman S, Benink HA, Worzella TJ, et al. Cell Viability Assays. Assay Guidance Manual. Eli Lilly & Company and the National Center for Advancing Translational Sciences; 2004.
19. Ahmad F, Halmi MIE, Baskaran G, Johari WLW, Shukor MY, Syed MA. Inhibitive bacterial MTT assay for river monitoring of heavy metals. Bioremediation Sci Technol Res. 2013;1(1):1–7.
20. Salari R, Salari R. Investigation of the Best *Saccharomyces cerevisiae* Growth Condition. Electron Physician. 2017 Jan 25;9(1):3592–7.
21. Vicent I, Navarro A, Mulet JM, Sharma S, Serrano R. Uptake of inorganic phosphate is a limiting factor for *Saccharomyces cerevisiae* during growth at low temperatures. FEMS Yeast Res. 2015 May 1;15(3):1–13.
22. Mazloun-Ardakani M, Barazesh B, Moshtaghion SM, Sheikhha MH. Designing and optimization of an electrochemical substitute for the MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) cell viability assay. Sci Rep. 2019 Dec 1;9(1).
23. Gong L, Yang G, Yang B, Gu J. Development of the yeast *Saccharomyces cerevisiae* as a biosensor for the toxicity detection of toxic substances. bioRxiv. 2020;1–18.
24. Fai PB, Grant A. Chemosphere A comparative study of *Saccharomyces cerevisiae* sensitivity against eight yeast species sensitivities to a range of toxicants. Chemosphere. 2009;75(3):289–96.
25. Diamantino TC, Guilhermino L, Almeida E, Soares AMVM. Toxicity of sodium molybdate and sodium dichromate to *Daphnia magna* Straus evaluated in acute, chronic, and acetylcholinesterase inhibition tests. Ecotoxicol Environ Saf. 2000;45(3):253–9.
26. Hsieh CY, Tsai MH, Ryan DK, Pancorbo OC. Toxicity of the 13 priority pollutant metals to *Vibrio fischeri* in the Microtox® chronic toxicity test. Sci Total Environ. 2004 Mar 5;320(1):37–50.
27. Wang CW, Liang C, Yeh HJ. Aquatic acute toxicity assessments of molybdenum (+VI) to *Daphnia magna*. Chemosphere. 2016 Mar 1;147:82–7.
28. Aidil MS, Sabullah MK, Halmi MIE, Sulaiman R, Shukor MS, Shukor MY, et al. Assay for heavy metals using an inhibitive assay based on the acetylcholinesterase from *pangasius hypophthalmus* (Sauvage, 1878). Fresenius Environ Bull. 2013;22(12):3572–6.
29. DOE. National Water Quality Standards For Malaysia [Internet]. 2019 [cited 2022 Jan 22]. Available from: <https://www.doe.gov.my/portaltv1/wp-content/uploads/2019/05/Standard-Kualiti-Air-Kebangsaan.pdf>
30. Witeska M. The effect of toxic chemicals on blood cell morphology in fish | Request PDF. Fresenius Environ Bull. 2004;13(12):1379–84.