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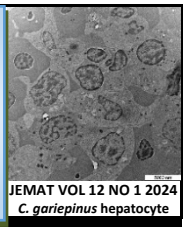

Biomonitoring Mercury Pollution in Juru Industrial Estate Using a Rapid Inhibitive Enzyme Assay

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ABSTRACT

Due to rapid industrialization coupled with indiscriminate release of pollutants, the number of polluted rivers in Malaysia has been steadily increasing over the years. Regular monitoring of contaminants, particularly heavy metals, is not feasible due to the steep cost of instrumental monitoring alone. In this study, a rapid inhibitive enzyme assay using the molybdenum-reducing enzyme from the bacterial isolate 34XW was developed for monitoring mercury. The Mo-reducing enzyme from this bacterium was very sensitive to mercury, with an IC_{50} confidence interval of 0.0025–0.0029. The inhibition model followed a log(inhibitor) vs. response with variable slope, enabling detection of mercury at the Maximum Permissible Limit (MPL) set by the Malaysian Department of Environment. Comparisons with established assays, using IC_{50} values and confidence intervals, confirmed the superior sensitivity of the developed method. Hierarchical clustering identified patterns of enzyme sensitivity, with assays grouped by IC_{50} intervals. Isolate 34XW was an outlier with exceptional sensitivity. K-Means clustering grouped assays into sensitivity tiers, with the clustering of the molybdenum-reducing enzyme from the bacterial isolate 34XW into a unique cluster due to the level of its sensitivity. Juru industrial estate water samples tested using the assay revealed its capability in detecting low concentrations of mercury. These findings underscore the assay's utility in detecting elevated mercury concentrations in environmental samples and its broader implications for environmental monitoring and biochemical applications. As a preliminary screening tool for heavy metal monitoring on a large scale, the assay is quick and easy to use.

INTRODUCTION

Water sources across the globe have been discovered to be contaminated with heavy metals like cadmium, mercury, chromium, and lead. This has led to the creation of numerous technologies that can detect and eliminate them. The majority of these heavy metals in the water supply originate from human activities [1–4]. The gastrointestinal tract is the primary route of heavy metal absorption in humans. A host of health problems can develop as a result of long-term exposure to heavy metals, including problems with the liver and kidneys, cognitive impairments, neurological disorders, infertility, exhaustion, gout, headaches, high blood pressure, and secondary microbial infections [5]. The abandoned copper mine in Mamut Sabah is one of the most notorious sites of metal pollution in Malaysia. The area is polluted due to the periodic breaks of pipes that carried metal [6]. There have been reports of metal pollutions at industrial sites, including those in the Juru and Prai Industrial Estates [7,8].

There are approximately 180 major river basins in Malaysia, making heavy metal monitoring a challenge at present. Around one hundred forty-seven basins are being closely watched at all times. Rivers are ranked from 1 to 5, with 5 indicating the highest level of pollution. Due to the high expense of monitoring heavy metal content for all rivers, only water parameters such as total suspended solid, turbidity, pH, biological oxygen demand (BOD), and chemical oxygen demand (COD) are currently reported annually. Class 5 rivers indicate severe pollution; nearly 43 of Malaysia's nearly 1800 rivers fall into this category. It is not considered safe to use the water from these rivers for farming or drinking [9]. Trace amount of heavy metals can cause a river to be classified as class 5. In the case of mercury, its value must not exceed the maximum permissible limit (MPL) of 0.001 mg/L.

Some rivers in Malaysia may have to be reclassified due to heavy metals content levels that are higher than the Maximum Permissible Level (MPL), according to independent, sporadic testing [7,10,11]. It is crucial to regularly monitor these rivers, preferably once a month, so we can evaluate their health and potentially reclassify them if necessary. By combining biomonitoring with instrumental validation, routine monitoring becomes economically feasible. The use of biological assays in biomonitoring can serve as a preliminary screening method; only samples that test positive are then sent for validation using instrumental methods like Flow Injection Mercury System (FIMS) or Atomic Emission Spectrometry (AES). One example of a microbial system used for heavy metal biomonitoring is the luminescence bacteria-based assays such as Xenoassay Light [12], Microtox [13] and MTT-based assays [14,15]. Enzyme-based assay systems include the use of enzymes such as proteases [7,8,16–19], urease [20], and acetylcholinesterases [21–23].

During our work with the molybdate-reducing bacterium *Serratia* sp. strain DRY5- a local isolate [24], we found that the molybdenum-reducing (Mo-reducing) enzyme or activity is strongly inhibited by heavy metals. The bacterium reduces molybdenum, a relatively nontoxic element to molybdenum blue, a nontoxic product, and this process can be potentially used in biorecycling and bioremoval of molybdenum. Previously, the Mo-reducing system from the bacteria has been employed to detect single element such as copper [25], mercury [26] and lead [27]. Screening for a more sensitive heavy metal detection system using the Mo-reducing system requires selecting a bacterium with minimal capacity to reduce Mo in the environment. This ensures heightened sensitivity to heavy metals. Despite its limited Mo-reducing ability, such a bacterium is highly suitable for developing an inhibitory heavy metal detection system. In this study we reported such as system.

MATERIALS AND METHODS

Screening for Mo-reducing bacterium sensitive to mercury

Mo-reducing bacteria were sourced from the BBE's laboratory culture collection. Growth and maintenance of the bacterium were carried out using low-phosphate medium supplemented with molybdenum, as described previously. The composition of the medium (w/v%, pH 7.0) is as follows: (NH4)2SO4 (0.30), glucose (1.0), MgSO4·7H2O (0.05), Na2HPO4 (0.05), Na2MoO4·2H2O (0.484), NaCl (0.5), and yeast extract (0.05). The bacterium was maintained on solid medium supplemented with 1.5% agar. The low-phosphate medium effectively preserved the molybdenum-reducing property of this bacterium [24]. An intense blue colony, formed after 48 hours of incubation at room temperature, was transferred into 50 mL of high-phosphate medium. This medium was identical to the original but had an increased phosphate concentration of 100 mM (pH 7.0). The bacterium was incubated overnight at 150 rpm in 50 mL of medium contained in 250 mL conical flasks. Cells were harvested by centrifugation at 10,000 × g for 10 minutes, washed three times with sterile deionized water, and recentrifuged. The cells were subjected to sonication using a Biosonik 111™ sonicator while kept on an ice bath. The total sonication time was 30 minutes with 3 min intermittent cooling on ice. Following sonication, the sample was ultracentrifuged at 105,000 × g for 90 minutes at 4°C. The resulting supernatant, containing the crude enzyme, was carefully collected.

Molybdenum-reducing enzyme assay

The enzyme assay in this study used NADH as the electron donor and 12-phosphomolybdate as the electron acceptor. Molybdenum blue is rapidly formed as 12-phosphomolybdate is reduced by the enzyme. A 50 mM stock solution of phosphomolybdic acid (sodium phosphomolybdate hydrate, Sigma, St. Louis, USA) was prepared in 10 mM phosphate buffer at pH 5.8 and diluted to a final concentration of 3 mM in a 1 mL reaction mixture. A 30 mM stock solution of NADH was also used, with a final concentration in the reaction mixture set at 2.5 mM. To initiate the reaction, 50 µL of crude molybdenum-reducing enzyme fraction (final protein concentration of 1 mg mL^{-1}) was added. The effect of mercury was studied at the final concentration of 0.005 mg/L (5 ppb) The total reaction volume was 1 mL. After precisely one minute of incubation at room temperature, the absorbance at 865 nm was measured to monitor the reaction. The activity of molybdenum reductase is defined as the amount of enzyme required to produce 1 nmole of molybdenum blue per minute at room temperature. The molybdenum blue concentration was quantified using its specific extinction coefficient of 16.7 mM^{-1} cm^{-1} at 865 nm. An increase in absorbance at 865 nm by 1.00 unit per minute per milligram of protein corresponds to the production of 60 nmoles of molybdenum blue in a 1 mL reaction mixture [28].

Real water samples monitoring works

River water samples were taken from the Juru River and nearby rivers surrounding the Juru Industrial Estates. The samples were collected using polycarbonate containers and transported to the lab in chilled conditions. The pH of these samples ranged from 6.5 to 7.5. Initially, the water samples were filtered through a Teflon membrane filter with a pore size of 0.45 µm. Volumes of up to 50 µL of heavy metals or river water samples were directly mixed with 50 µL of enzyme and incubated for 5 minutes at room temperature. After incubation, the mixtures were assayed under the same conditions as previously described. Mercury levels, however, were specifically determined using a Perkin Elmer Flow Injection Mercury System (FIMS). All experiments were repeated three times to ensure reliability.

Calculation for percent of inhibition

The percent inhibition was computed according to following formula:

$$\% \text{ Inhibition} = \frac{\text{Absorbance of control - Absorbance of sample}}{\text{Absorbance of control}} \times 100\%$$

K-Means Clustering and Hierarchical Clustering methods

Data Collection and Processing

IC_{50} values for 10 enzyme assays were collected to assess their sensitivity to heavy metal exposure. The enzyme assays included Urease, 15-min Microtox™, 48-hour Daphnia magna, 96-hour Rainbow trout, Papain, AChE from *E. electricus*, and Mo-reducing enzymes from *Serratia sp.* strains (DRY5, DRY8, DRY6), as well as Isolate 34XW. Each IC_{50} was expressed as a confidence interval with lower and upper bounds, from which the midpoint was calculated to represent average sensitivity. The dataset was normalized using MinMax scaling to ensure all features contributed equally to the clustering algorithms.

K-Means clustering was applied to the normalized data using the midpoint, lower bound, and upper bound of IC_{50} values as input features. The optimal number of clusters (k=3k=3) was determined based on domain expertise and inspection of cluster separability. The clustering results were visualized using scatter plots, with each point labeled by the corresponding enzyme assay for clarity. Clusters represented distinct sensitivity tiers, allowing for categorization of the assays based on their IC_{50} values. Agglomerative hierarchical clustering was performed on the normalized dataset using the Ward linkage method to minimize intra-cluster variance. A dendrogram was generated to visualize hierarchical relationships among the assays, with branch lengths representing Euclidean distances between observations. The dendrogram provided a clear visualization of groupings and dissimilarities, highlighting similarities within clusters and distinct outliers.

Software and Tools

All clustering analyses and visualizations were performed using Python programming language. The scikit-learn library was used for K-Means clustering and data normalization, while the scipy library was employed for hierarchical clustering and dendrogram generation. Data visualizations were created using the matplotlib library. Cluster groupings were validated against known sensitivity trends from the literature, confirming that enzymes with lower IC_{50} values exhibit higher sensitivity to heavy metal inhibition. The clustering outputs provided meaningful insights into enzyme sensitivity tiers, which were further compared and corroborated with patterns reported in previous studies.

RESULTS AND DISCUSSION

The Mo-reducing enzyme is a new enzyme that can be considered as a novel discovery even though it has been purified but not characterized at the molecular level and its encoded protein has not been identified. This enzyme allows bacteria to counteract the toxicity of soluble molybdenum through the formation of colloid. This colloidal molybdenum which is synthesized in this work can be trapped inside semi-permeable membrane such as dialysis tubing. This mechanism has a great perspective; not only as a remediation strategy for molybdenum polluted environments, but also as a way to recover and reuse this metal from wastewater. In this way, recycling molybdenum could help to support the management of resources in industries that use this element a lot, including steel and chemical industries. Even though the enzyme was purified, more research is needed in order to determine the details of its architecture, the way it works and its genetic background; this could be useful for the analysis of the enzyme's potential in the field of environmental biotechnology and industrial ecology.

Enzyme inhibition studies

The best isolate with the Mo-reducing enzyme that is the most sensitive to mercury was isolate 34XW (**Fig. 1**). The model for describing the mode of inhibition of mercury was the log(inhibitor) vs. response with variable slope (**Fig. 2**). Based on the LOD values for mercury (**Table 1**), the developed assay could detect mercury at the Maximum Permissible Limit as outlined by the Malaysian Department of Environment [9]. Reproducibility of the developed assays was assessed by repeated measurement of the enzyme inhibition by the heavy metals The resulting CV (coefficient of variation) of the replicated data was from 8 to 15% suggesting adequate reproducibility. Mercury affects enzymes in a way that is structural in that it interacts with the enzyme at the molecular level and binds to the thiol group of cysteine amino acid.

This binding causes the mercury to interact with the active site of the enzyme which affects its function and conformation. It can also interact with other amino acids including selenocysteine and histidine which makes it difficult for the enzyme to function efficiently. Also, it causes oxidative stress by increasing the level of reactive oxygen species that can cause damage to proteins and reduce their functionality. These disruptions affect very important processes in the body including DNA replication, cellular metabolism and antioxidant defense systems and this in turn results to cell damage and toxicity [29–35].

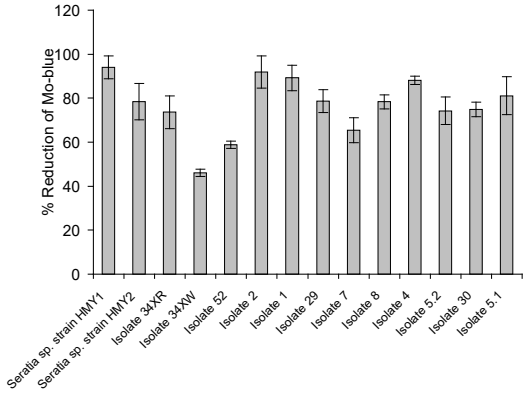

Fig. 1. Screening results for the inhibitory effect of 0.005 ppb mercury on the Mo-reducing enzyme assay. Data is mean± standard error (n=3).

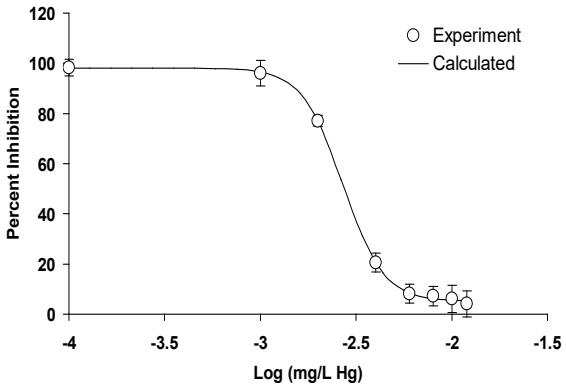

Fig. 2. Fitting mercury inhibition to the Mo-reducing enzyme of using the log(inhibitor) vs. response with variable slope. Data is mean± standard error (n=3).

Table 1. Data compared to the Maximum Permissible Limit (MPL) specified in Malaysia's interim national water quality standards, as well as the developed assay's Limits of Detection (LOD), most effective nonlinear model, and correlation coefficient [36].

Regression model	R^2	IC_{50} (mg/L) (95% CI)	LOD (mg/L) (95% CI)	MPL (Class IIA and IIB)
log(inhibitor) vs. response -- Variable slope	0.99	0.0027 (0.0025—0.0029)	0.0012 (0.0015—0.0010)	0.001

Subsequently, IC_{50} was used to compare the developed method's sensitivity to other well-established assays. In order to determine if a newly-developed method outperforms an existing one, one must examine the confidence interval of the IC_{50}.

We can conclude that the two IC_{50} values differ significantly at the $p<0.05$ level if their confidence intervals do not overlap. However, additional experiments are required to prove non-significance if the confidence intervals overlap, which does not mean that the two values are not significant [37]. The most sensitive assay in the developed method was the Mo-reducing enzyme from isolate 34XW, as shown by the IC_{50} value. In order to get consistent results from any experiment involving living organisms, a temperature-controlled water bath and facilities for housing and raising the creatures were required [14]. There are a number of stages involved in developing the urease and papain assays [7,20], Additionally, the urease assay necessitates a water bath, which makes it impractical for use in field trials due to its minimum 2-hour completion time. Field trials may benefit from this assay because it is a one-step inhibitory assay at room temperature.

Hierarchical Clustering Dendrogram

The hierarchical clustering dendrogram is a powerful tool that offers a systematic way of arranging the enzyme assays' IC_{50} confidence intervals and thereby identify patterns of sensitivity [39]. The assays with close IC_{50} values are placed closely at lower linkage distances and thus form meaningful clusters. For example, the Mo-reducing enzymes namely DRY5, DRY8, and DRY6 show high degree of clustering which indicates that their IC_{50} midpoints are quite close (0.154-0.178 range); on the other hand, Isolate 34XW is quite different from the rest as it has the lowest IC_{50} range (0.0025-0.0029) thus being very sensitive to heavy metals. On the other hand the papain assay whose IC_{50} range is quite wide (0.24-0.412) is quite different from the other enzymes and this could be an indication that it is less sensitive or more resistant to metal inhibition. Moderate sensitivity enzymes like Urease, 5 min Microtox™ and 48 hours Daphnia magna are also seen to form another cluster, this is because their IC_{50} values are within the same range but within the lower spectrum.

The branch lengths in the dendrogram represents the differences, where short branches of the Mo-reducing enzymes show the high similarity while long branches for papain and Isolate 34XW exhibit the large differences in sensitivity. These groupings have practical implications; for instance, the Mo-reducing enzymes can be substituted one for the other in applications that call for moderate sensitivity; this is because they are all alike in sensitivity. In their natural environment, the Mo-reducing enzymes can be replaced with each other as they have similar sensitivity and can be used in applications that are not extremely sensitive to the presence of metals; Isolate 34XW as an outlier with high sensitivity can be used as a very sensitive biosensor for the detection of metals in the environment; papain's resistance will be advantageous in situations where it is desirable to reduce the sensitivity to metal inhibition although as far as mercury pollution is concerned, the level is too high for meaningful detection.

There are some limitations of the clustering; the results are based on midpoint values of the clusters which group the data in a simple manner and may not capture the variation within the confidence intervals; the results may also be different in the real world for instance when there is a change in pH or temperature, especially pH where heavy metals solubility and bioavailability is highly dependent. Despite this the buffered incubation pH at pH 5.5 is generally favorable for the solubility of cationic metal ions including mercury. In general, the dendrogram presents a distinct classification of enzyme sensitivity by levels, which is useful for the selection of suitable enzymes for biochemical assays [40] as well as for the development of environmental monitoring systems.

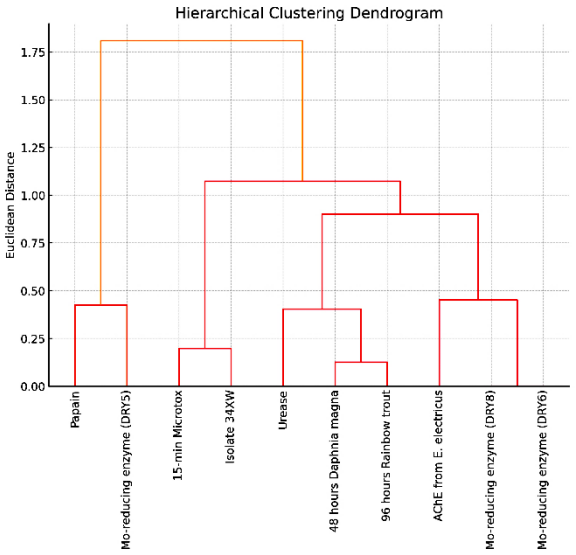

Fig. 3. Hierarchical Clustering Dendogram of enzyme assays based on IC_{50} values (lower bound and midpoint) under mercury exposure.

Table 2. Evaluation of the newly-created test against a battery of existing tests. The range is C.I. (95% Confidence Interval).

	LC_{50} or IC_{50} (mg/l)									
Metals	Urease[a]	15-min. Microtox™ [a, c]	48 hours *Daphnia Magna*[a]	96 hours Rainbow trout[a c]	Papain[b]	AChE from *E. electricus*[d]	Mo-reducing enzyme *Serratia* sp. strain DRY5[h]	Mo-reducing enzyme *Serratia* sp. strain DRY8[i]	Mo-reducing enzyme *Serratia* sp. strain DRY6[h]	This study
Hg^{2+}	0.021-0.33	0.029-0.050	0.0052-0.21	0.033-0.21	0.240	0.084-0.1	0.224-0.272	2.516-2.824	0.154-0.178	0.0025—0.0029

Note
[a][20]
[b][7]
[c][13]
[d][22]
[h][38]
[i][24]

K-Means Clustering

Mercury assays was clustered according to the IC_{50} confidence intervals utilizing K-Means and classified clustering methods (Fig. x). The object of this study was to find sensitivity levels and potential outliers for environmental monitoring purposes. We collected the lower bound, upper bound, and midpoint values of IC_{50} for 10 different enzyme assays using the MinMax scaling. These assays included: Urease, 15-minute Microtox™, 48-hour Daphnia magna, 96-hour Rainbow trout, Papain, AChE from *E. electricus*, Mo-reducing enzymes from *Serratia* sp. strains (DRY5, DRY8, DRY6), and Isolate 34XW.

Each data point in the scatter plot is labeled with its corresponding enzyme assay, and three separate clusters are shown: Cluster 0, Cluster 1, and Cluster 2. The assays in Cluster 0 have moderate sensitivity; for example, there is the 15-minute Microtox™ and the outlier Isolate 34XW, which has incredibly low IC_{50} values. Papain, found in Cluster 1, has a low sensitivity to metal inhibition and a high IC_{50} range. Assays like Urease and 96-hour Rainbow Trout, which are moderately sensitive, and Mo-reducing enzymes (DRY5, DRY8, DRY6), which exhibit comparable IC_{50} values, are included in Cluster 2. This grouping brings attention to patterns of enzyme sensitivity, which has biochemical and environmental implications.

Cluster 0, which included moderately sensitive assays like 15-minute Microtox™, Cluster 1, which contained lowly sensitive Papain with high IC_{50} values, and Cluster 2, which contained moderately to highly sensitive enzymes like Urease and Mo-reducing enzymes, as well as the extremely sensitive outlier Isolate 34XW, were identified through K-Means clustering with three clusters (k=3k=3), with sensitivity tiers visualized using labeled scatter plots. A dendrogram was generated by agglomerative hierarchical clustering using Ward linkage, which confirmed these groupings. The Mo-reducing enzymes clustered closely together because of their high similarity, while Isolate 34XW and Papain formed separate branches, indicating their dissimilarity. The evaluations summarized a robust strategy for classifying enzyme assays according to patterns in IC_{50} sensitivity [41]. This plan is in line with what is already known from the literature, which states that enzymes with lower IC_{50} values are more susceptible.

Fig. 4. K-Means clustering of enzyme assays based on IC_{50} values (lower bound and midpoint) under mercury exposure.

Clustering methods can be useful for understanding sensitivity patterns in biochemical assays, and the results provide practical advice for choosing assays for environmental monitoring, such as using highly sensitive enzymes like Isolate 34XW to detect trace contaminants and low-sensitivity assays like Papain in robust contamination scenarios.

Evaluation of developed assay

Juru industrial estate water samples were subsequently used to test the developed assay. More than half of the water samples inhibited the enzymes. These water samples tested positive for heavy metals, surpassing the legal limit, according to the results of the analysis. Class IIB (body contact for recreational purposes) and Class IIA (water supply) mercury MPLs are 0.001 mg L-1 and 0.001 mg/L, respectively [36]. The location of N 05° 20.96, E 100° 24.17' was inhibitory despite showing no detectable mercury (**Table 3**). This is likely due to the Mo-reducing enzyme being known to be inhibited by other heavy metals such as copper and silver that is present in the wate samples. This suggests that the developed assay was able to detect high concentrations of heavy metals in real water samples, since the majority of the samples that inhibited the test had heavy metals above the MPL. Additionally, the results demonstrated that heavy metal pollution was present at least during the sampling period in multiple locations within the Juru industrial estate. This work's elevated heavy metal levels may be the result of a one-time incident of pollution or the result of a persistent pollution problem caused by nearby industries; more frequent sampling will show us.

One of Malaysia's oldest industrial sites is the Juru industrial estate. There are a lot of factories here, including metal works. There have been numerous reports of heavy metal pollution in water bodies near this location. This area has been the focus of biomonitoring efforts with the expectation that they will serve as an early detection system, hence lowering the cost of the more costly instrumental-based approach [19]. At present, class 5 rivers are automatically designated as having extremely polluted water because their heavy metal levels are higher than the Maximum Permissible Level (MPL). Regrettably, heavy metal concentrations in 180 river basins are only determined when absolutely necessary. Multiple studies have shown that pollution has been steadily increasing over the years, and there seems to be no end in sight. Heavy metals in these rivers have both short-term and long-term consequences, and many of them are utilized for agricultural and irrigation purposes [7,12,19,23,36,42,43].

Table 3. Assessment of the Mo-reducing enzyme inhibitive assay test on contaminated water samples taken from the Prai industrial estate in Penang.

locations	% Inhibition Enzyme Activity	Concentrations of mercury (mgl^{-1})
N 05° 20.447 E100° 26.403'	0	n.d.
N05°20.665' E100°26.364'	1.21	n.d.
N05°20.601' E100°26.427'	2.45	n.d.
N05°20.640' E100° 26.470'	100	0.009±0.001
N05°18.947' E100°26.348'	100	0.008±0.001
N 05° 20.96, E 100° 24.17'	100	n.d.
N 05° 20.96, E 100° 17.25'	100	0.0102±0.001
Tap water, University Putra Malaysia	0	n.d.

[1] 20% Inhibition is considered significant toxicity. Data were presented as whole number.
[2] n.d. = not detected

CONCLUSION

The molybdenum-reducing enzyme has been utilized to create a heavy metal assay that is both sensitive and quick. This inhibitory assay has excellent repeatability and sensitivity that is on par with many of the ones that are now in development. Polluted waters from the Juru Industrial estate were successfully tested for heavy metals using an enzyme that is sensitive to mercury, silver, copper, and chromium. Instrumental analysis has verified the presence of heavy metals. As a biomonitoring tool, the created assay is quick, easy, and cheap to run. Applying clustering methodologies, we ranked enzyme assays according to their sensitivity, providing useful information for choosing enzymes for biochemical or environmental monitoring. Finding enzymes that can detect trace contaminants (like Isolate 34XW) or that can function in more demanding environments (like Papain) was a key finding of the analysis. This methodology demonstrates how sensitivity analysis can benefit from integrating clustering methods with biochemical data.

AI AND AI-ASSISTED TECHNOLOGIES DECLARATION

We relied heavily on robust AI-based tools like Grammarly, ScholarAI, and ChatGPT during the initial stages of our research and paper preparation. As supplementary resources, these tools aided in data collection, analysis, and manuscript editing; they had no role in drawing scientific conclusions or interpreting data. We are solely responsible for all scholarly work, including all interpretations, conclusions, and argument presentation and consistency.

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