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A Near-Real-Time Achromopeptidase-Based Enzyme Assay for Biomonitoring of Zinc Pollution in Kuah's Jetty in Langkawi

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ABSTRACT

Enzyme-based assay in near-real-time biomonitoring provides high sensitivity to bioavailable contaminants, leading to quick results that support prompt action. It is essential to use this method to reduce pollution in drinking, agriculture and marine waters and protect the health of both humans and animals. This work applies a previously discovered enzyme test in biomonitoring to identify contaminants, particularly zinc, in marine water samples from the Langkawi Island, an island that harbors the UNESCO's Geoforest Park status. We used the achromopeptidase dye binding assay, specifically designed for detecting mercury zinc at levels below one part per million, to identify trace amounts of these metal successfully. The test demonstrated a sensitive, quick, and cost-efficient monitoring method with little inhibition (<10%) during a 6-hour field trial for three consecutive days, suggesting low pollution levels and confirmed by instrumental analysis. This method allows for the prompt identification of environmental pollutants, which helps take appropriate actions and safeguard ecotourism locations by offering data-driven information for policy development. Enzyme tests are simple and visually appealing, making them effective instructional tools that help raise environmental awareness and support conservation initiatives. Our research highlights the need to use enzyme tests for broad environmental evaluation, harmonizing local monitoring methods with global standards, and promoting international cooperation in environmental conservation. This work enhances our comprehension of ecological well-being in marine and brackish waterways and underscores the significance of ongoing monitoring to protect natural environments.

INTRODUCTION

Langkawi was designated as a UNESCO geopark in July 2007, signifying a significant step in the island's transformation from a peaceful Malaysian destination to an internationally renowned tourist hotspot. The recognition from the United Nations Educational, Scientific and Cultural Organization has led to significant growth in coastal development and tourism sectors, increasing its attractiveness to global tourists. The rise in tourism has led to notable environmental issues, as seen in the increasing reports of pollution in Langkawi, with one of its rivers classified as a class IV (polluted). The Department of Environment reported in 2017 that Sg Ulu Melaka was classified as Class IV due to contamination, continuing a trend of increasing pollution occurrences in Langkawi [1]. The existence of heavy metals, including cadmium (Cd), cobalt (Co), lead (Pb), and zinc (Zn),

probably originating from boat emissions and construction-related activities, present a significant threat to the natural balance of the region [2–7]. Chronic toxicity results from prolonged exposure to zinc at levels above dietary recommendations. Unlike acute toxicity, the effects of chronic exposure manifest over an extended period and can be subtle initially. Chronic ingestion of excessive zinc can interfere with the absorption of copper and iron, leading to deficiencies of these critical nutrients. Symptoms of chronic zinc toxicity include lethargy, neurological disorders such as neuropathy, immune system dysfunction, and alterations in cholesterol and lipid metabolism. Long-term exposure to high levels of zinc can also lead to copper deficiency, which in turn may cause anemia and weakening of bones. Additionally, there is evidence to suggest that excessive zinc intake might be linked to an increased risk of prostate cancer [8–10].

Biomonitoring through enzyme assays emerges as a powerful approach in environmental management, providing a sensitive, cost-effective, and rapid means for detecting pollutants, which greatly benefits both public awareness and authoritative action. These assays are adept at identifying low concentrations of contaminants like heavy metals and organic compounds, facilitating authorities' early implementation of remedial measures. Their cost efficiency and the minimal requirement for sophisticated equipment enable widespread and frequent environmental assessments, contributing to a detailed understanding of ecological health across vast areas [11–14].

The swift processing of enzyme assays ensures timely interventions critical for preventing environmental degradation and safeguarding public health. Additionally, some enzyme assays' simplicity and visual appeal serve as excellent resources for educational initiatives to enhance environmental consciousness in the community, thereby promoting active conservation efforts. The accurate data generated from these assays support evidence-based policymaking, enabling authorities to establish precise pollutant thresholds, assess the effectiveness of environmental protections, and make necessary adjustments. Moreover, enzyme activity indicators offer early warnings of ecological distress, allowing for interventions before visible damage occurs, thereby preventing long-term ecological damage [15–18].

By aligning local monitoring practices with international standards through enzyme assays, authorities can effectively foster global collaboration in environmental protection, addressing transboundary challenges. In essence, enzyme assays for biomonitoring equip both the public and decision-makers with essential tools and knowledge for more effective environmental stewardship and public health protection. We have developed several near-real-time monitoring of pollution, especially heavy metals, using enzymes from microorganisms and plants and utilize these assays to monitor various potential and polluted sites in Malaysia including an UNESCO site in the river Malacca [19–25]. Of the heavy metals often reported at levels above the Maximum Permissibility Limit in Langkawi waters and sediment is zinc [26–30], which have led to a higher concentration of the metal in fish near the area [31]. In this study, we explore the feasibility of using the achromopeptidase dye binding assay we previously developed for zinc biomonitoring in Kuah's Jetty waters in Langkawi with a Limit of Detection value of 0.124 mg/L [22]

MATERIAL AND METHODS

Preparation of casein and achromopeptidase solution

Casein, procured from Sigma, was precisely measured to 2 grams and blended with 100 milliliters of deionized water. To achieve a pH level of 8.0, the solution was titrated with 5N solutions of NaOH and/or HCl. This mixture was then continuously agitated at 60°C throughout the night to ensure thorough dissolution. To separate insoluble particles, the solution was strained through multiple cheesecloth layers. Subsequent clarification was achieved by centrifuging the solution at 10,000×g at a temperature of 4°C. The protein concentration in the resulting clear supernatant was determined via the Bradford method, employing crystalline BSA from Sigma as a reference. This prepared solution was preserved at 4°C for immediate use or frozen at -20°C for long-term storage. Achromopeptidase (EC 3.4.21.50), from *Achromobacter lyticus,* is an extracellular protease with a mol wt of 30 kDa, procured from Sigma, was dissolved at 4 °C in a 20 mM sodium phosphate buffer with a pH of 8.0, creating a 1.0 mg/mL stock solution. From this stock, working solutions of achromopeptidase (1.0 mg/mL) and casein (1 mg/mL) were prepared daily.

Achromopeptidase inhibition studies

The initiation of the positive control experiment involved combining 50 µL of achromopeptidase (0.06 mg/mL final concentration) in 20 mM phosphate buffer at a pH of 8.0, as determined from a previous experiment [22]. Zinc at 1 mg/L final concentration served as a positive control. This mixture was then incubated for 30 minutes at a temperature of 30°C. Following this substitution, 50 µL of casein solution was introduced to the mixture, resulting in a final concentration of 0.1 mg/mL, and was thoroughly mixed. An aliquot of 20 µL from this mixture was immediately combined with 200 µL of Bradford dye-binding reagent. The resulting solution was allowed to stand at ambient temperature for 5 minutes, after which the absorbance was recorded at 595 nm, marking the initial absorbance reading. After an additional incubation period of 30 minutes, a second aliquot of 20 µL was extracted, mixed with the Bradford dye reagent in the same manner, and the absorbance at 595 nm was measured following a 5-minute incubation, mirroring the initial procedure.

Near real-time field trials

Every hour for six hours, water samples were collected into acid-washed HDPE bottles, each supplemented with a few drops of 1% (v/v) HNO_3, from the Kuah's Jetty in Langkawi specifically at the coordinates 6°18'18.4"N 99°51'01.0"E (refer to **Fig. 1**). Initially, these samples underwent filtration through a 0.45 µm syringe filter to obtain a clear filtrate. Subsequently, 50 microliters of this filtrate were assayed for zinc content using the achromopeptidase assay at a controlled temperature of 30 °C. This temperature control was achieved using a portable egg incubator (30 Watt, generic brand) powered by a DC12V to AC220V car inverter (ZTE Avid Plus, China), ensuring a stable environment of 30 ± 1°C. The absorbance measurements were conducted with a portable mini-spectrophotometer (Model M6+, Axiom, Germany). Post-assay, the samples were stored in a Coleman® ice cooler for preservation until they could be analyzed further in the laboratory.

Zinc was determined using Atomic Emission Spectrometry, specifically on a Perkin Elmer ICP OES (Optima 8300, PerkinElmer, Inc., 940 Winter Street, Waltham, MA, USA). This comprehensive approach allowed for the precise quantification of these metals, providing critical insights into the water quality near the geoforest park.

Fig. 1. Location of water sampling (red pinned) in Kuah's Jetty in Langkawi, Malaysia . (Source Google Earth image).

Data and Statistical Analysis

The per cent inhibition was calculated according to the following formula:

$$\% \text{ Inhibition} = \frac{\text{Test activity of sample} - \text{test activity of control x } 100}{\text{Test activity of control}}$$

RESULTS AND DISCUSSION

Ecotourism, especially water-based activities, can unintentionally increase heavy metal levels in aquatic environments through many interrelated paths. Boats and watercraft engines used in ecotourism activities emit exhaust pollutants including heavy metals such as lead, cadmium, and mercury due to fuel combustion. Older and inadequately maintained vessels are especially responsible for this pollution. Boats commonly use antifouling coatings to inhibit the growth of barnacles and algae, which release copper and other heavy metals as they break down [32]. These boats' maintenance can introduce heavy metals into the water through operations including paint stripping, engine repairs, and replacing metal parts.

Anchoring disrupts sediment, releasing heavy metals and making them more accessible to aquatic food systems. Constructing docks and marinas for ecotourism infrastructure can disrupt soil and sediment, which may release accumulated heavy metals. Tourist arrivals result in elevated wastewater and runoff, potentially transporting heavy metals from different origins into aquatic environments. Recreational equipment like jet skis and motorboats can cause erosion and sediment resuspension, releasing heavy metals that had previously deposited in the sediments. While marketed as a sustainable tourism option, ecotourism requires careful supervision to reduce its environmental effects, such as the possible increase in heavy metal concentrations in water sources [2,33–35].

Near real-time field trials

We conducted a near real-time field trial over a six-hour duration, with measurements taken at hourly intervals for three consecuttive days. The results demonstrated minimal inhibition (less than 10%) on the achromopeptidase assays utilized. Instrumental analyses further revealed that the levels of zinc were below the maximum permissible limits (MPL) set at 0.4 mg/L for class III waters. In this context, the threshold for significant inhibition was established at 20%. This minimal inhibition suggests the effectiveness of the achromopeptidase assay in these environmental conditions. Previous near real-time studies utilizing enzymatic methods in riverine settings [19,21,22,25,36] have demonstrated the efficacy of these bioassays in tracking the temporal fluctuations of heavy metal concentrations, suggesting a broad applicability for monitoring environmental contaminants. Building on this foundation, a subsequent investigation was conducted using samples from marine and brackish waters [24]. These expansive aquatic systems act as significant reservoirs, quickly diluting heavy metals originating from terrestrial sources. Despite this rapid dilution, research has consistently shown elevated levels of heavy metals in these environments, particularly within sedimentary fractions [37–40].

The tendency of sediments to accumulate heavy metals highlights the intricate dynamics of pollutant distribution across different aquatic environments. Sediments often serve as environmental sinks, capturing and retaining heavy metals over extended periods, which can lead to long-term ecological impacts. This phenomenon underscores the necessity of implementing comprehensive monitoring strategies that encompass various environmental compartments, including both water columns and sediments, to provide a holistic assessment of heavy metal pollution in marine and brackish waters [37–40].

Such assessments are crucial as they allow for the identification of pollution sources and the evaluation of contaminant dispersion patterns, which are vital for the development of effective environmental policies and remediation strategies. Moreover, the use of bioassays, as demonstrated in riverine and maritime studies, offers a promising approach to enhance real-time monitoring capabilities in these complex aquatic systems, potentially leading to more proactive environmental management and protection measures [41–44]. This observation of an absence in response to achromopeptidase suggests that this area remains comparatively pristine. To protect this ecotourism destination, it will be essential to implement increased monitoring measures going forward in the future.

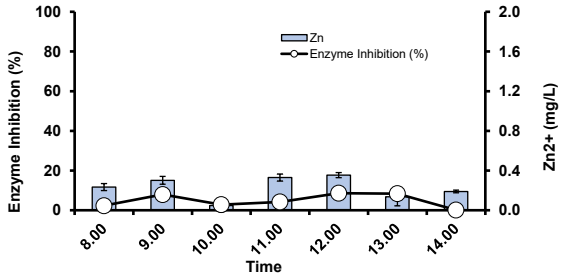

Fig. 2. Near real-time detection of zinc in Kuah's Jetty waters in Langkawi waters using the achromopeptidase inhibitive enzyme assay on day 1. Error bars represent mean ± standard deviation (n=3).

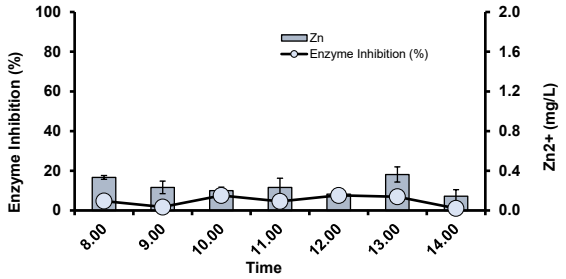

Fig. 3. Near real-time detection of zinc in Kuah's Jetty waters in Langkawi waters using the achromopeptidase inhibitive enzyme assay on day 2. Error bars represent mean ± standard deviation (n=3).

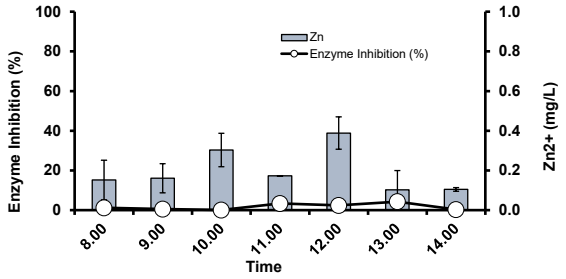

Fig. 4. Near real-time detection of zinc in Kuah's Jetty waters in Langkawi using the achromopeptidase inhibitive enzyme assay on day 3. Error bars represent mean ± standard deviation (n=3).

The fact that heavy metal concentrations in rivers and oceans can change over time is evidence of the ever-changing character of these contaminants. Heavy metal concentration in sediments also varies across time and space, highlighting the intricate patterns of environmental pollution [45]. An essential part of environmental forensics, addressing these variations calls for fast detection technologies that can follow changes in heavy metal concentrations.

Batch processing of samples, which necessitates collection and transportation to a laboratory for analysis, has traditionally been the mainstay of heavy metal detection procedures [46–48]. This method is laborious and can miss quick shifts in pollution levels. A move towards real-time or near real-time monitoring approaches has occurred in reaction to these restrictions. Emerging as potential options for quick environmental evaluation are innovations in bioassays that utilize plants, microbes, and enzymatic reactions [49–51].

Enzyme assays are ideal for on-site assessment because they provide rapid results. The entire process, from sample collection to detection, may be finished in about an hour using portable spectrophotometry. Monitoring drinking water systems quickly and in near-real-time is essential to preserve public health. Monitoring is a crucial early warning system that helps minimize health risks and prevent exposure to hazardous substances by quickly identifying chemical and biological contaminants. This prompt response adheres to stringent legislative standards that ensure water quality remains within acceptable consumption limits and empowers water providers to address any arising concerns promptly. Furthermore, it enhances operational efficiency by allowing you to adjust your water treatment processes in real-time, optimizing resource use and reducing costs associated with excessive treatment or emergency pollutant removal. Public confidence in the water supply is crucial, and the transparency and promptness of near-real-time biomonitoring assure customers that their drinking water is safe. It also helps prevent infrastructure damage from contaminants and allows for quick responses to changing environmental conditions that may affect water quality, such as weather events or industrial accidents. Ensuring the safety and purity of drinking water is crucial for public health, regulatory adherence, and the efficient functioning of water treatment plants. Swift biomonitoring is crucial in this procedure [52–55].

The research has demonstrated that enzyme-based tests effectively detect changes in heavy metal levels over time in water bodies located in industrial areas. The achromopeptidase test monitors mercury in marine habitats, showcasing a unique method and demonstrating the technique's potential. Future studies will build upon this foundation by discovering more sampling locations and conducting thorough field experiments. This trend confirms the effectiveness of enzyme tests for environmental monitoring. It paves the way for broader use and advancement of real-time detection systems to improve our capacity to respond to environmental toxins.

CONCLUSION

Enzyme tests, namely the achromopeptidase dye-binding assay, are a significant tool in environmental management for identifying contaminants, notably zinc, in Kuah's Jetty in Langkawi. Showcasing responsiveness to minimal levels of zinc for early detection of the metal, allowing for immediate corrective measures. Enzyme tests are cost-effective and easy to use, making them excellent for broad environmental evaluations and greatly enhancing our comprehension of ecological health. Furthermore, the minimal inhibition seen in our tests indicates that the region being studied is still reasonably clean, emphasizing the necessity of continuous monitoring to protect this ecotourism destination. The data obtained from these evaluations can improve government and increase public awareness and conservation efforts. This research highlights the importance of enzyme tests in environmental conservation by providing a quick, precise, and easily available method to protect both ecological balance and public health.

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