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Synechocystis, photo by BASF

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Introduction is about 600 words, covering the aims of the research and provide an adequate background, avoiding a detailed literature survey or a summary of the results. **Materials and Methods** should emphasize on the procedures and data analysis. **Results and Discussion** should be written as a series of connecting sentences, however, for a manuscript with long discussion should be divided into subtitles. Thorough discussion represents the causal effect mainly explains why and how the results of the research were taken place, and do not only re-express the mentioned results in the form of sentences. **Concluding** sentence should be given at the end of the discussion. **Acknowledgements** are expressed in a brief; all sources of institutional, private and corporate financial support for the work must be fully acknowledged, and any potential conflicts of interest are noted.

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Saharjo BH, Nurhayati AD. 2006. Domination and composition structure change at hemic peat natural regeneration following burning; a case study in Pelalawan, Riau Province. Biodiversitas 7: 154-158. DOI: 10.13057/biodiv/d070213.

The usage of "et al." in long author lists will also be accepted:

Smith J, Jones M Jr, Houghton L et al. 1999. Future of health insurance. N Engl J Med 965: 325-329. DOI: 10.10007/s002149800025.

Book:

Rai MK, Carpinella C. 2006. Naturally Occurring Bioactive Compounds. Elsevier, Amsterdam.

Chapter in the book:

Webb CO, Cannon CH, Davies SJ. 2008. Ecological organization, biogeography, and the phylogenetic structure of rainforest tree communities. In: Carson W, Schnitzer S (eds.). Tropical Forest Community Ecology. Wiley-Blackwell, New York.

Abstract:

Assaeed AM. 2007. Seed production and dispersal of *Rhazya stricta*. 50th annual symposium of the International Association for Vegetation Science, Swansea, UK, 23-27 July 2007.

Proceeding:

Alikodra HS. 2000. Biodiversity for development of local autonomous government. In: Setyawan AD, Sutarno (eds.). Toward Mount Lawu National Park; Proceeding of National Seminary and Workshop on Biodiversity Conservation to Protect and Save Germplasm in Java Island. Universitas Sebelas Maret, Surakarta, 17-20 July 2000. [Indonesian]

Thesis, Dissertation:

Sugiyarto. 2004. Soil Macro-invertebrates Diversity and Inter-Cropping Plants Productivity in Agroforestry System based on Sengon. [Dissertation]. Universitas Brawijaya, Malang. [Indonesian]

Information from the internet:

Balagadde FK, Song H, Ozaki J, Collins CH, Barnet M, Arnold FH, Quake SR, You L. 2008. A synthetic *Escherichia coli* predator-prey ecosystem. Mol Syst Biol 4: 187. DOI: 10.1038/msb.2008.24. www.molecularsystembiology.com.

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Hexavalent chromium [Cr(VI)] tolerance and reduction activity of *Synechococcus* sp. and *Synechocystis* sp. isolated in West and South Bay of Laguna de Bay, Philippines

ERICKA CRISTINA O. PUJALTE, KRISTA DENISE B. POSADAS, TRISHA C. MORALES♥, AIMEE CAYE G. CHANG

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Abstract. *Pujalte ECO, Posadas KDB, Morales TC, Chang ACG. 2024. Hexavalent chromium [Cr(VI)] tolerance and reduction activity of* Synechococcus *sp. and* Synechocystis *sp. isolated in West and South Bay of Laguna de Bay, Philippines. Asian J Trop Biotechnol 21: 1-9.* Cyanobacteria are prevalent in terrestrial and aquatic ecosystems which can tolerate stress caused by heavy metals. In the Philippines, various anthropogenic activities have contributed to the heavy metal contamination in water systems. Laguna de Bay is the largest inland body of water in the Philippines that functions as a multipurpose lake; however, heavy metal contamination such as hexavalent chromium [Cr(VI)] has progressed through the years due to various anthropogenic activities. This study evaluated the capability of cyanobacterial strains isolated from Laguna de Bay to tolerate and reduce varying concentrations of Cr(VI) using different parameters. Cyanobacterial isolates from Tadlac and Jamboree Lake were subjected to tolerance assay in varying Cr(VI) concentrations, followed by the reduction assay utilizing 1,5-Diphenylcarbazide (1,5-DPCZ) at OD_{540}. Through morphological characterization, two genera were identified: *Synechococcus* sp. from West Bay and *Synechocystis* sp. from South Bay. This study revealed that both isolates could tolerate and reduce high Cr(VI) levels within optimum pH of 7 and 8, respectively. The data acquired from the tolerance assay showed that a Cr(VI) concentration of 1000 mg/L still permitted the growth of the two cyanobacteria genera. Percentage reduction of the isolates at their respective optimal pH showed variation wherein *Synechococcus* sp. at pH 7 exhibited a 58% Cr(VI) average reduction compared to *Synechocystis* sp. at pH 8, which then exhibited a 66% Cr(VI) average reduction. The present study's findings indicate the potential of the two indigenous cyanobacteria in the bioremediation of Cr(VI) in Laguna de Bay.

Keywords: Bioreduction, biosorption, Cr(VI), cyanobacteria, tolerance

INTRODUCTION

Microorganisms such as cyanobacteria have long been acknowledged for their ability to remove heavy metals in terrestrial and aquatic environments. These microorganisms undergo oxygenic photosynthesis, allowing them to amalgamate and produce algal blooms in extreme environmental conditions (Huertas et al. 2014). Cyanobacteria are cost-effective, eco-friendly, low maintenance, and fast-growing; thus, they are excellent agents in bioremediation (Kulal et al. 2020). Aside from being highly diverse organisms, cyanobacteria are also known to inhabit various aquatic systems, such as those with heavy pollution, owing to their simple growth requirements and ability to acclimate to changing environmental factors (Abed et al. 2009).

Cyanobacteria, under the presence of heavy metals in a water source, can experience an exerted impact on their physiological processes; however, these species can adopt strategies at the cellular and molecular level to be able to combat the stress caused by heavy metal ions (Al-Amin et al. 2021). In addition, the ability to excrete heavy metal ligands, allow cyanobacteria to adapt to high metal concentrations (Huertas et al. 2014).

Heavy metals are a group of metals and metalloids characterized by their relatively high atomic number and atomic density, such as Mn, Pb, As, Cr, and Cu (Raychaudhuri et al. 2021). Even in low concentrations, most heavy metals are classified as toxic, carcinogenic, and naturally mutagenic. During prolonged exposure, humans and animals can acquire heavy metal poisoning from contaminated sources via dermal contact, inhalation, and consumption of contaminated food (Kumar and Bharadvaja 2020). Due to their toxicity, heavy metals are known environmental pollutants, and contamination can be transmitted to humans by consuming contaminated organisms such as Nile tilapia (*Oreochromis niloticus*) (Alam et al. 2019). Heavy metal pollution from anthropogenic activities such as agriculture and urban runoff increases public health risks due to their persistence in aquatic systems and bioaccumulation in aquatic flora and fauna (Ahmad et al. 2020).

Moreover, with the increase of pollutants in both aquatic and terrestrial ecosystems, biotechnologists and researchers innovated the use of bioremediation–a process in which chemical reactions facilitated by microorganisms and/or biological organisms reduce or transform contaminants into less toxic forms; this process can either be performed in situ or in vivo (Fennell et al. 2011).

Cyanobacteria have a high binding affinity to metal ions due to the presence of negatively charged groups which act as metal-binding sites. Hence, these microorganisms are expected to be effective bioremediation agents against heavy metals as they can detoxify wastewater through phytoremediation (Al-Amin et al. 2021).

In the Philippines, Laguna de Bay is Southeast Asia's largest freshwater lake and the third largest freshwater lake (LLDA 2016). The lake offers importance for its unique ecosystem and high economic importance; however, its water quality has continuously declined due to waste contaminants such as heavy metals brought by anthropogenic activities along its tributary rivers (Sacdal et al. 2022). Heavy metal runoff has been one of the main pollutants of surface and groundwater in areas with high urbanization and industrialization, especially in the western and southern parts of the lake (Vardhan et al. 2019). The presence of metal ion pollutants enabled several species of microalgae to develop resistance against metal ions found in Laguna de Bay (Rai et al. 1981; Nacorda et al. 2007).

Therefore, with the potentiality of cyanobacteria isolated from the West and South Bays of Laguna de Bay as a phytoremediation agent, this present study intends to detect and further investigate its capability to thrive in harsh environments, particularly with Cr(VI) exposure. Lastly, this study aims to evaluate the biosorption activity of the harvested cyanobacterial cultures in varying Cr(VI) concentrations and pH levels. These objectives prompt the need to conduct the study and further evaluate the nature of cyanobacterial strains as phytoremediation agents.

MATERIALS AND METHODS

Sampling site

The sampling site was in Laguna de Bay (14.3935° N, 121.1939° E), Philippines, particularly in the West Bay (Jamboree Lake; 14°23′8″N 121°2′8″E) and South Bay (Tadlac Lake; 14°10′57″N 121°12′23″E) (Figure 2) which are active sites for industrial, household, and agricultural pollutants. Laguna de Bay is known to be the largest inland body of water in the Philippines, having a total surface area of 900 km^2 with a highest elevation point of 12.50 meters and lowest elevation point of 10.50 meters (LLDA 2016).

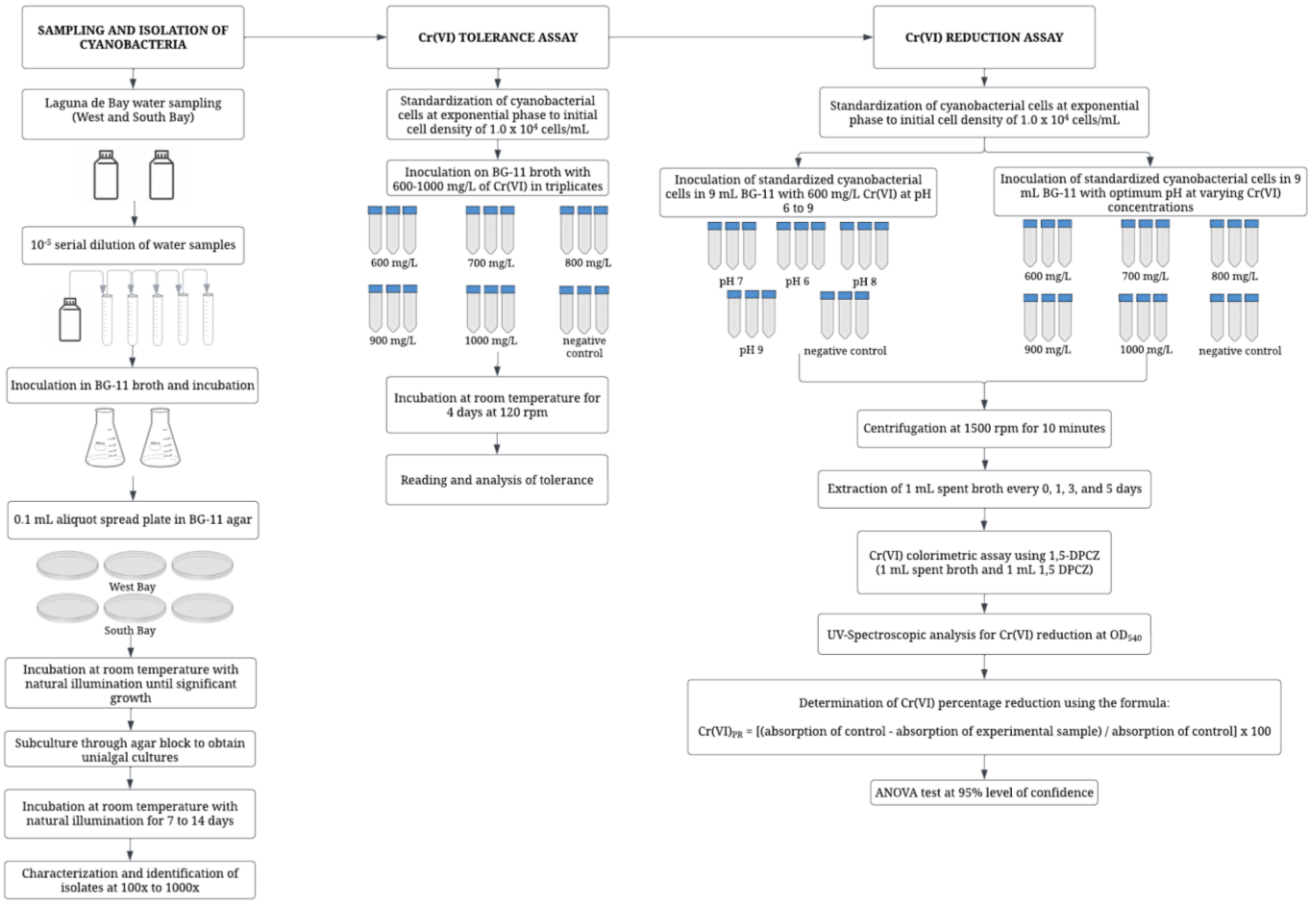


Figure 1. An experimental framework for the Cr(VI) tolerance and reduction activity of *Synechococcus* sp. and *Synechocystis* sp. isolated in the West and South Bay of Laguna de Bay, Philippines

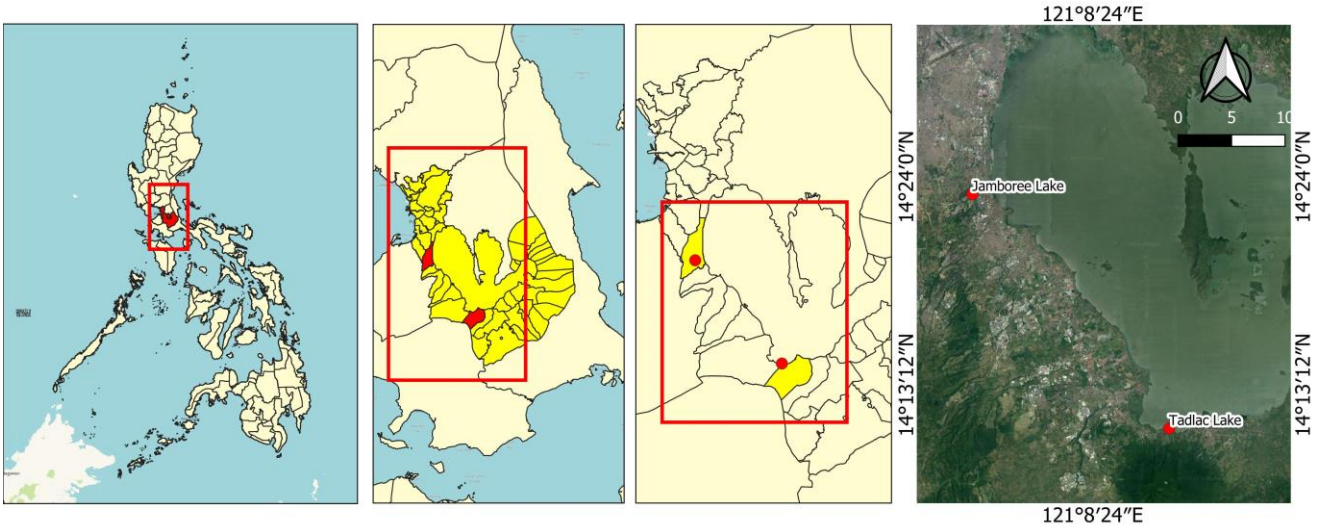


Figure 2. Map of Laguna de Bay, Philippines, and the sampling sites of the cyanobacterial strains: Jamboree Lake, Muntinlupa, and Tadlac Lake, Los Baños, Philippines

Standard sampling protocols

The standard procedure for sampling cyanobacteria by the Center for Freshwater Biology (2010) was followed throughout the collection. Water sampling was conducted in October, mid-day between 10 AM and 3 PM. It was collected in Jamboree Lake (West Bay) and Tadlac Lake (South Bay) at a 5-20 cm depth using a plastic bucket, which was then transferred into four 500 mL sterile HDPE bottles with proper labeling. The presence of visual surface "blooms" of cyanobacteria served as an indication of cyanobacterial accumulation in the location. Handling of water samples was conducted aseptically. Lakewater pH from both sampling sites was determined in duplicates using pH test strips.

Isolation and characterization of cyanobacteria species

The isolation method was derived with modification from De Sotto et al. (2015) study. One milliliter (1 mL) of the collected water sample was serially diluted to 10^{-5} (Figure 1). The standard formula for BG-11 was used for broth and plate media preparation that contained: 1.5 g/L $NaNO_3$, 0.04 g/L K_2HPO_4, 0.075 g/L $MgSO_4•7H_2O$, 0.036 g/L $CaCl_2•2H_2O$, 0.006 g/L citric acid, 0.006 g/L ferric ammonium citrate, 0.001 g/L $Na_2•EDTA•2H_2O$, 0.02 g/L Na_2CO_3, 2.86 g/L H_3BO_3, 1.81 g/L $MnCl_2•4H_2O$, 0.222 g/L $ZnSO_4•7H_2O$, 0.39 g/L $NaMoO_4•2H_2O$, 0.079 g/L $CuSO_4•5H_2O$, 0.0494 $Co(NO_3)_2•6H_2O$ (Kirrolia et al. 2012). Adjustment of pH for broth and plates via the addition of sterilized 0.1 M NaOH and/or 0.1 M HCL was performed to maintain a pH value of 7-8. Next, 1000 µL serially diluted water samples were extracted from the BG-11 broth and plate cultures for incubation and characterization. Prepared samples were done in triplicates and incubated at room temperature for 7-14 days.

A natural illumination cycle was followed during the cultivation. After successful cultivation, cyanobacterial colonies were purified via spread plate inoculation and agar block method. Inoculated cyanobacterial isolates were then viewed through a compound light microscope at 100x to 1000x magnification and identified through the use of dichotomous keys from Nienaber and Steinitz-Kannan (2018) and Casamatta and Hašler (2016). After identification, one cyanobacteria genera from West Bay and South Bay were selected to evaluate Cr(VI) tolerance and reduction.

Growth studies and measurements

The cultivated cyanobacterial strains were cultured and maintained in 300 mL Erlenmeyer flasks using BG-11 broth medium in pH 7-8 and were agitated at 100-120 rpm using an orbital shaker at room temperature under natural illumination. Growth of the samples was measured using a UV-visible spectrophotometer (at 650 nm every 5 days) and terminated for microscopic examination of the cells and cell quantification using a hemocytometer and a compound light microscope.

Cr(VI) tolerance

Nine (9) mL of BG-11 medium and 1 mL of sample from each bay at the exponential growth phase were subjected to serial dilution of $1x10^4$ cells/mL for standardization through hemocytometer cell counting. The standardized cells were then subjected to varying concentrations (600, 700, 800, 900, and 1000 mg/L) of Cr(VI) in triplicates. The prepared mixtures were placed in an orbital shaker at 100-120 rpm at room temperature for 4 days. Unexposed cyanobacterial isolates inoculated in BG-11 broth were used as a negative control. Tubes were observed for tolerance through turbidity and quantified by UV-visible spectroscopy analysis and viable cell count.

Cr(VI) reduction

One (1) mL of cyanobacterial culture from each bay was vortexed and extracted into a falcon tube containing 9 mL BG-11 medium to perform the standardization of cyanobacterial cells to a serial dilution value of $1x10^4$

cells/mL through hemocytometer cell counting. Optimization of pH for Cr(VI) reduction was done by drawing out 1 mL of the standardized cyanobacterial cells into falcon tubes containing 600 mg/L Cr(VI) with varying pH (6, 7, 8, and 9). All prepared samples were done in triplicates. The second part of the reduction assay was performed by inoculating 1 mL of standardized cyanobacterial cells into 9 mL BG-11 medium under optimum pH at varying Cr(VI) concentrations (600, 700, 800, 900, and 1,000 mg/L). All prepared samples were also done in triplicates. The prepared samples from both reduction assay parts were centrifuged at 1,500 rpm for 10 minutes. Cr(VI) colorimetric assay was performed by extracting 1 mL of spent broth on days 0, 1, 3, and 5, followed by the addition of 1 mL of 1,5-Diphenylcarbazide (0.5 g 1,5-DPCZ in 100 mL absolute ethanol and 400 mL 3.6 N H_2SO_4) to achieve a ratio of 1:1 in each solution. Those solutions were mixed by pipetting up and down before transferring to a cuvette for UV-visible spectroscopy analysis of Cr(VI) reduction at OD_{540}. One (1) mL of standardized cyanobacterial cells was inoculated into 9 mL of BG-11 medium without Cr(VI), which served as the negative control. The percentage reduction of Cr(VI) was determined using the formula (Bennett et al. 2013):

$$Cr(VI)_{PR} = \frac{(absorbance\ of\ control - absorbance\ of\ experimental\ sample)}{absorbance\ of\ control}\ x\ 100$$

The obtained percentage reduction of Cr(VI) was further evaluated using the ANOVA test at a 95% confidence level and the Tukey Honest Significant Difference (HSD) and post-hoc test.

RESULTS AND DISCUSSION

pH lake water sampling

The lake water pH from the sampling sites was measured twice, wherein the water in Tadlac Lake, Los Baños (South Bay), had a pH of ~8 during both trials, while the pH of the water from Jamboree Lake, Muntinlupa (West Bay) had a pH of ~7 on both trials (Table 1).

Species description and morphology

The cyanobacterial isolates from West and South bays, Laguna de Bay, were observed on a compound light microscope at 100x to 1000x magnification, as shown in Figure 3. Identification through morphology was done using dichotomous keys from Casamatta and Hašler (2016) and Nienaber and Steinitz-Kannan (2018).

***Synechococcus* sp.** This species was isolated from West Bay, Laguna de Bay. The cells are unicellular, rod-shaped, light-green to blue-green, and without a mucilaginous sheath.

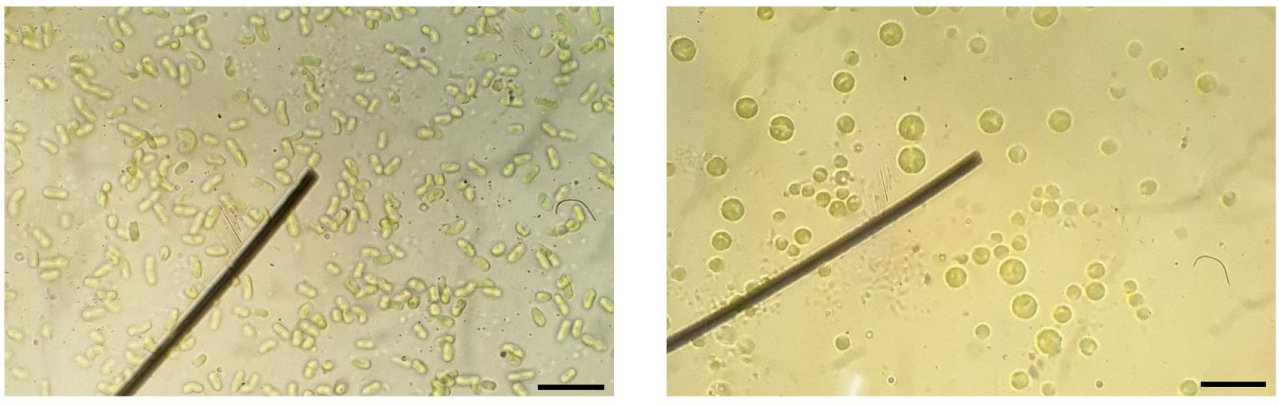
***Synechocystis* sp.** This species was isolated from South Bay, Laguna de Bay. The cells are unicellular, spherical, bright green in color, and have a colorless mucilaginous sheath.

Growth studies and measurements

Cyanobacterial cells at their exponential phase were utilized in this study. *Synechococcus* sp. and *Synechocystis* sp. were subjected to a UV-Visible spectrophotometer at 650 nm to verify their growth phase. Both isolates were found to be at their exponential phase at day 35 (Figure 4), which makes the cells viable for experimentation.

Table 1. Physico-chemical parameters in the sampling sites in Laguna de Bay, Philippines

Parameters	West Bay	South Bay
Exact location	14°23'8" N 121°2'8" E	14°10'57" N 121°12'23" E
pH	6.5 - 7.0	7.5 - 8.0



A. *Synechococcus* sp. 1000 X

B. *Synechocystis* sp. 1000 X

Figure 3. Microscopic observation of cyanobacterial isolates from A. West and B. South Bay, Laguna de Bay, Philippines (each scale bar = 10 µM; magnification = 1000x)

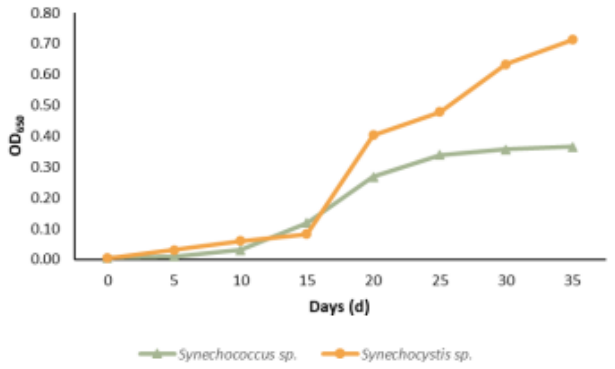


Figure 4. Growth curve of *Synechococcus* sp. and *Synechocystis* sp. isolated from West and South Bay, Laguna de Bay, Philippines

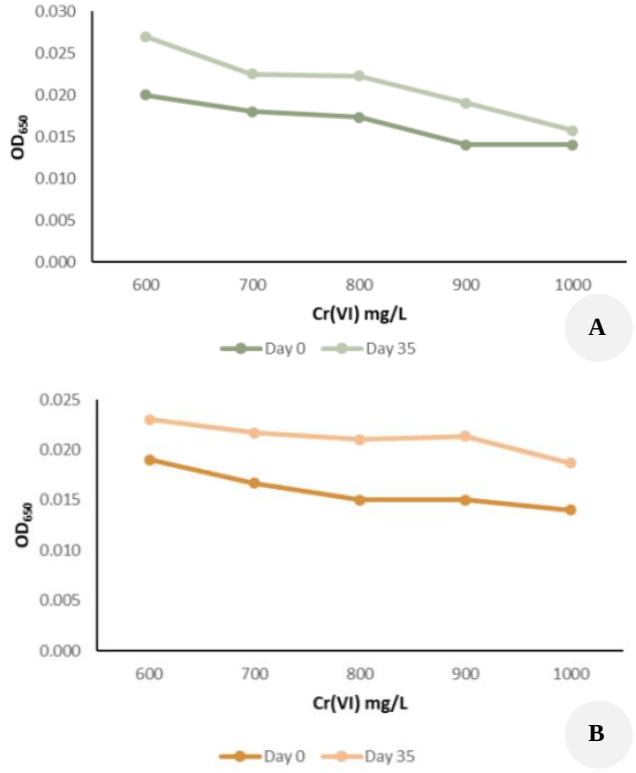


Figure 5. Comparison of Cr(VI) tolerance of A. *Synechococcus* sp. and B. *Synechocystis* sp. in varying Cr(VI) concentrations at Day 0 and Day 35

Tolerance of cyanobacterial isolates against Cr(VI)

After 35 days, the cyanobacterial isolates that were amended with varying Cr(VI) concentrations were observed wherein visible cyanobacterial growth was observed in all tubes amended with varying Cr(VI) concentrations viz. 600, 700, 800, 900, and 1000 mg/L for *Synechococcus* sp. and *Synechocystis* sp. After this, tubes were observed for turbidity, and 1 mL aliquots of each tube were quantified through absorbances at OD_{650} to indicate cyanobacterial cell growth (Figure 5.A and 5.B). From the acquired data, it can be inferred that a Cr(VI) concentration of 1000 mg/L still permitted the growth of the cyanobacterial cultures isolated from the West and South Bays of Laguna de Bay. This verifies the isolated cyanobacterial cells' tolerance capacity against heavy metals such as Cr(VI).

Optimization of pH for Cr(VI) reduction

Moreover, to assess the optimum pH for Cr(VI) reduction, the cyanobacterial isolates were exposed to varying pH levels of 6, 7, 8, and 9 using the lowest experimental Cr(VI) concentration at 600 mg/L. As shown in Figure 6, pH 7 showed the highest Cr(VI) reduction for *Synechococcus* sp. (West Bay), while pH 8 had the highest Cr(VI) reduction for *Synechocystis* sp. (South Bay). The results illustrate similarity with the pH values obtained during water collection in both bays (West Bay: pH 7; South Bay: pH 8). This may indicate that the Cr(VI) reduction of cyanobacteria may be ideal at the pH of the natural environment where the species thrived. Statistically, both isolates showed no significant difference ($p > 0.05$) in varying pH levels at 600 mg/L Cr(VI).

Cyanobacterial Cr(VI) reduction

The results showed the Cr(VI) percentage reduction of the isolates in varying heavy metal concentrations at optimal pH 7 for *Synechococcus* sp. and pH 8 for *Synechocystis* sp. isolates. There was an 8% higher Cr(VI) removal for *Synechococcus* sp. (58% Cr(VI) average reduction) over *Synechocystis* sp. (66% Cr(VI) average reduction). This can be correlated to the significant differences in Cr(VI) reduction for both isolates ($p < 0.05$); post-hoc analysis was performed for verification wherein percentage reduction between 600 mg/L and 1000 mg/L, along with 800 mg/L and 1000 mg/L of Cr(VI) concentrations showed significant differences. Therefore, it can be elucidated that Cr(VI) reduction in *Synechococcus* sp. (42-65% Cr(VI) reduction) and *Synechocystis* sp. (45-78% Cr(VI) reduction) is constant at 600-900 mg/L and starts to decrease at 1000 mg/L significantly. Additionally, Figures 8.A and 8.B show the Cr(VI) percentage reduction of both isolates from days 0 to 5, which generally shows a trend of decreasing heavy metal reduction across all Cr(VI) concentrations (600-1000 mg/L) as contact time increases (Figures 7.A and 7.B).

Discussion

Heavy metals in Laguna de Bay are caused by various anthropogenic sources such as urban and industrial activities, which are common most especially on the western side of the lake. The southern bay, specifically Tadlac Lake, is a volcanic crater lake that is now used for recreational purposes (Sacdal et al. 2022). Hexavalent chromium [Cr(VI)] is amongst these toxic heavy metals found in the lake and is considered carcinogenic. Moreover, an association between Cr(VI) exposure and lung cancer was found through inhalation (World Health Organization 2017). The study of Sacdal et al. (2022) recorded the presence of other heavy metals present in Laguna de Bay such as Ni, Co, Cu, Mn, and As. Elevated concentration of these heavy metals can resulted in

deterioration of cell morphology and also competition for essential nutrients and binding sites for enzymes and transporters being threatened, eventually causing lysis and cell death (Tottey et al. 2012; Tiwari et al. 2019; Kalita and Baruah 2023). Among the mentioned heavy metals, Cr was found to be the most distributed heavy metal in Laguna de Bay, hence the researchers opted for this to be used in the study. One of the toxic characteristics of Cr(VI) is its ability to bind extracellularly to various functional groups found in the microbial cell wall, which can damage microbial enzymes. Consequently, microbes, including cyanobacterial communities, have developed defensive systems to cope with heavy metal stress (Sharma et al. 2022). Cyanobacterial communities in mining sites in the Philippines were detected via isolation-dependent methods, which heavily suggests the potential of cyanobacteria as a phytoremediation agent due to their tolerance in extreme environments contaminated with heavy metals (Damatac II and Cao 2022).

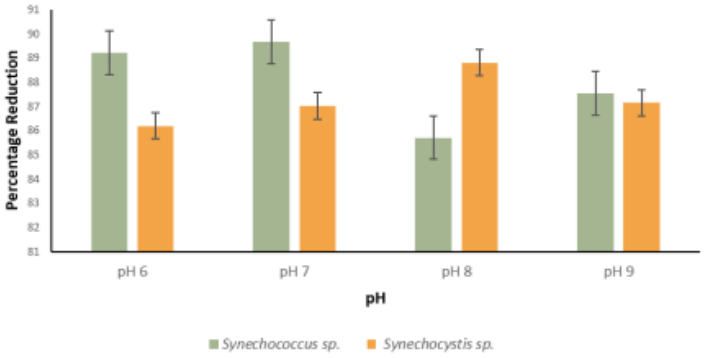


Figure 6. Cr(VI) percentage reduction of *Synechococcus* sp. and *Synechocystis* sp. in varying pH levels at 600 mg/L Cr(VI)

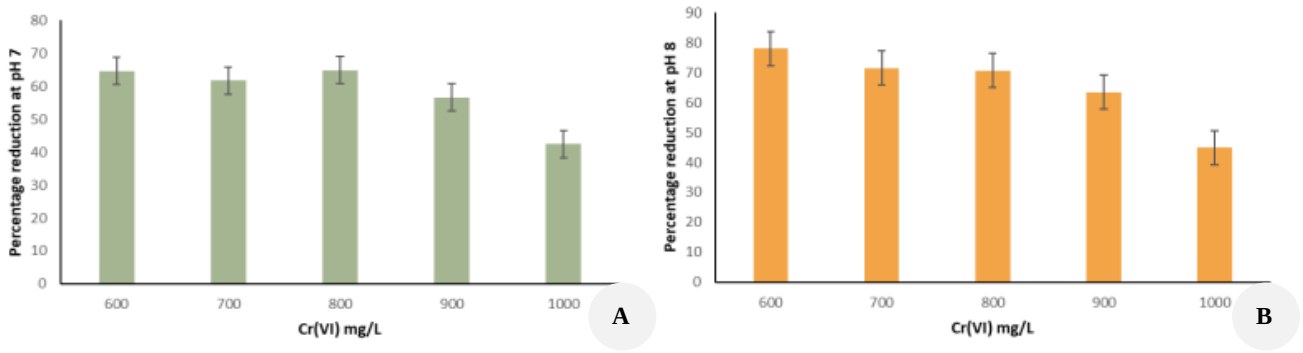


Figure 7. A. Cr(VI) percentage reduction of *Synechococcus* sp. in varying Cr(VI) concentrations at pH 7 and B. Cr(VI) percentage reduction of *Synechocystis* sp. in varying Cr(VI) concentrations at pH 8

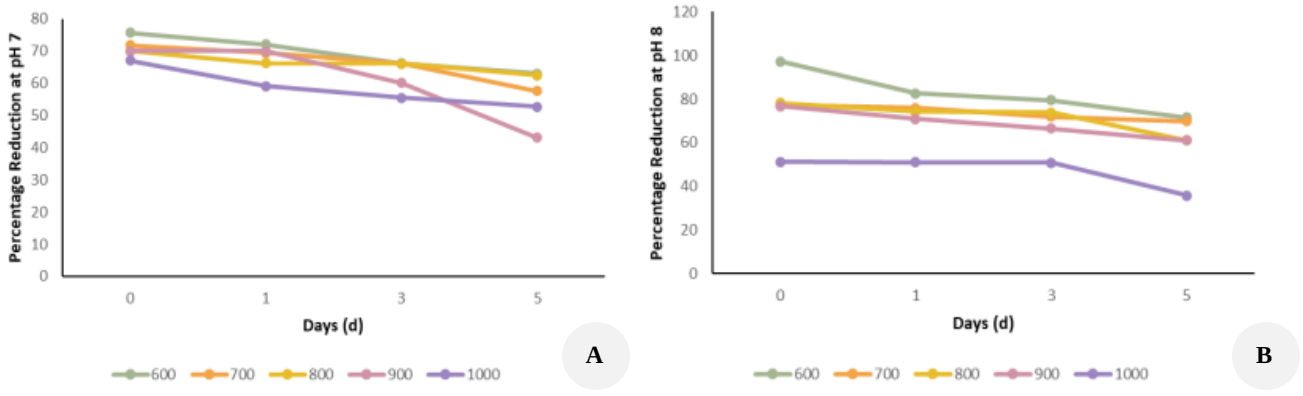


Figure 8. A. Cr(VI) percentage reduction of *Synechococcus* sp. at pH 7 from Day 0 to 5 and B. Cr(VI) percentage reduction of *Synechocystis* sp. at pH 8 from Day 0 to 5

Cr(VI) is known to cause oxidative stress; hence, it is essential to understand cyanobacterial cells' ability to tolerate Cr(VI) levels. The characteristics of cyanobacteria, such as their ubiquity, rapid growth rate, simple growth conditions, and heavy metal tolerance and removal, contribute to these microorganisms' potential as bioremediation agents. Tolerance to heavy metals such as Cr(VI) can be influenced by the type of cyanobacterial species used. Additionally, *Synechococcus* sp. was found to be the most sensitive to Cr(VI); however, sensitivity gradually decreased as the cyanobacterial strains acquired tolerance to heavy metal ions due to prolonged exposure (Munagamage et al. 2016). This further implicates the existence of specificity regarding the sensitivity of cyanobacteria to different types of heavy metal ions present in the environment.

Laguna de Bay is one of the most important freshwater lakes in the country due to it being a multipurpose lake, however, the presence of heavy metal contaminations such as Cr(VI) can potentially threaten its function. In this study, the different cyanobacterial strains, *Synechococcus* sp., and *Synechocystis* sp., were amended with varying Cr(VI) concentrations to determine their tolerance against the heavy metal. The test result showed that at 1,000 mg/L of Cr(VI) concentration, cyanobacterial isolates from both bays could still grow under such harsh conditions (Figures 5.A and 5.B). Along with this, previous studies suggested that *Synechococcoccus* and *Synechocystis* cultures can tolerate high levels of Cr(VI) concentrations, wherein *Synechococcus* showed twice the number of cells compared to *Synechocystis* while both were found to be photosynthetic-dependent. Additionally, *Synechococcus* had higher photosynthetic activity and stronger oxidative stress tolerance mechanisms (Gupta et al. 2013; Khattar et al. 2014; Gupta and Ballal 2015; Gupta et al. 2021).

It is also important to note that since the cultures were isolated from polluted environments, the possibility that the organisms have adapted to heavy metal toxicity and their continuous exposure to heavy metals, such as chromium, may have led them to tolerate high levels of concentrations (Khattar et al. 2014). This further verifies the alarming chromium contamination in the surface water of Laguna de Bay, particularly in West Bay and South Bay, along with the potentiality of cyanobacteria to pave the way for in-situ bioremediation of heavy metals in the environment (Al-Homaidan et al. 2015; Kwak et al. 2015; Cui et al. 2020).

Among the mechanisms that enable these species to thrive under harmful conditions and maintain cellular homeostasis is their ability to perform biosorption. In recent studies, ion exchange has become an emerging factor concerning the factors that influence the biosorption process of these microorganisms. During the process of ion exchange in cyanobacteria, proton and metal ions compete with each other for the binding site, which is governed by the endogenous pH of the cell (Schiewer and Volesky 2000; Tiwari et al. 2019).

Chromium is known to have a pH-dependent equilibrium in aqueous solutions (Mendam et al. 2022). The pH facilitates the metal binding in aqueous mediums (Dixit and Singh 2014). This study investigated the effects of varying pH levels 6, 7, 8, and 9 regarding the Cr(VI) reduction efficiency of *Synechococcus* sp. and *Synechocystis* sp. at 600 mg/L (Figure 6). It was found that the highest Cr(VI) reduction was at pH 7 for *Synechococcus* sp. (90%) and at pH 8 for *Synechocystis* sp. (88.8%); this may be attributed to the favorable growth conditions of the cyanobacterial biomass in the respective pH levels. *Synechocystis* sp. possesses heavy sheaths known to be chelating agents for the biosorption of positively charged heavy metal ions in aqueous solutions (De Philippis et al. 2011). On the other hand, *Synechococcus* sp., though lacking the presence of a heavy sheath, has also been demonstrated to have biosorption abilities towards heavy metal ions such as lead and uranium (Acharya et al. 2008; Anburaj et al. 2020). At a higher pH, there are fewer concentrations of H^+ ions compared to low pH, resulting in the reduced competition of H^+ ions and Cr(VI) ions existing in the solution that would bind in the cellular structure of the cyanobacteria (Sen et al. 2018). In addition, with increased pH, adsorbing sites may undergo deprotonation due to low concentration of H^+ ions that causes cation exchange of H^+ with Cr(VI) ions, allowing better metal removal capability (Miranda et al. 2012; Sen et al. 2018). This may explain why the present study's optimum Cr(VI) reduction for obtained cyanobacterial isolates was at pH 7 and 8. A trend was observed in several studies wherein pH beyond the optimum results in a decrease in metal adsorption due to the formation of hydroxylated complexes of the metal that would compete with the active adsorbing sites of cells (Volesky and Holan 1995; Pardo et al. 2003; Gabr et al. 2008).

In this study, it was also found that at optimum pH (7 and 8), there was efficient Cr(VI) reduction from *Synechococcus* sp. (42-65%) and *Synechocystis* sp. (45-78%) at Cr(VI) concentrations of 600-1,000 mg/L (Figures 7.A-B). At the highest concentration of 1000 mg/L, there was a significant decrease in Cr(VI) reduction. It can be inferred that cyanobacterial isolates can effectively reduce Cr(VI) at their respective optimum pH; however, there is a specific maximum value of initial Cr(VI) wherein isolates can effectively bind Cr(VI) ions existing in the solution, beyond this threshold value can have detrimental effects to the organism leading to reduced adsorption (Sen et al. 2018). An increasing number of studies have been conducted on the subject of transformation of the water-soluble and eminently toxic chromium (VI) to a less toxic form and insoluble chromium (III) (Shukla et al. 2012). Furthermore, as shown in Figures 8.A and 8.B, the percentage reduction of varying Cr(VI) concentrations generally decreased from days 0 to 5 for both isolates. Similar trends were observed from Gupta and Rastogi (2008) and Sen et al. (2018), wherein there was a gradual decrease in Cr(VI) biosorption as contact time increased. This may be due to the uptake of Cr(VI) on the adsorption sites of the cell surface; as metal ions progressively cover more sites, the adsorption rate becomes slower until equilibrium uptake is achieved (Miranda et al. 2012). The results of the present study show that *Synechococcus* sp. and *Synechocystis* sp. isolated from Laguna de Bay possess

a high reduction capability for Cr(VI) at optimum pH 7 and 8 and may be efficiently used as biosorbents for the removal of heavy metal from aquatic systems. Various studies from other countries presented the tolerance and removal capacity of *Synechocystis* sp. wherein Ozturk et al. (2009) concluded that the cyanobacterial exopolysaccharide from cyanobacteria isolated in Mogan Lake and Bafa Lake, Turkey, including its monomer components, contributes to its tolerance and reduction capacity. Additionally, *Synechocystis* strains isolated from Lahore, Pakistan, showed chromium resistance, reduction, and the production of non-protein thiols when exposed to chromium stress (Hameed and Hasnain 2012). On the other hand, the Cr(VI) tolerance and bioreduction capacity of *Synechococcus* sp. has not been widely studied.

This study revealed *Synechococcus* sp. and *Synechocystis* sp. isolated from the West and South Bays of Laguna de Bay, Philippines, to tolerate and efficiently reduce Cr(VI) under unfavorable conditions. As such, the study provided further evidence regarding the potential of cyanobacteria as a phytoremediation agent, particularly against heavy metal pollution such as Cr(VI). Furthermore, the isolates can be used in biotechnological applications to reduce heavy metal pollutants in contaminated aquatic environments. Cyanobacterial species can be genetically modified to increase and enhance growth, photosynthetic efficiency, and tolerance against environmental factors (Priyanka et al. 2020). Furthermore, the photoautotrophic capacity of cyanobacteria makes them advantageous as a long-term bioremediation agent due to the absence of secondary pollution during the reutilization cycle of biomass (Mulbry et al. 2008; Brar et al. 2017). However, the study by Al-Amin et al. (2021), highlighted that the use of cyanobacteria as bioremediation agents can jeopardize indigenous aquatic systems if toxic cyanobacterial species are used.

However, this study is only limited to the isolated cyanobacterial strains viz. *Synechococcus* sp. and *Synechocystis* sp. were isolated in the West and South Bays of Laguna de Bay, Philippines. Hence, using other cyanobacterial strains from other parts of the lake could further help assess the potentiality of cyanobacteria in tolerating and reducing Cr(VI). Other heavy metals could also be assessed to verify further their capability in reducing heavy metal pollutants. In addition, the measurement of Cr(VI) uptake and the effect of the inoculum size are also important parameters to be considered for heavy metal reduction which could be done in future studies.

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Review: Current perspectives on enzyme applications in medicine, agriculture, and industries

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Abstract. *Dahiru MM, Abdulhamid AA, Abaka AM. 2024. Review: Current perspectives in enzyme applications in medicine, agriculture, and industries. Asian J Trop Biotechnol 21: 10-25.* The conventional use of chemicals worldwide in different industries has significantly increased, impacting the environment. The need to improve industrial processes led to prospects into enzymes from various sources, including microbial sources to substitute the toxic chemicals and processes with environmentally friendly processes and biodegradable waste products. Enzyme applications are notably getting more attention in medicine, agriculture, and industries, including pharmaceuticals, food, detergents, leather, and cosmetics. They are utilized in these industries to enhance durability of product, shorten process time, improve efficiency and stability, and properly manage waste. Medicine mainly uses them for diagnostic and treatment purposes, including proteases and amylases. The pharmaceutical industries explore the specificity of enzymes to bind target substrates, yielding specific and desired products via reactions, including hydrolysis, acylation, and esterifications. Waste and pathogen treatments, in addition to soil and plant improvements, are the major applications of enzymes in agriculture, employing pectinases, cellulases, and xylanases. In the food industry, amylases are used for starch degradation and dough improvement, while proteases are used for juice beer production clearance. The detergent industry adds enzymes to its formulations, including lipases, proteases, and cellulases, to improve stain removal by modifying fabric fibers to release dirt and soil particles. Enzymes, including proteases and lipases, are used in the leather industry for curing, soaking, dehairing, degreasing, and tanning, in addition to waste elimination. In comparison, an immobilized lipase is utilized in the cosmetic industry to produce retinoids, protein disulfide isomerase, glutathione sulfhydryl oxidase, and transglutaminase are applied in hair waving. Furthermore, developing the cost-effective technique of enzyme immobilization technology further promotes the application of enzymes in these endeavors attributed to their stability and recyclability. Thus, there is a significant improvement in research towards green and eco-friendly applications of enzymes. This review discusses how enzymes have been successfully deployed and utilized in medicine, agriculture, pharmaceutical, food, detergent, leather, and cosmetics industries. Enzymes in these industries exhibited beneficial applications, though the recyclability of the enzymes remains challenging for some endeavors. However, immobilization techniques have been gaining attention which might present a solution to the challenges associated with enzyme reusability.

Keywords: Agriculture, cosmetics, detergent, industries, leather

INTRODUCTION

Enzymes, otherwise called biocatalysts, have for centuries been in use due to their high efficiency and substrate specificity to yield valuable products for several uses (Madhavan et al. 2017; Prasad and Roy 2018). The human body, which comprises cells and tissues, requires a constant supply of nutrients in food containing both plant and animal-sourced enzymes. The most important advantage of enzymes is their chemical and stereoselectivity during catalysis, in which they catalyze and convert specific reactions and substrates into products (Choi et al. 2015; Kaushal et al. 2022). Furthermore, these specificity covers conditions, including temperature and pH, attributed to the optimal functional conditions of their sources, such as microbes (Kaushal et al. 2022); enzyme specificity is a high requirement in industries that use them. Enzymes catalysis de novo reactions in the body, vital for the body's normal functioning, in which food is converted to energy used for synthesis and degradation (Kaur and Sekhon 2012). The inherent characteristics of enzymes to catalyze synthesis, change, or break down molecules are the main reason why they are exploited by several industries and partly due to their most environmentally friendly byproducts (Mata et al. 2010; Huisman and Collier 2013; Kapoor et al. 2017; Madhavan et al. 2017; Sun et al. 2018). Biocatalysts from different sources, including microbial, plant, and animal, have been used in medicine, agriculture, and industries to yield products such as wine, food, and drugs (Gupta et al. 2017; Mariyam et al. 2024). The growing interest in enzyme applications in industries is driven by the increased demand for sustainable and eco-friendly industrial processes (Ranjbari et al. 2019)

Microbial sources especially bacterial and fungal, are the most explored enzyme sources due to the diversity in the optimum conditions to use, ease of production, and optimization (Okpara 2022). Moreover, these sources can be engineered to produce efficient enzymes that withstand harsh conditions, readily on their availability and higher replication rates (Okpara 2022). Different organisms can produce isoenzymes that function in different pH and temperature conditions. These enzymes are utilized in medicine, agriculture, food, pharmaceutical, cosmetics, leather, and detergent industries, with future biotechnologies promoting their advancements and utilization in these industries (Okpara 2022). In those industries, enzymes are crucial in various processes, especially in food industries, exhibiting their significance, with notable examples including alpha-amylase, protease, lipase, pectinase, and glucose oxidase. Moreover, enzymes from microbial sources offer advantages over animal and plant sources, including genetic modification (over-expression of specific genes) and large-scale production via solid-state and submerged fermentation techniques; however, many remain unexplored. Protein engineering provides an avenue to improve and modify enzymes to suit our needs and overcome the challenges associated with the naturally available enzymes including instability to harsh industrial scale conditions (Baweja et al. 2016). These challenges can be lowered by editing specific residues within the enzyme structure via mutation to improve the enzyme's version (Baweja et al. 2016). Moreover, exploring alternative sources of microbial enzymes from natural environments or habitats with extremely harsh conditions offers other alternative methods to obtain industrial stable enzymes (Baweja et al. 2016). Furthermore, some techniques are employed to improve the activities of enzymes and proteins, including rational redesign and random mutagenesis (Baweja et al. 2016). The rational redesign requires the knowledge of the enzyme's mechanism of action and entails reconfiguring the active site of the known 3D structure of the enzyme that has been formulated to achieve the desired product. For the latter, modifications are via direct evolution mimicking the natural evolutionary process, employing variant generation molecular techniques such as chemical agents, error-prone PCR, and repeated oligonucleotide-directed mutagenesis.

Emerging trends show the exploitation of enzyme immobilization techniques in bioprocessing and catalysis, reccently gaining attention (Kaushal et al. 2022; Mohidem et al. 2023). This technique overcomes the challenges encountered using free enzymes, including instability, poor shelf-life, and sensitivity to harsh conditions of industrial processes (Razzaghi et al. 2018; Liu and Dong 2020). Free enzymes are not immobilized; thus, they are free reaction, medium, and cheaper than their immobilized counterpart. However, enzyme recovery for the free enzyme technique has been reported to be challenging (Ribeiro et al. 2011). This cost-effective technique allows enzyme recovery from the reaction mixture, preventing enzyme degradation and allowing reusability (Mohidem et al. 2023). The application of these techniques, including adsorption, entrapment, and cross-linking, improves enzyme properties, such as stability and reusability (Jesionowski et al. 2014; Cao et al. 2016; Cipolatti et al. 2016; Mehta et al. 2016; Grigoras 2017; Mohidem et al. 2023). These methods rely on the nature of the substrate, enzyme employed, and operating conditions for optimum output (Chauhan 2014; Mohidem et al. 2023). The advantages, including high output and efficiency of these techniques, are considered to identify a suitable one for high product specificity (Chauhan 2014; Jesionowski et al. 2014). Furthermore, a comparable catalytic activity was reported previously for both immobilized and bound enzymes (Ge et al. 2023). Additionally, immobilized enzymes offer advantages over free enzymes, including reusability and stability towards reaction conditions (Somu et al. 2022). Thus, a perfect eco-friendly substitute for both conventional catalysis and free enzyme techniques. Notably, advancement in the immobilized enzyme technology is the nanocarriers in the form of encapsulated enzymes further enhancing enzyme reusability and stability to harsh conditions (Cao et al. 2016; Mehta et al. 2016; Grigoras 2017; T.sriwong and Matsuda 2022; Aziz and Abdel-Karim 2023). Moreover, encapsulation is a dose-release control, leading to extended enzyme use (Aziz and Abdel-Karim 2023). Although previous studies highlight the applications of enzymes in different endeavors, emphasizing their advantages over conventional techniques, more research gaps open more opportunities to explore, notably the enzyme immobilization technique, which has been gaining attention recently. The emergence of new applications in enzyme technology is reported in the present study. The present review is focused on the applications of enzymes in medicine, pharmaceutical, agriculture, food, detergent, leather, and cosmetics industries regarding how these enzymes have been utilized to improve different processes to improve industrial processes from toxic chemicals and processes to environmentally friendly processes and biodegradable waste products.

MEDICINE AND PHARMACEUTICAL INDUSTRIES

Medical application of enzymes considers enzymes' therapeutic and diagnostic roles, including alleviating enzyme deficiencies, improving the immune, detoxifying, aiding digestion, and detecting some substances in body fluid levels (Meghwanshi et al. 2020). The use of enzymes in medicine can be singly or combined with other drugs or vaccines to work synergistically (Mane and Tale 2015). Several ailments, such as metabolic and genetic disorders (fibrosis conditions, ocular pathologies), infectious diseases with antibiotic-resistant capabilities, cancer, cardiovascular diseases, and digestive problems, have been reported as targets of enzyme therapy (Gupta et al. 2017; de la Fuente et al. 2021). These enzymes are administered due to their specificity and high affinity, though sometimes they present challenges, including immune response to their presence, short life, and difficulty reaching their targets (Lenders and Brand 2018; de la Fuente et al. 2021). These

challenges hamper the potential application of enzymes, which must be improved for effective application in this endeavor. Although the ELISA technique has been well implemented in the medical field, emerging techniques such as microarray have recently gained attention (de la Fuente et al. 2021). Additionally, angiotensin-converting enzyme 2 has been studied as a potential option for COVID-19 therapy (de la Fuente et al. 2021). Typical examples of enzymes used to treat metabolic disorders include amylase, lipase, and protease (Mane and Tale 2015). Enzymes play key roles in therapeutic medicine; a typical example is using RNases in chemotherapy; antitumor activity of RNase from *Bacillus licheniformis* (baliface) has also been reported (Sokurenko et al. 2016). Moreover, another lysozyme, RNase U, was reported to exert antiviral activity via selective degradation of viral RNA and is regarded as a potential anti-HIV drug candidate (Lee-Huang et al. 1999). A chondroitin sulfate ABC endolyase chemonucleolysis therapy for cervical and lumbar intervertebral disc herniation yielded positive results. It was regarded as a novel approach for many treatments, attributed to its high nucleus pulposus specificity (Ishibashi et al. 2019).

Enzymes diagnose different medical conditions, including diabetes and other health disorders. Typical examples are glucose oxidase for glucose, urease and glutamate dehydrogenase for urea, lipase, carboxyl esterase, glycerol kinase for triglycerides, urate oxidase for uric acid, creatinase, and sarcosine oxidases for creatinine (Le Roes-Hill and Prins 2016; Singh et al. 2016). Glucose oxidase combined with catalase is utilized in test kits, notably biosensors, to detect and estimate glucose levels in biological fluids (Khatami et al. 2022). Cholesterol oxidase has been implicated in the detection of cholesterol, while putrescine oxidase is employed for detecting biogenic amines, such as putrescine, a marker for food spoilage (Le Roes-Hill and Prins 2016). Enzymes are also utilized as therapeutic drugs in ailments characterized by enzyme deficiencies, problems associated with digestion, and diagnostic procedures, such as ELISA and diabetes test kits (Mane and Tale 2015). Another enzyme benefit with medical applications is proteases with several subclasses with different uses. Alkaline protease produced by *B. subtilis* has some therapeutic properties, such as fibrin degradation, and is suggested to have future anticancer potential (Jaouadi et al. 2011). In another study, oral administration of combined aceclofenac and the proteolytic enzymes bromelain and papain via supplementation to patients with lower back pain led to a significant decrease in alkaline phosphatase and serum creatine with prolonged beneficial effects (Naeem et al. 2020). Elastoterase immobilized on bandages has been used for burns and wound treatments (Palanivel et al. 2013; Harish and Uppuluri 2018). Tyrosinase is involved in melanogenesis and the production of L-Dihydroxy phenylalanine (L-DOPA), a precursor for dopamine synthesis, used for the management of Parkinson's disease and control of myocardium neurogenic injury (Zaidi et al. 2014). Enzymes applied in medicine are the target for removing cytotoxic substances from circulation, anticoagulants, and disorders arising from metabolic deficiencies (Kaur and Sekhon 2012).

Adenosine deaminase (pagadamase), which was the first enzyme utilized to treat an inherited immune disorder, acts by cleaving excess molecules of circulating adenosine, thereby reducing the immune toxicity associated with a high level of adenosine (Tartibi et al. 2016; Fejerskov et al. 2017). Pagadamase cleaves the enzyme leading to an increase in the half-life of ADA and decreasing the immune response due to the bovine origin of the enzyme. Another enzyme, α-galactosidase, is vital for digestion and breaks down α-galactoside residues in carbohydrates, with its absence leading to gastrointestinal disturbances. However, this enzyme is now supplemented in food to remedy its absence (Shang et al. 2018). The deficiency of lysosomal acid lipase is associated with metabolic disease; Wolman disease has been reported to be remedied via sebelipase alfa, a recombinant form of the enzyme (Pastores and Hughes 2020). Other recombinant replacement therapies include porphobilinogen deaminase, sacrosidase, and lactase administered for acute intermittent porphyria (Fontanellas et al. 2016), congenital sucrase-isomaltase (Puntis and Zamvar 2015), and lactase deficiencies (Catanzaro et al. 2021), respectively. Chronic total occlusion characterized by coronary artery blockage leads to collagen plaque buildup, obstructing blood flow to the heart. However, catheter administration of type IA collagenase from *Clostridium histolyticum* digest the collagen plagues (Strauss et al. 2003). Moreover, collagenase is employed in fasciotomy to remove the fibrotic fascia of the fingers and palm (Degreef 2016). Furthermore, it is applied in the degradation of fiber plaques associated with Peyronie's disease (Randhawa and Shukla 2019). Tumor cells are linked with increased amino acid metabolism for survival and proliferation. PEGylated kynureninase was reported to exhibit anti-tumor activity via a prolonged depletion of the L-tryptophan metabolite, kynurenine, which can be readily eliminated. Additionally, the enzyme reversed the immune-suppressing effect of interferon-γ-inducible indoleamine 2,3-dioxygenase (Triplett et al. 2018).

Asparaginase, an effective enzyme for managing acute lymphocytic leukemia, has been reported previously (Gupta et al. 2017). Asparagine is a normal nonessential amino acid synthesized in the body; however, tumor cells are short of the enzyme aspartate-ammonia ligase required for the synthesis of asparagine (Radadiya et al. 2020; Wang et al. 2021). Administration of asparaginase doesn't affect normal cellular concentration but leads to a decrease in the extracellular concentration of asparagine, preventing its entry into tumor cells. Hyaluronidase cures heart attack and increases in activity of enzymes such as aldolase, malic dehydrogenase, and isomerase due to myocardial infarction (Chowdhury et al. 2017). Lytic enzymes from isolated bacteriophages effectively treat drug-resistant strains of bacteria and remove dead skin from burns (Gurung et al. 2013). Collagenase has also been reported to facilitate the healing mechanism due to skin burn by removing dead cells, thus working synergistically with antibiotics (Ostlie et al. 2012). Lysozyme as an antimicrobial agent has also

shown potential against HIV (Chan et al. 2006). Extracts isolated from fungi, consisting of amylase, proteases, and cellulases, have been applied to prevent dyspepsia and flatulence as they break down indigestible fiber in food such as cabbage (Fieker et al. 2011). Nattokinase has been previously reported to demonstrate beneficial effects in cardiovascular ailments, including preventive and treatment effects, and is regarded as an alternative cardiovascular disease therapy (Chen et al. 2018). Congenital sucrase-isomaltase associated with the indigestion of sucrose has been reported to be remedied by the administration of *Saccharomyces cerevisiae* β-fructofuranoside fructohydrolase, otherwise called sacrosidase which aids in the digestion of sucrose (Lwin et al. 2004; Matta et al. 2018). Phenylketonuria, characterized by the deficiency of phenylalanine hydroxylase, has been reported to be treated using phenylalanine ammonia-lyase produced from a recombinant yeast, which aids in the digestion of phenylalanine in the gastrointestinal tract (Wallig et al. 2017).

Lysosomal storage diseases present a group of inherited and rare pathologies associated with either lysosomal enzyme deficiencies or molecular transport alterations. Notable examples include Hunter's syndrome, Fabry's disease, and Gaucher's disease. Iduronate 2-sulfatase, which catalyzes the breakdown of glycosaminoglycans dermatan- and heparan-sulfate, is deficient in Hunter's syndrome, accumulating metabolites in tissues and organs. This impairs physical and mental development, which can be improved by intravenous enzyme administration (Whiteman and Kimura 2017; Mohamed et al. 2020; Ueda and Hokugo 2020). Another deficient enzyme associated with lysosomal storage disease (Fabry's disease) is the α-galactosidase A, which leads to cellular accumulation of metabolites, potentially leading to renal and cardiac failures (Chan and Adam 2018; McCafferty and Scott 2019; Azevedo et al. 2021). However, intravenous administration of α-galactosidase A ameliorates symptoms (El Dib et al. 2016). β-glucocerebrosidase cleaves the β-glucosidic linkage of glucocerebroside, an intermediate in glycolipid metabolism. Genetic mutation in the gene for the enzyme results in a lysosomal disease known as Gaucher's disease, characterized by the accumulation of excess glucocerebrosides (Erdem et al. 2018). In Gaucher's disease, the glucocerebrosidase enzyme deficiency leads to the buildup of lipids in the liver, spleen, and bone marrow with symptoms including swelling of these tissues, anemia, and thrombocytopenia. Similarly, exogenous intravenous injection of the enzyme improves the enzyme level (Charrow 2009; Shemesh et al. 2015). Additionally, administration of exogenous α-L-iduronidase, N-acetylgalactosamine-6-sulfate sulfatase, arylsulfatase B, β-glucuronidase, α-D-mannosidase, tripeptidyl peptidase 1 and acid α-glucosidase presents potential treatment alternatives for Hurler's syndrome (Tolar and Orchard 2008; Bie et al. 2013), Morquio syndrome type A (Regier and Tanpaiboon 2016; Lee et al. 2022), Maroteaux-Lamy syndrome (Harmatz et al. 2017), Sly syndrome (Harmatz et al. 2018; Wang et al. 2020), α-mannosidosis (Ceccarini et al. 2018; Lund et al. 2018), Batten disease (Brudvig and Weimer 2022) and Pompe's disease (Kishnani and Beckemeyer 2014; Bellotti et al. 2020), respectively.

The pharmaceutical industries achieved large-scale enzyme production via fermentation using bacteria and fungi strains *Escherichia coli*, *B. subtilis*, *Aspergillus oryzae*, and *A. niger* that give high enzyme yield (Brahmachari et al. 2016; Yang et al. 2017). The ability of enzymes to specifically bind to their target substrate and transform it into a desired useful product is heavily exploited by pharmaceutical industries (Choi et al. 2015). The major targets of enzymes in pharmaceutical industries include hydrolysis, diacylation, acylation, group protection and deprotections, esterification, and others (Meghwanshi et al. 2020). In these industries, enzyme application highly relies on substrate and product specificity, including stereo-, regio-, and chemo-selectivity (Choi et al. 2015). Pharmaceutical synthesis often produces undesirable and environmentally toxic products and harsh process conditions, including high temperatures, which are significantly lowered or abolished in enzyme-catalyzed routes (Choi et al. 2015). Biocatalysts, especially those of microbial origins, are utilized by these industries due to their high specificity and affinity for their substrate which are transformed into specific products under optimum conditions. Enzyme-catalyzed reaction pathways reduce the requirement of harsh chemicals to speed up reactions or the need to apply high temperatures. Thus reducing risk and improving the safety of the processes (Choi et al. 2015). Enzymes are utilized to produce important precursors and intermediates such as amines, alcohols, carbonyl, and carboxylic acid derivatives required for synthesizing different pharmaceuticals via various processes (Wu et al. 2021). A novel β-glucosidase obtained from *Pseudomonas lutea* combined with *S. cerevisiae* was reported to efficiently convert the conversion of cellobiose to ethanol at 4℃ with 91.42% efficiency via simultaneous saccharification and fermentation. The technique was considered energy-saving compared to the commercial β-glucosidase (Tiwari et al. 2014).

A good example of the application of enzymes in the pharmaceutical industry is in the manufacture of sitagliptin, used to treat type II diabetes. Sitagliptin is a dipeptidyl peptidase-4 inhibitor that prevents the blood-retinal barrier increase and inhibits diabetes-induced tight junction disassembly (Gonçalves et al. 2018). Conventionally, this drug is produced by a rhodium-based catalyst through hydrogenation of enamine at high pressure to produce sitagliptin at 97% enantiomeric excess (Sheldon 2011). However, engineered R-selective transaminase from *Arthrobacter* sp. can produce the drug through the conversion of prositagltiptin ketone to sitagliptin with more than 99.95% enantiomeric excess (Sheldon 2011). Furthermore, (R)-3-amino-4-(2,4,5-triflurophenyl) butanoic acid, an intermediate for the drug synthesis is synthesized in dual steps initially requiring the conversion of β-ketoester to β-keto acid by *Candida rugosa* lipase followed by its subsequent conversion *Ilumatobacter coccineus* ω-Transaminase to its equivalent β-amino acid (Kim et al. 2019). A route was previously proposed for the synthesis of the neuroactive drug pregabalin, which exerts

anticonvulsant, pain-killing, and anti-anxiety effects used to treat epilepsy, anxiety, and social phobia (Martinez et al. 2008; Chen et al. 2011; Marouf 2018). Here, lipolase, which belongs to the class of lipase, is employed to synthesize by hydrolyzing selectively and separating the *S*-enantiomer intermediate from the *R*-enantiomer to pregabalin via decarboxylation (Martinez et al. 2008; Chen et al. 2011). The conventional route, bisphosphine rhodium, and nitrilase are employed for the asymmetric synthesis of (S)-3-cyano-5-methylhexanoate. However, both routes are neither cost-effective nor eco-friendly (Hoge et al. 2004; Xie et al. 2006; Zheng et al. 2014). The new route was reported to be more efficient and cost-effective than the classic route by increasing product yield by up to 40-45% with a purity of up to 99.5% and reducing waste generation by 20% (Zheng et al. 2014).

Alcohols with chirality are important precursors and intermediates in the pharmaceutical industries, taking advantage of their chiral moieties for drug synthesis (Prier and Kosjek 2019; Hollmann et al. 2021). Dulox alcohol, which is the precursor for the synthesis of the antidepressant drug Duloxetine, was reported to be synthesized by alcohol dehydrogenase from *Lactobacillus brevis* and EbN1 strain from *Aromatoleum aromaticum* (Leuchs and Greiner 2011). A new route was proposed for (*S, S*)-reboxetine succinate synthesis used for managing the late stage of development of fibromyalgia acting as an antidepressant (Krell et al. 2005; Hayes et al. 2011). Synthesis of this drug requires acetylation of a diol intermediate, whereas, in the conventional route, this process is accompanied by generating unwanted products due to poor enantioselectivity and inefficiency (Choi et al. 2015). In the new route, lipase B from *Candida antarctica* is used, which allows for acetylation of the diol intermediate with good enantioselectivity, resulting in 99% yield and 98% regioselectivity and enzyme recovery and reuse. Thus, a cost-effective route (Hayes et al. 2011). Xemilofiban which is an antiplatelet agent, is synthesized via enantioselective acylation of ethyl 3-amino-5-(trimethylsilyl)-4-pentynoate mediated by *E. coli* penicillin G amidohydrolase to yield the *S*-isomer which act as a chiral synthon for xemilofiban synthesis (Topgi et al. 1999). Nanokitanase employed as a cardiovascular drug, is a bacteria serine protease that demonstrated improved activity and stability as a therapeutic agent following modification via site-directed mutagenesis (Weng et al. 2015). Similarly, Harobin, another serine protease, exhibited improved anti-fibrinolytic and anti-thrombosis activities following genetic modifications. The mutant was hypothesized to be a therapeutic candidate for thrombosis and hypertension due to its high expression and activity level (Li et al. 2017). Furthermore, *Bacillus pumilus* serine alkaline protease employed for harsh conditions due to its high catalytic activity, thermoactivity, and pH stability demonstrated a shifted optimum temperature from 65°C to 75°C and increased activity following its genetic mutation (Jaouadi et al. 2010).

In the conventional synthesis of bicyclic proline, an intensive process requires an excess of metal-based oxidant and reductant through 8 reaction steps. The enantioselective, MAO-catalyzed (monoamine oxidase) synthesis of the intermediate is an attractive alternative that might reduce operation time and waste generation (Li et al. 2012b). However, there has been research for the scale-up of a monoamine oxidase (MAO)-catalyzed process for enantiomerically pure desymmetrization of a bicyclic proline intermediate. This is an important precursor in synthesizing boceprevir, an NS3 protease inhibitor used to treat chronic hepatitis C infections (Kjellin et al. 2018). The MAO-catalyzed method is more convenient and faster, generating less waste (Li et al. 2012b). The properties of MAO are improved through a series of protein engineering through subsequent evolution and mutation. Bisulphate is added to alleviate its inhibition while capturing the imine compounds. Significant improvements in MAO activity, solubility, and thermostability are achieved through protein engineering via 4 rounds of evolution involving random mutations and subsequent screening for desired phenotypes. Adding bisulfate to the MAO-catalyzed process to capture imine compounds mitigated its irreversible inhibition (Li et al. 2012b).

Table 1. Enzymes in medicine and pharmaceutical industries

Enzymes	Applications
Amylase, lipase, and protease	Treatment of metabolic disorders (Mane and Tale 2015)
RNases	Chemotherapy (antitumor) (Sokurenko et al. 2016)
Glucose oxidase, urease, glutamate dehydrogenase, lipase, carboxyl esterase, glycerol kinase, urate oxidase	Diagnostic purposes (Le Roes-Hill and Prins 2016; Singh et al. 2016; Khatami et al. 2022)
Proteases, tyrosinase, pagadamase, β-glucocerebrosidase, α-galactosidase, asparginase, hyaluronidase, collagenase	Therapeutic purposes (Ostlie et al. 2012; Gurung et al. 2013; Chowdhury et al. 2017; Fejerskov et al. 2017; Erdem et al. 2018; Shang et al. 2018; Khatami et al. 2022)
R-selective transaminase	Sitagliptin production (Sheldon 2011)
Lipolase	Pregabalin synthesis (Martinez et al. 2008; Marouf 2018)
Alcohol dehydrogenase	Duloxetine and statin production (Leuchs and Greiner 2011)
Lipase B	Reboxetine succinate synthesis (Krell et al. 2005; Hayes et al. 2011)
Monoamine oxidase	Boceprevir production (Li et al. 2012b)
Penicillin acylase	Penicillin analogs production (Martens and Demain2017)
Lipase	Sitagliptin, retagliptin, and evogliptin production (Kim et al. 2019)
Leucine dehydrogenase and glucose dehydrogenase	Boceprevir, atazanavir, and telaprevir production (Li et al. 2014; Patel 2018)

The human body is designed to prevent humans from digesting cellulose, which certain foods such as fruits and vegetables contain, because they lack the enzyme cellulase as found in other mammals. Microbes such as *B. licheniformis* and *Trichoderma reesei* produce cellulases sold commercially to blend enzymes under different brands (Jayasekara and Ratnayake 2019). Penicillin acylase produced by yeast, bacteria, and fungi breaks penicillin into 6-amino penicillanic acid, offering an advantage over the conventional method for synthesizing 6-amino penicillanic acid (Brahmachari et al. 2016). This is faster and more cost-effective, especially considering the enzyme immobilization techniques (Brahmachari et al. 2016; Zhang et al. 2017; Meghwanshi et al. 2020). Penicillin acylase also catalyzes the condensation of the β-lactam nucleus with the appropriate D-amino acid while producing other penicillin analogs (Martens and Demain 2017). Furthermore, penicillin acylases were previously reported to be employed in synthesizing semisynthetic penicillin, a more stable with better pharmacological properties than penicillin G and V. Moreover, they are a better alternative against antibiotic-resistant microbial targets (Grulich et al. 2016). This process is achieved by condensing the β-lactam nucleus with the suitable D-amino acid. Synthesis of the carbacephalosporin antibiotic Loracarbef was also reported *via* kinetic enantioselective acylation of azetidinone intermediate catalyzed by penicillin G acylase (Cainelli et al. 1997). Moreover, Glutaryl-7-ACA hydrolase catalyzes a key step in synthesizing 7-aminocephalosporanic acid, a vital intermediate for β-lactam antibiotic synthesis (Groeger et al. 2017). Lipase from *C. rugosa* plays a crucial role in the conversion of β-ketoester to β-keto acid, which is converted to the corresponding β-amino acid (R)-3-amino-4-(2,4,5-triflurophenyl) butanoic acid (3-ATfBA) by ω-Transaminase (ω-TA) from *I. coccineus*. This is a vital intermediate in the production of sitagliptin, retagliptin, and evogliptin (Anjibabu et al. 2016; Kim et al. 2019).

Two key enzymes, leucine dehydrogenase, and glucose dehydrogenase from Exiguobacterium sibiricum and Bacillus megaterium, respectively, synthesize L-tert-leucine. L-tert-leucine is the chiral backbone for the production of antiviral drugs boceprevir, atazanavir, and telaprevir, which are potent inhibitors of Hepatitis C genotype 1 protease, HIV protease, and Hepatitis C NS3-4A serine protease, respectively (Li et al. 2014; Patel 2018). Leucine dehydrogenase converts trimethylpyruvic acid to L-tert-leucine, while glucose dehydrogenase regenerates NADH from NAD+ to continue the process. The intermediates of the hypolipidemic drug statin which is also employed as a prevention strategy for cardiovascular diseases are enzymatically synthesized in *E. coli* host by alcohol dehydrogenase (KleADH) from *Klebsiella oxytoca* and ketoreductase of *Acinetobacter calcoaceticus* for t-butyl 6-chloro-(3R,5S)-dihydroxyhexanoate and (3S,5R)-dihydroxy-6-(benzyloxy) hexanoic acid, ethyl ester, respectively (Patel 2009, 2018). Table 1 summarizes the applications of enzymes in the medicine and pharmaceutical industry.

AGRICULTURE

Enzymes have a very wide range of applications in agriculture, such as soil and plant improvement, waste treatment, and treatments against pathogens (Gupta and Seth 2020; Kumar et al. 2023; Mariyam et al. 2023; Kim et al. 2024; Kumar et al. 2024). Application of different cellulase and pectinases is common in agricultural practices, such as promoting plant growth through rhizobacteria rather than chemical fertilizers (Asmare 2014; Mori et al. 2014), thus decreasing fertilizers. Although such microbes' exact mode of action is not yet understood, certain fungi species are also used to improve yield (Jayasekara and Ratnayake 2019). Both fungi and bacteria used in agriculture can produce cellulase, and possible synergy is suspected between cellulase and antibiotics (active against plant pathogens) to improve plant growth and development (Jayasekara and Ratnayake 2019). Among the cellulolytic microorganisms, there have also been reports of soil improvement through soil decomposition and nutrient accessibility. However, as mentioned earlier, no supporting evidence exists for such a mechanism.

During animal feed formulation, some enzymes are added to improve the quality of livestock feed to break down non-nutritional and harmful feed components (Singh et al. 2016). A notable example is the enzyme addition to improve the overall protein quality of feed. Xylanases are used during feed formulation to improve fiber content and degrade arabinoxylan, cellulose, and hemicellulose by hydrolyzing them to easily digestible form. Thus, improving digestion by both ruminant and non-ruminant animals (Bhat 2000). Amylases were also previously reported to be used during animal feed pretreatment to improve digestibility (Sivaramakrishnan et al. 2006). Enzymes used in poultry include phytase (used in cereal-based feed to liberate phosphorus), proteases (degrade protein in feed), amylases, and gluconases (Selle and Ravindran 2007; Adrio and Demain 2014). Phytase acts on phytic acid to liberate phosphorus, an important mineral for bone formation in growing animals (Selle and Ravindran 2007). This enzyme liberates phosphate in situ, eliminating the need to add phosphorus as a feed supplement, subsequently reducing the cost (Jarvie et al. 2015; Scanlon et al. 2018).

The enzyme keratinase are hydrolase groups that disrupt keratin's disulfide hydrogen bonds (Kalaikumari et al. 2019). They are utilized in animal waste treatments, which constitute plenty of keratins that would have been left undegraded due to their complexity, leading to environmental pollution if untreated. This enzyme converts such waste into simple biodegradable substances (Hossain et al. 2017). Keratinase is also utilized in the degradation of feathers from poultry which contains keratin structure accounting for 5% of the body weight and is a rich source of proteins for feed and food. Thus, it can be degraded into feed and food by the keratinolytic process (Lasekan et al. 2013). Although these enzymes directly affect feed, they are also useful for reducing feed costs and increasing meat yield (Adrio and Demain 2014). The application of

enzyme-immobilized carrier nanomaterials and agro-waste was recently reported to show promising results with a potential for exploration attributed to their reusability, stability, and improved enzyme activity (Mohidem et al. 2023). Moreover, previous studies showed improved enzyme activities and recyclability using agro-waste carriers, including rice husk and onion skins (Ganonyan et al. 2021; Spennato et al. 2021; Khataminezhad et al. 2023). In another study, different enzymes were reported employed in bioremediation processes, including dehydrogenase, oxygenase, esterase, and laccase (Cirino and Arnold 2002; Park et al. 2006; Bhatt et al. 2021). Sobucki et al. (2021) reported the implication of soil enzymes in the ecosystem's normal function, including soil mineralization, plant decomposition, and nutrient cycling. These enzymes include cellulase, protease, phosphatases, and arylsulfatase, contributing to carbon, nitrogen, and phosphorus cycles to maintain soil ecology (Sobucki et al. 2021). The agricultural applications of enzymes are summarized in Table 2.

FOOD INDUSTRY

Enzyme use in the food industry has been on the rise for centuries due to the ease of use and the inherent characteristics of enzymes that make them specific regarding product yield. Key applications in the food industries include protease, amylase, lipases, and glucanases. Globally, enzymes are employed in different food industries, including dairy, brewery, meat, baking, juice and beverages, vegetable processing, dietary supplements, oils, and fats (Robinson 2015). Alpha-amylase is used in the baking industry to improve the quality of bread and degrade the starch in wheat flour into small dextrins to improve the activity of yeast during dough fermentation (Singh and Kumar 2019). Another significant application of α-amylase is beer and fruit juices clarification and the pretreatment of animal feed to improve fiber digestibility (Singh and Kumar 2019). During beer production, amylases hydrolyze starch into smaller particles easily fermented by *S. cerevisiae* to produce beer and for clarification (Okpara 2022). The qualities of acidic proteases, including stability and activity around acidic pH, are exploited in manufacturing digestive aids, soy sauce, and seasonings (Razzaq et al. 2019). Furthermore, these proteases are crucial in manufacturing beer and fruit juices for clearing the mixtures (Zhang et al. 2010). Alkaline protease immobilized on mesoporous silica and mesoporous ZSM-5 zeolite materials was reported to increase the catalytic properties of the enzyme (Kumari et al. 2015). Moreover, the technique presented a novel bioprocess approach for milk coagulation to produce cheese. Gluten-reduced beer was previously produced from chitosan cross-linked immobilized *A. niger* prolyl endopeptidase, exhibiting improved thermal stability with comparable activity to the free enzyme. The result revealed a decreased gluten level from 65 mg/kg to 15 mg/kg after 10 hours of treatment (Benucci et al. 2020). In another study, fungal amylase covalently immobilized on a chitosan-containing cellulose exhibited improved resistance to pH inactivation and increased thermal stability up to 350% in addition to an augmented stability of the enzyme compared to its native counterpart. Moreover, the enzyme for the hydrolysis of barley malt demonstrated an increased product yield by a factor of 1.5 (Raspopova and Krasnoshtanova 2016).

In the beverage industry, amylases are employed to hydrolyze polysaccharides in raw juice to improve juice extraction, clarification, and yield (Sivaramakrishnan et al. 2006). Lipase and xylanase improve dough stability and conditioning, while glucose oxidase and lipoxygenase achieve dough strengthening and whitening. Aspartame is a popular artificial sweetener synthesized from L-aspartic acid, which itself is synthesized by adding ammonia to fumarate by aspartase. L-aspartate-α-decarboxylase is utilized to produce L-aspartic acid, a precursor for the synthesis of β-alanine through decarboxylation (Qian et al. 2018). Food industries exploit the acyl groups, transferring the ability of lipases from esters to other nucleophiles to give end products flavors and aromas (Sá et al. 2017). Lipases are continuously utilized in baking industries in the manufacture of bread, where aeration is a requirement that contributes to the quality of the end product. Egg whites are used during baking, which affects the dough by reducing dough quality; lipases are employed to hydrolyze the lipids in the egg whites to improve quality and act as a preservative to the finished product (Okpara 2022); here, phospholipases that degrade phospholipids in flour with a low tendency to generate off-flavors are used. In another study, *Rhodothermus marinus* extremophile lipase covalently immobilized on chitosan was employed to synthesize aroma ester methyl acetate with potential application to improve food flavors (Memarpoor-Yazdi et al. 2017). Moreover, the enzyme's catalytic activity was retained; up to 78.6% of the initial activity for up to 60 minutes post-incubation at 70°C and pH 8.5 with 67% recovery of the immobilized enzyme compared to the free enzyme (22%). Additionally, the enzyme showed stability to high temperatures and organic solvent with potential application for organic synthesis in harsh industrial conditions.

Table 2. Agricultural applications of enzymes

Enzymes	Applications
Cellulase	Promoting plant growth (Jayasekara and Ratnayake 2019)
Xylanases	Feed formulation (Bhat 2000; Sivaramakrishnan et al. 2006)
Keratinase hydrolases	Poultry waste degradation and feed formulation (Hossain et al. 2017)
Phytases	Feed formulation of cereal-based feed to liberate phosphorus (Selle and Ravindran 2007; Adrio and Demain 2014)
Proteases	Protein degradation in feed formulation
Amylases	Feed pretreatment to improve digestibility (Sivaramakrishnan et al. 2006)
Dehydrogenase, oxygenase, and esterase	Bioremediation processes (Cirino and Arnold 2002; Park et al. 2006; Bhatt et al. 2021)

Table 3. Enzymes in food industries

Enzymes	Applications
Alpha-amylase	Bread, juice, and beer production (Singh and Kumar 2019)
Lipase and xylanase	Dough stability and strengthening (Qian et al. 2018)
Glucose oxidase and lipoxygenase	Dough whitening (Qian et al. 2018)
Aspartase	Synthesis of aspartame (Qian et al. 2018)
Lipases	Flavoring (Sá et al. 2017) and preservation (Okpara 2022)
Laccase	Discoloration, stabilization, and flavoring (Singh and Kumar 2019)
Glucoamylase	Dough stalling and appearance and beer production (Okpara 2022)
Proteases	Flavoring and cheese production (Allegrini et al. 2017)
β-galactosidase	Probiotic production (Panesar et al. 2013; Fernández-Lucas et al. 2017)
Papain	Protein hydrolysates production (Elavarasan and Shamasundar 2016)
Pectinases	Color and aroma improvement in beverages (Kårlund et al. 2014)
Cellulase and xylanases	Fruit juice extraction (Garg et al. 2016)
Naringinase	Debittering agent (Gallage et al. 2014)
Pepsin and renin	Cheese production (Qureshi et al. 2015)
Transglutaminase	Milk protein polymerization (Okpara 2022)
Catalases	Preservative (Röcker et al. 2016)

Another application of phospholipases is seen in cake baking, where there is the stabilization of bubbles on egg yolk lipids within the batter due to the availability of a bubble-batter interface created by the monoacylglycerols (Borrelli and Trono 2015). Laccase is applied in discoloration, haze, wine stabilization, baking, and flavoring during food processing (Singh and Kumar 2019). During baking, laccase oxidizes and improves the dough's and baked products' strength, enhancing crumb structure and increasing softness and volume. Glucose oxidase is employed during production to produce D-glucono-δ-lactone, an additive that improves color development, flavor, and texture and acts as a preservative (Khurshid et al. 2011). Glucose oxidase also increases bread size during baking (Ge et al. 2020). In wine production, it is added to decrease the alcohol level in wine (Röcker et al. 2016). Glucoamylase is another enzyme of microbial origin used for dough stalling in the baking industry to improve the quality and appearance of bread (Okpara 2022). Additionally, this enzyme is employed in beer production to produce light beer via addition to the wort during fermentation (Blanco et al. 2014).

Proteases have a broad operational temperature (10-80°C), which favors their applications in the processing of cheese and dairy products and enhances the quality of bread, baked goods, and crackers (Singh and Kumar 2019). A typical example is aminopeptidases, which significantly improve the flavor of fermented milk products. Proteases are also applied during cheddar production to lower the time for cheese aging by accelerating flavor modifications via the enzymatic breakdown of proteins (Allegrini et al. 2017). Microbial alkaline protease is used in synthesizing highly nutritive protein hydrolysate preparation used in food preparation for infants, drug production, and fortification of soft drinks and juice (Mótyán et al. 2013; Singh et al. 2016). Probiotics beneficial to the gut's health by stimulating bacteria growth can also be enzymatically produced in the form of non-digestible dietary supplements. A typical example is galactooligosaccharides, which are used as a low-calorie sweetener and are synthesized via simultaneous transgalactosylation and hydrolysis of lactose by β-galactosidase (Panesar et al. 2013; Fernández-Lucas et al. 2017). Moreover, β-Galactosidase finds its use in the dairy industry, exhibiting significant activity improvement of up to 99% in the enzyme immobilization technique with potential application in lactose hydrolysis in milk or whey (Panesar et al. 2010). In another study, β-galactosidase immobilized on silica/chitosan support exhibited high stability with potential dairy industry application (Ricardi et al. 2018). Moreover, operational stability was observed after 200 hours of continuous use on a fixed-bed reactor with up to 90% activity. The proteolytic enzyme papain from papaya has been identified as a key enzyme in fish industries, minimizing pollution by converting the waste from these industries to protein hydrolysates employed as flavor enhancers and food supplements (Elavarasan and Shamasundar 2016). Proteases are utilized for the hydrolysis of protein hydrolysates into free amino acids to synthesize antioxidants against the autoxidation of linoleic acid (Gómez-Guillén et al. 2011).

In the beverage industry, pectinase preparations decrease maceration time by enzymatically breaking pectin and bringing about quick dissolution, improved aroma, color, and product (Kårlund et al. 2014). During juice processing, cellulases, pectinases, and amylases soften fruits by hydrolyzing cellulose and hydrocellulose found in fruits and vegetables to improve the procedures. Thus enhancing yield and cost-effectiveness (Garg et al. 2016). The main sources of cellulase for industries are made up of cellulase from fungi and bacteria, including *A. niger*, *T. reesei*, and *B. licheniformis* (Yadav et al. 2020). Cellulase and pectinase enhance juice yield, cloud stability, and texture. During debittering, enzymes such as Naringinase and lemonade degrade bitter substances in citrus juice (Bhardwaj et al. 2019). Esterases are another class of enzymes employed to synthesize ferulic acid, the precursor of vanillic acid used to improve flavor and taste in the beverage industry (Gallage et al. 2014). The beverage industries also utilize xylanases to hydrolyze hemicellulose and improve juice extraction, clarification, and yield (Bhardwaj et al. 2019). In another study,

pectinases immobilized on polyvinyl alcohol gel were tested for fruit juice clarification. The immobilized enzyme demonstrated stability to low pH with 20% initial activity retention after eight cycles of reuse, decreasing the turbidity of apple juice up to 80% after 3 cycles (Cerreti et al. 2017). Laccases from fungal sources are employed in the brewery industry to remove haze from beer and wine via phenol oxidation improving the appearance and acting as a preservative by removing oxygen from beer (Okpara 2022). In juice making, laccases clarify and increase yield (Yin et al. 2017). Pectinases mainly produced by fungi are employed in the cocoa industry to increase yield, improve flavor and aroma, and decrease processing time at the curing stage of wet processing (Oumer 2017; Okpara 2022). Additionally, pectinases are employed in coffee production to remove the mucilage layer of the cocoa beans (Oumer 2017), while in tea production, they are used to remove pectin from the leaves to enhance fermentation time (Suhaimi et al. 2021). In baking, enzymes improve the text, color, softness, and appearance of crumbs and increase shelf life. These enzymes are used for the product's consistency; a typical example is amylase, which softens the dough and enhances shelf life (Singh et al. 2016).

Another key enzyme in the baking industry is xylanase, which is employed to hydrolyze arabinoxylan in wheat to solubilize and make it extractable thereby improving the qualities of the dough (Courtin and Delcour 2002). Additionally, a recombinant form of this enzyme produced by *Pichia pastoris* was reported to decrease the sugar content of bread (de Queiroz Brito Cunha et al. 2018). Glucoamylases are used in breaking down maltose for utilization during fermentation, leading to a rise in the dough for bakeries and the production of ethanol in beer or wine (Raveendran et al. 2018). Laccases are used to cross-link milk proteins in skimmed milk, thus improving the quality of the yogurt (Struch et al. 2016). These enzymes are also used to produce soy sauce and light beer. Probiotics are not digestible, hence their usage in enhancing the bacteria growth in the gut (Choi et al. 2015). Lipase enhances the flavor and shelf-life extension of bakery products (Adrio and Demain 2014). In another study, lipase immobilized on α-lactalbumin nanotube carriers remarkably increased the enzyme's activity (by up to 68%) and catalytic efficiency and promoted free fatty acids and flavor release (Guan et al. 2021). Enzymes are employed in the dairy industry to enhance the organoleptic properties (flavor, aroma, and texture) and yield increase. Diary enzymes, including lactases, lipases, proteases, and catalases, produce cheese, milk, and yogurt (Qureshi et al. 2015). Milk coagulation is achieved during cheese production by combining pepsin and chymosin, known as rennet. Proteases are also used to produce cheese and decrease allergic reactions to milk. People who are unable to take milk due to lactose intolerance can now take milk due to the use of lactase, which breaks down lactose, thus promoting the digestibility and sweetness of milk (Qureshi et al. 2015). Transglutaminase from bacterial sources (Okpara 2022) is another vital enzyme of the dairy industry that is added to polymerize milk proteins to improve the quality of some dairy products (Kieliszek and Misiewicz 2014). Catalases act as a preservative that removes peroxide and oxygen from milk, wine, and other food products to prevent rancidity, thereby acting as a preservative (Röcker et al. 2016). Table 3 summarizes the enzymes utilized in the food industry.

DETERGENT INDUSTRIES

The enzymes used in the detergent industry are on the rise due to their stain-removal ability. Fabrics made up of cotton are cleaned with cellulases by modifying the fibers within the fabric to remove dirt and soil particles within the fabric, thus brightening and softening it (Jayasekara and Ratnayake 2019). A recent advance has shown the use of different enzyme formulations to increase cleaning efficiency. A typical example is the combination of lipase, cellulase, protease, and amylase used to clean blood, fats, lipids, and carbohydrates from surgical equipment (Jayasekara and Ratnayake 2019). Alkaline yeast lipases are preferable in a cold wash than those from bacteria and molds due to their suitability for lower temperatures, hence, being used as components of detergents. Moreover, applying enzyme immobilization techniques significantly improves the yield of the efficiency and catalytic effect of the enzyme formulations in detergents. Immobilized serine protease, esperase retained its catalytic activity following 20 minutes of incubation in anionic and non-ionic surfactants, while the free enzyme lost 50% of its activity (Vasconcelos et al. 2006). The immobilized enzyme exhibited a superior stain removal effect from cotton than the free enzyme, with no recorded damage to wool. In addition, *Bacillus* sp. NPST-AK15 alkaline protease immobilized on hollow core-mesoporous shell silica nanospheres retained its catalytic efficiency for twelve consecutive reaction cycles. Although the catalytic temperature of both the immobilized and free enzyme remained at 60°C, an insignificant pH change from 10.5 to 11.0 was observed for the immobilized enzyme with a 1.5-fold increase in substrate affinity and enhanced organic solvent stabilities (Ibrahim et al. 2016).

The nature of lipids makes fatty stains difficult to remove from fabrics and glassware. These stains might easily be removed at high temperatures; however, washing at a lower temperature is favored. Lipases in laundry and dishwashing detergents remove fatty stains such as butter, margarine, fats, fat-containing sauces, salad oil, soups, human sebum, or certain cosmetics. Several thermostable lipases have been successfully used as detergent additives (Naganthran et al. 2017; Tang et al. 2017). Lipase isolated from *Bacillus methylotrophicus* was previously reported to show thermostability at a broad pH range, making it a good alternative for the detergent industry for the removal of grease, oil, and other oily stains (Sharma et al. 2017). Mannans are frequently used as thickening agents or stabilizers in ice cream, chocolate, ketchup, and personal care products. Mannan-containing soils also easily adsorb to the cellulose fibers of cotton fabrics by hydrogen bonds and are difficult to remove. Mannanases are specifically

supplemented with detergent to remove mannan-based dirt from clothes (Chauhan et al. 2012). Pectinases in detergents break the pectin backbone in pectin-based stains caused by fruits, vegetables, sauces, jams, and jellies for easy removal from fabrics during a wash. Thus, pectin-based stains and pectinase detergents are used (Sarmiento 2015). In another study, lipase immobilized on woolen fabric demonstrated enhanced oily stain removal after staining with olive oil after 24 hours of room temperature storage. Furthermore, an 80% activity was retained following storage of the immobilized woolen fabric for more than 80 days in pH 8.5 of tris buffer in a refrigerator (An et al. 2014). Similarly, lipase immobilized on arylamine glass beads showed promising results in oil stain removal without considerable activity loss after 100 cycles (Sharma et al. 2008).

The specificity of the enzymatic effect reduces damage to fabrics and surfaces notably associated with chemically synthesized detergent (Singh et al. 2016). A typical example is dishwashing detergents, often containing varying degrees of amylase and lipase intended to remove starch food deposits and fats and oils, respectively (Li et al. 2012a; Sarmiento 2015). *Laceyella sacchari* TSI-2 α-amylase immobilized on diethylaminoethyl cellulose via glutaraldehyde crosslinking showed high operation stability, increased shelf-life, and improved solvent stability, exhibiting high efficiency in removing starch stains from cotton (Shukla and Singh 2016). Although the pH optima remains intact, the temperature optima and thermal stability changed from 60 to 70°C, while the pH stability of the immobilized enzyme changed from 6 to 7.

LEATHER AND COSMETICS INDUSTRIES

In the leather industry, challenges include preparing the leather and eliminating waste. The conventional leather processing method entails using toxic chemicals, generating and releasing pollutants with significant environmental effects, including total solids accumulation in water bodies and oxygen deprivation (Kanagaraj et al. 2020). These challenges can be lowered by applying enzymes that offer an eco-friendly, efficient, and cost-effective alternative. Enzymes are used to enhance the quality of the leather and also minimize waste (Adrio and Demain 2014). Enzymes facilitate procedures and enhance leather quality at different stages in leather processing, such as curing, soaking, liming, dehairing, bating, pickling, degreasing, and tanning (Mojsov 2011). These enzymes include alkaline proteases, neutral proteases, and lipases. Proteases, lipases, and amylases are used in the dehairing process to preserve the hair, which is a challenging step in leather preparation. This excludes the conventional use of chemicals like amines and limes (De Souza and Gutterres 2012). Therefore, to make leather soft during soaking, alkaline proteases eliminate nonfibrillar proteins (composed of aggregated protein structures that don't form fibrils) from leather (Singh et al. 2016). Different enzymes are being investigated for their application in leather processing, such as the soaking step, including chondroitinases, hyaluronidases, phospholipases, amidases, and lignocellulases (Kanagaraj 2009). In the conventional method, the dehairing process entails using lime sulfide, leading to sulfide contamination. However, enzyme technology employs proteases, which eliminate this dehairing problem. Ugbede et al. (2023) reported the dehairing process of animal skin using *B. subtilis* and *A. flavus* proteases in a medium containing hair, feathers, and agro-waste. Moreover, both enzymes exhibited 71.5% and 94.8% recovery with 1.5 and 2.0 fold purifications, respectively. Moujehed et al. (2022) reported a chemical-free alternative for degreasing sheep skins using *Yarrowia lipolytica* LIP2 lipase. Here, 6 mg of lipase/kg of raw skin was used for the degreasing in 15 minutes at pH 8 and 30°C, yielding superior quality leather compared to the chemically treated.

Enzymes are also utilized in the cosmetic industry. An example is using an immobilized enzyme technique applied to lipase to synthesize retinoid's water-soluble derivative, used in skin care products (Gurung et al. 2013). The use of superoxide dismutase in sunscreen cream, mouthwash, and toothpaste as free radical scavengers was previously reported (Li et al. 2012a). Damages such as those caused by microbes are minimized by superoxide dismutase. Protein disulfide isomerase, glutathione sulfhydryl oxidase, and transglutaminase are applied in hair-waving (De Souza and Gutterres 2012). In another study, *Marasmiellus palmivorus* VE111 laccase produced after hydrolysis and alcoholic fermentation in a medium of lignocellulosic residues of *Araucaria angustifolia* degraded almost 33% of melanin in 8 hours, using vanillin mediator (Polesso et al. 2022). It demonstrates potential eco-friendly applications for producing anti-hyperpigmentation skin care products. A combined laccase and natural phenol redox mediators cocktail effectively degraded eumelanin from *Sepia officinalis*, offering an alternative to traditional skin whitening agents (Gigli et al. 2022). Moreover, the combined cocktail demonstrated a synergistic effect and degrading eumelanin sub-units better than single-mediator counterparts. In another study, cetstearyl stearate, a widely employed substance in the cosmetic and hygiene personal industry, was enzymatically synthesized *via* esterification of stearic acid and cetostearyl alcohol with high conversion values of 99% (Holz et al. 2018). Moreover, the optimum reaction condition was 75°C, 1:1.5 acid to alcohol molar ratio, 600 mmHg vacuum, and 760 rpm agitation. At the same time, the final product characterization showed acidity index, iodine index, hydroxyl index, and saponification index values of 0.6 mg KOH g^{-1}, 0% of iodine absorbed/g of the sample, 17.06 mg KOH.g^{-1}, 133.68 mg KOH g^{-1}, respectively. The *C. antarctica*, lipase B, was used to synthesize amphiphilic fatty amides from linoleic acid and salicylic acid in a solvent-free process at 65°C and reduced pressure (50 mbar) with high conversion rates, up to 95% via aminolysis reactions (Mouad et al. 2016). Furthermore, adjusting the enzyme concentration showed an increased yield of fatty amide 3 from 30% to 88%. Thus, this result demonstrates that a solvent-free enzymatic synthesis is an attractive method for producing

fatty amides with potential applications in the cosmetic industry.

CONCLUDING REMARKS

Conventional industrial processes employ chemicals for production, leading to increased environmental toxic waste accumulation, inefficiency, and high cost. The application of enzymes presents a link for transformation into eco-friendly industrialization, minimizing the environmental impact and providing efficient and cost-effective techniques. Although using enzymes offers a better alternative to conventional methods, there are also shortcomings associated with enzymes, including short life, instability to harsh industrial bio-processing conditions, and reusability. However, biotechnology technology advancement presents opportunities to overcome these shortcomings via genetic engineering and enzyme immobilization technology. The use of enzymes on a commercial scale is applied in different endeavors, encompassing medicine, agriculture, and industries, to improve efficiency and sustainability processes. This brings about processes under mild conditions with lesser energy requirements due to the environmentally friendly and biodegradable nature of enzymes and their byproducts. Thus, enzymes will continuously be employed in industries due to their low cost and safety for both individuals and the environment, leading to higher performance and improved production processes with lower environmental influence.

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Isolation, identification, and characterization of heavy metal-resistant bacteria from soil samples collected at a cement company in Nigeria

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Abstract. *Martins AL, Silas TV, Abah MA, Adebisi AK, Sunday AM, Emochonne RY, Iheanacho CC. 2024. Isolation, identification, and characterization of heavy metal-resistant bacteria from soil samples collected at a cement company in Nigeria. Asian J Trop Biotechnol 21: 26-32.* Many heavy metals, including cadmium, chromium, copper, lead, and zinc, are produced during cement-making. Even in low quantities, most of the heavy metals released are known to be harmful to plants and animals. The objective of this study was to isolate, identify and characterize heavy metal-resistant bacteria from soil samples collected at Benue Cement Company, Nigeria. Soil samples taken near the Gboko facility of Nigerian cement manufacturer Benue Cement Plc were tested for heavy metal-resistant bacteria. Results from the study revealed that compared to other metals (Pb, Cr, and Cd), the concentration of copper and zinc was consistently quite high across all sites. The levels of all components examined were determined to be higher than the limits allowed by the World Health Organization In this study, Cd, Zn, Cr, Cu, and Pb were found in soil samples taken from the Benue cement industry, according to the analysis of heavy metals. The results obtained from this study further revealed that out of 20 isolates only five (5) bacterial isolates, namely *Staphylococcus aureus*, *Escherichia coli*, *Proteus* sp., *Bacillus cereus*, and *Lactobacillus* which showed high levels of heavy metal resistance were selected for further studies in secondary screening. Based on the biochemical tests, *S. aureus* reacted positively to catalase and coagulase test. They were also seen to ferment lactose, sucrose and glucose. The *E. coli* reacted negatively to citrate, catalase, coagulase tests, and did not ferment sucrose. However, the bacteria fermented lactose and glucose. *Lactobacillus* reacted positively to only citrate but fermented the three sugars. *Proteus* sp. fermented glucose and sucrose and also reacted positively to citrate, catalase and urea tests. The *B. cereus* fermented glucose and sucrose. The bacteria also reacted positively to only citrate and catalase tests. Results of showed that *S. aureus* had MIC values between 12 and 16 mg/L, *E. coli* between 20 and 50 mg/L, *Proteus* species between 15 and 64 mg/L, and *B. cereus* between 10 and 18 mg/L, all against various metals. The *B. cereus* showed the lowest resistance to several heavy metals, while *E. coli* showed the highest resistance. Additionally, *E. coli* demonstrated a significant resistance level to all ten antibiotics examined. Antibiotic resistance was highest in *E. coli* and lowest in *Proteus* species. The findings of this study revealed that the four isolates that showed high tolerance to heavy metals could be used as inoculants to bioremediate cement sites that polluted by heavy metals.

Keywords: Antibiotic resistance, bacteria, cement, heavy metals, soil

INTRODUCTION

In pursuing better living conditions, man has increasingly contributed to environmental challenges through industrialization and urbanization (Adekola et al. 2012). Animals and people alike suffer due to the disruption of nature's natural balance that he brings about through his exploitation of it (Udiba et al. 2012). Like many other developing nations, Nigeria is experiencing rapid urbanization and industrialization. With the increase in manufacturing and other service sectors, the country is realizing the importance of diversifying its economic base to secure its future (Afolabi et al. 2012). Cement manufacturing is a major contributor to the emissions of heavy metals such as Cd, Cr, Cu, Pb, and Zn (Al-Khashman and Shawabkeh 2006; Aman et al. 2008). The wind speed and particle size determine the distance that these heavy metals are carried in the soil by cement dust and stack fumes (Udiba et al. 2012; Tatah et al. 2016). Most of the heavy metals in cement dust come from the ingredients themselves. Approximately half of the total Cd, Cu, and Zn loads in cement are introduced via raw materials, with typical raw materials containing 25 mg/kg of Cr, 21 mg/kg of Cu, 20 mg/kg of Pb, and 53 mg/kg of Zn (Achternbosch et al. 2003). Even at low concentrations, most emitted heavy metals are toxic to plants and humans (Sherene 2010). The health effects of soil and water contamination can vary greatly depending on the type of pollutant, the exposure route, and the susceptibility of the exposed population (e.g., children, adults, or elderly) (Adekola et al. 2012). Anthropogenic heavy metal deposition can occur via several processes, including cement manufacture, although the main sinks for these metals are soil and water (Al-Khashman and Shawabkeh 2016; Tatah et al. 2017; Otitoju et al. 2022). Contamination of soil with heavy metals poses multiple problems due to

their inability to break down biologically, a constant source of environmental anxiety to ecological preservation (Emmanuel et al. 2009; Tatah et al. 2020; Otitoju et al. 2022). Animals and humans are at risk when they consume plants, fruits, and vegetables with bioaccumulated harmful metals from polluted soil and water (Turner 2009; Udiba et al. 2012; Abah et al. 2021; Olawale et al. 2023).

In most cases, heavy metals kill microbes by blocking vital functional groups, displacing vital metal ions, or changing the active conformation of biological components. Nevertheless, microbes rely on certain metals at low concentrations as co-factors for enzymes and metalloproteins (Olawale et al. 2023). Microbes that are able to withstand the effects of heavy metals have begun to proliferate in the soil and water of industrialized areas (Emmanuel et al. 2009). The ability to produce new microbial strains with potent heavy metal detoxification activities is often determined by plasmids (Abimbola et al. 2019). Plasmid and transposon genes encode metal resistance in bacteria; this resistance can be transmitted between genera and even between different types of bacteria and in situ and indigenous microflora (Zhema et al. 2022). There is a strong correlation between metal and antibiotic resistance (Ansari and Malik 2007); heavy metal resistance is often far more prevalent than antibiotic resistance. Heavy metal-polluted settings often have bacteria resistant to antibiotics and heavy metals, as studies have shown that these genes tend to be located on the same plasmid (Malik and Jaiswal 2000). The organic compounds included in heavy metal contaminants can be broken down by bacteria and other microbes employed in bioremediation, leading to the harmless release of carbon dioxide and water (Rashed 2010). Therefore, bacteria have evolved multiple coping methods to withstand heavy metal uptake in settings polluted with metals. Consequently, these organisms can be bio-indicators to detect environmental heavy metal pollution (Das et al. 2016). The survival of these microorganisms relies on their inherent biochemical, structural, and physiological characteristics and genetic adaptations (Ozer et al. 2013). Therefore, the present research aimed to identify, isolate, and characterize heavy metal-resistant bacteria from soil samples at and around the Benue Cement Company in Nigeria. These bacteria could be useful in bioremediation efforts in areas where metals have been polluted.

MATERIALS AND METHODS

Study area

Gboko is home to Benue Cement Company Plc. (now Dangote Cement Company Plc.) in Nigeria's north-central area, specifically in the Gboko Local Government Area (LGA) of Benue State. Approximately 532 feet above mean sea level, it can be found with these coordinates: 7°24 42.45N, 8°58 31.28E (Figure 1). The research site was in a tropical subhumid zone where the average yearly temperature ranges from 23 to 34°C.

Collection of soil samples

Soil samples were collected from five separate farmlands within a five-kilometer radius of the cement industry, down to a 10 cm depth. The cement factory's surrounding water sources were also sampled using sterile glass bottles: stagnant water, flowing water, abandoned pits, stream water, and underground water. For analysis, the soil and water samples were brought to the laboratory in an ice box.

Metal analysis of the collected soil samples

The concentrations of metals in all samples were determined according to the AOAC (2010) using an Atomic Absorption Spectrometer (Varian SpectrAA 220 USA).

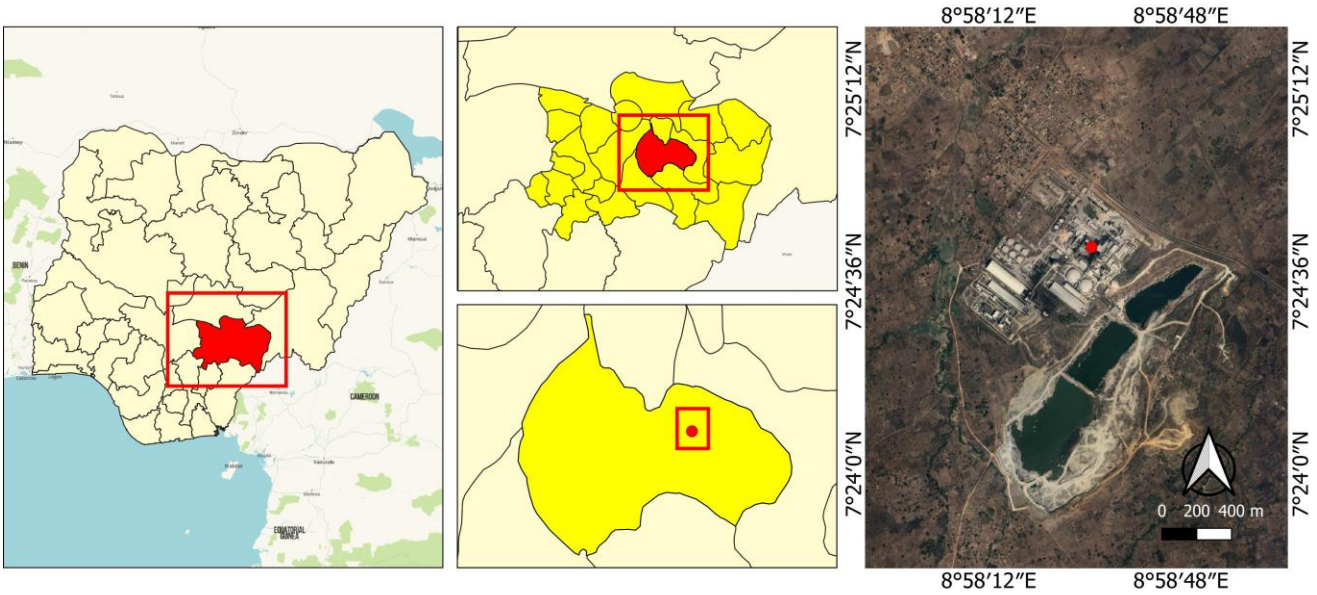

Figure 1. The location of sampling site in Gboko Local Government Area (LGA) of Benue State, Nigeria (Source: www.google.com)

Bacterial isolation from soil samples

To isolate bacterial isolates from the obtained soil samples, enrichment technique was used via added 5 g of soil to 500 mL of mineral salts media in sterilized Erlenmeyer flasks. Sole carbon source was applied as 2000 µL of crude oil and incubated with shaking for 7 days at room temperature. Then, ten-fold serial dilutions were performed for enrichment sample suspension and one mL of each dilution was poured into oil agar plates to isolate bacterial isolates and incubated at 30°C for 3-7 days. Sub-culturing repeated for selected pure colonies on agar plates and transferring on Nutrient agar slants for morphological, biochemical and molecular identification.

Morphological characterization

Morphological characteristics, include color and shape of colony, cell size, and motility (Cheesbrough 1991). Microscopic features were recorded for all isolates via Gram stain protocol.

Biochemical characterization

Biochemical tests were carried out according to the method of Cheesbrough (1991).

Catalase test

Gas bubbles detecting within 10 s after added purified bacterial culture to 5 mL of hydrogen peroxide solution, considered as a positive catalase test.

Urea test

Slanted two mL of urea medium which placed in bijou bottles applied for the incubated bacterial colony at room temperature. Red-pink colour in the medium was considered as a positive test for urease induction.

Indole test

Appearance of bright red and yellow color which composed after added 0.5 mL of Kovac's reagent to incubated bacterial culture at 35°C for 24 h on SIM media indicated a positive and negative results respectively.

Citrate test

Simmons citrate test was performed via inculcate Simmons Citrate Agar plates (TSBA, Himedia) surface with bacterial cultures then, incubated at 37°C up to 48 h. changing media colour from green to bright blue indicate positive reaction.

Carbohydrate fermentations

Some of the isolates showed a color change from pink to yellow, indicating the production of fermented sugars such as glucose, sucrose and lactose of a gas bubble in the Durham's tube.

Coagulase test

A drop of sterile distilled water was placed on each end of a sterile slide. A colony of test organism was emulsified on each spot to make thick suspensions. A loopful of plasma was added to one of the suspension and mixed gently. The slide was examined for clumping or clotting of the organism within 10 seconds. Plasma was not added to the second suspension which serves as control.

Preparation of stock solutions for metals

All of the solutions utilized in the study were prepared using chemicals of a spectroscopic grade. In 1,000 mL of deionized water, sufficient quantities of $CdCl_2$, $PbCl_2$, $ZnCO_3$, $CrCl_3$, and $CuCl_2$ were dissolved to produce a synthetic stock solution of Cd (II), Pb(II), Zn (II), Cu (II), and Cr (II) effluent (1,000 mg/L). The stock solutions of the metal ions were serially diluted to prepare all other concentrations. Therefore, to balance the pH of the water-based solution, 0.1 M hydrochloric acid and 0.1 M sodium hydroxide were utilized.

Determination of Minimum Inhibitory Concentrations (MICs) of heavy metals

The plate dilution method was used to calculate MIC of the metals for each isolate as described by Bauer et al. (1966). Minimum Inhibitory Concentrations (MICs) of heavy metals were given as the concentrations at which microbiological growth might be inhibited.

Determination of antibiotic resistance

The disc diffusion method was employed according to the method of Bauer et al. (1966), to identify the antibiotic resistance pattern in various bacterial isolates. Strained strains were defined as those with a diameter of 12 mm or greater.

Data analysis

The results were analyzed statistically using One-Way Analysis of Variance (ANOVA), followed by Duncan multiple comparisonsusing Statistical Package for Social Science (SPSS) version 21. Significance between means was determined at a p-value of less than 0.05 ($p < 0.05$). The results for each treatment were presented as mean ± standard deviation.

RESULTS AND DISCUSSION

Heavy metal analysis of soil samples

Results of heavy metals analysis in and around the Benue Cement Factory is summarized in Table 1. Compared to other metals (Pb, Cr, and Cd), the concentration of copper and zinc was consistently quite high across all sites. The levels of all components examined were determined to be higher than the limits allowed by the World Health Organization (WHO 2010). In this study, Cd, Zn, Cr, Cu, and Pb were found in soil samples taken from the Benue cement industry, according to the analysis of heavy metals.

Isolation and identification of heavy metal-resistant bacteria

Results showed that total 20 isolates were isolated from the samples. Out of 20 isolates only five (5) bacterial isolates, namely *Staphylococcus aureus*, *Escherichia coli*, *Proteus* sp., *Bacillus cereus*, and *Lactobacillus* which

showed high levels of heavy metal resistance were selected for further studies in secondary screening (Table 2).

Based on biochemical assays (Table 3) and morphological examination, *S. aureus* showed positive result to catalase and coagulase test. They were also seen to ferment lactose, sucrose and glucose. The *E. coli* reacted negatively to citrate, catalase, and coagulase test. It fermented lactose and glucose but did not ferment sucrose. *Lactobacillus* reacted positively to only citrate but fermented the three sugars. *Proteus* sp. fermented glucose and sucrose and also reacted positively to citrate, catalase and urea tests. The *B. cereus* fermented glucose and sucrose. The bacteria also reacted positively to only citrate and catalase tests.

Minimum Inhibitory Concentrations (MICs) of metals

The study investigated the resistance of four (4) of the five (5) isolates to the heavy metals Cd (II), Pb (II), Zn (II), Cu (II), and Cr (II). The *E. coli* and *Proteus mirabilis*, two of the most metal-resistant bacterial species, had the highest Minimum Inhibitory Concentration (MIC) values of all the isolates tested. Table 4 shows the results of separate determinations of Minimum Inhibitory Concentrations (MICs) of different metals. The *S. aureus* had MICs values between 10 and 16 mg/L, *E. coli* between 20 and 50 mg/L, *Proteus* species between 15 and 64 mg/L, and *B. cereus* between 10 and 18 mg/L against various metals.

Resistance to antibiotics

Table 5 shows the estimated antibiotic resistance of the most common ten tested antibiotics against the multiple metals resistant isolates (*E. coli* and *Proteus* species) to study the correlation between metals and antibiotic resistance in bacteria.

Table 1. Heavy metal concentration (mg/L) of soil

Samples	Pb	Cu	Zn	Cr	Cd
1	0.235±0.00^{a}	4.670±0.01^{a}	8.4600±0.01^{a}	0.016±0.00^{a}	0.6220±0.00^{a}
2	0.131±0.00^{b}	3.020±0.00^{b}	9.5350±0.02^{b}	0.021±0.00^{b}	0.5210±0.00^{b}
3	0.222±0.00^{c}	3.775±0.00^{c}	6.6400±0.01^{c}	0.035±0.00^{c}	0.4215±0.00^{c}
4	0.145±0.00^{d}	5.110±0.01^{d}	6.5300±0.01^{d}	0.025±0.00^{d}	0.3160±0.00^{d}
5	0.317±0.00^{e}	4.850±0.00^{e}	7.6150±0.01^{e}	0.036±0.00^{c}	0.5155±0.00^{e}

Note: Mean values on the same column having different letters of the alphabet are statistically significant $p \leq 0.05$

Table 2. Morphological characteristics of isolated bacteria

Bacteria Isolates	*Staphylococcus aureus*	*Escherichia coli*	*Proteus* sp.	*Bacillus cereus*	*Lactobacillus*
Colony colour	Golden yellow	Pink	Cream	White	Yellow
Gram nature	Positive	Negative	Negative	Positive	Positive
Cell morphology	Cocci	Rod	Rod	Rod	Rod
Motility	Negative	Positive	Positive	Positive	Negative

Table 3. Biochemical analysis of isolated bacteria

Biochemical Parameters	*Staphylococcus aureus*	*Escherichia coli*	*Proteus* sp.	*Bacillus cereus*	*Lactobacillus*
Catalase	+	-	+	+	-
Citrate	-	-	+	+	+
Coagulase	+	-	-	-	-
Glucose	+	+	+	+	+
Indole	-	+	-	-	-
Lactose	+	+	-	-	+
Sucrose	+	-	+	+	+
Urea	-	-	+	-	-

Note: (+): Positive, and (-): Negative

Table 4. Minimum inhibitory concentration (MIC) of bacterial isolates to lead (Pb), copper (Cu), cadmium (Cd), zinc (Zn), and chromium (Cr)

Bacterial Isolates	MIC (mg/L)				
	Pb	Cu	Cd	Zn	Cr
Staphylococcus aureus	12.00	15.00	14.00	10.00	16.00
Escherichia coli	35.00	20.00	40.00	50.00	30.00
Proteus sp.	33.00	15.00	35.00	64.00	35.00
Bacillus cereus	15.00	18.00	10.00	15.00	12.00

Table 5. Antibiotic sensitivity of heavy metals resistant isolates

Antibiotics	Disc codes	Inhibition zone diameter (mm)		Resistance/sensitivity	
		Escherichia coli	*Proteus* sp.	*Escherichia coli*	*Proteus* sp.
Streptomycin	S	6	11	R	R
Gentamycin	Gen	9	28	R	S
Pefloxacin	PEF	34	7	S	R
Ciprofloxacin	CPX	5	30	R	S
Septrin	SXT	6	12	R	R
Chloramphenicol	C	8	19	R	S
Augmentin	AUG	12	22	R	S
Ofloxacin	OFX	30	17	S	S
Sparfloxacin	SP	10	8	R	R
Amoxicillin	AM	25	10	S	R

Note: S: Sensitive; R: Resistant

Discussion

In particular, the emissions of heavy metals such as Cd, Cr, Cu, Pb, and Zn from cement manufacture are a major cause of environmental contamination (Aman et al. 2008; Chattopadhyay and Grossart 2011). The primary source of these heavy metals in the soil is raw materials, but they can also be found in cement dust and stack fumes (Das et al. 2016). Because of their persistence in the environment and bioaccumulation in the food chain, heavy metals are hazardous to plants and people, even in low quantities (Ozer et al. 2013). Heavy metal contamination is a global concern; resolving it is important (Akhter et al. 2017).

How microbial communities react to heavy metals is conditional on the metals' concentration and accessibility (AOAC 2010). Heavy metals frequently inhibit microorganisms, essential for the biological remediation of heavy metal-polluted locations (Cheesbrough 2005). Therefore, finding microbes that can survive in environments with high concentrations of heavy metals is crucial for creating efficient bioremediation systems.

The results of present study showed that heavy metal pollutants, such as Cd, Zn, Cr, Cu, and Pb was found in soil samples from the Benue Cement Company in Nigeria. Bacteria resistant to heavy metals were isolated and identified. The *S. aureus, E. coli, B. cereus, Lactobacillus*, and *Proteus* species were the five strains chosen for additional analysis from 20 isolates. Three similar bacterial species, namely *E. coli, S. aureus*, and *Proteus* species have been identified from industrial effluents in Punjab, India (Summers and Silver 1972). Nath et al. (2012) also found *S. aureus* and *Proteus* species in sewage from Asam, India, garages, and petrol pumps. Because of their adaptability and favorable conditions, these bacterial species are likely be found in areas contaminated with heavy metals from cement dust.

The results of heavey metal resistance revealed that *E. coli* and *Proteus* species had varying Minimum Inhibitory Concentrations (MICs) for heavy metals, such as Pb, Cu, Zn, and Cr. The resistance pattern exhibited by *E. coli* and *Proteus* species usually forms in reaction to environmental stresses caused by heavy metals (Gullberg et al. 2014). Research by Bauer et al. (1966) shows that certain bacteria, such as *E. coli* and *Proteus* species, can withstand and even eliminate heavy metals. Therefore, to mitigate heavy metal toxicity, other studies have investigated biosorption and microbial production of metal-binding compounds (Kabata-Pendias and Mukherjee 2007).

The varying degrees of toxicity that various metals have on bacterial cells may explain why their Minimum Inhibitory Concentrations (MICs) vary among different strains of bacteria. Zinc wasthe least poisonous metal, with a maximum concentration of 64 mg/L in one isolate. In contrast, copper was the most toxic, with no isolated organism having a MIC higher than 20 mg/L in *E. coli*.

Ansari and Malik (2007) measured several metals' Minimum Inhibitory Concentrations (MICs) against soil-isolated *E. coli* bacteria (Bauer et al. 1966). Maximum MICs for mercury (32 mg/L), cadmium (200 mg/L), copper (400 mg/L), nickel (800 mg/L), and lead (1600 mg/L) were observed. In addition, MICs for Cd (4-7 mM), Cr (0.7 mM), Ni (6.75-8.5 mM), Pb (6 mM), As (6.5-15 mM), and Hg (0.75 mM) were found in sewage multi-metal resistance isolates such *Proteus* species, *Acinetobacter radioresistens*, and *Pseudomonas aeruginosa* (Nath et al. 2012). Species of *Pseudomonas*, in contrast, showed remarkable resistance to copper, zinc, lead, arsenate, and mercury (Mohammad et al. 2015). Metal resistance was rather high among our bacterial isolates compared to other strains reported to be metal-resistant.

Bacteria have evolved various resistance mechanisms in response to environmental heavy metal stress. According to Das et al. (2016), when exposed to high concentrations of harmful metals, microbes create a wide variety of metal-binding compounds, some particular and others more general, which can reduce the impact of the metals and facilitate their uptake. Detoxification and heavy metal removal from polluted environments could use these processes (Raja et al. 2009). Therefore, considering the prevalence of heavy metal contamination at numerous sites, bioremediation using multiple metal-resistant bacteria holds great promise (Wei et al. 2009).

Therefore, Summers and Silver (1972) mention a correlation between metal resistance in microbes and antibiotic resistance. Since efflux pumps in bacterial membranes mediate both forms of resistance, microbial populations can develop antibiotic resistance when heavy metals and antibiotics are present in specific settings (Abskharon et al. 2010). The *P. mirabilis* and *E. coli* were

resistant to most antibiotics tested in the present investigation. The *E. coli*, the most antibiotic-resistant strain, was also the most metal-resistant. These results point to typically high Minimum Heavy Metal Concentrations (MHCs) or the concentration needed to cause antibiotic resistance While both isolates exhibited some degree of antibiotic resistance, the resistance level was most pronounced in *E. coli* and lowest in *Proteus* species. Ciprofloxacin showed inhibitory zone of 5 mm against *E. coli* and 7 mm against *Proteus* specie when tested with perfloxacin. From the 10 antibiotics tested, *E. coli* showed no resistance to Perfloxacin (PEF), Ofloxacin (OFX), and Amoxacillin (AM) with 34, 30, and 25 mm zone diameters, respectively. On the other hand, *Proteus* species did not show resisance to GEN CPX, C, AUG, and OFX. According to Raja et al. (2009), along with resistance to ampicillin, tetracycline, chloramphenicol, kanamycin, erythromycin, streptomycin, and nalidixic acid, *P. aeruginosa*, *A. radioresistens*, and *Proteus* species, all have multiple metal resistances.

In conclusion, the results showed that the four characterized isolates, namely *B. cereus*, *S. aureus*, *E. coli*, and *Proteus* species exhibited an impressive tolerance to heavy metals. These findings exhibited that microorganisms can adapt to polluted heavy metal environments and be utilized in bioremediation initiatives. More importantly, environmental managers must address the correlation between heavy metal pollution and antibiotic resistance.

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The impacts of rock quarry activities on physicochemical and bacterial diversity of air in Akpuoha and Ishiagu communities of Ebonyi State, Nigeria

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Abstract. *Agwaranze DI, Ogodo AC, Nwaneri CB. 2024. The impacts of rock quarry activities on physicochemical and bacterial diversity of air in Akpuoha and Ishiagu communities of Ebonyi State, Nigeria. Asian J Trop Biotechnol 21: 33-40.* This study aims to evaluate the impacts of rock mining (quarry) activities physicochemical and bacterial diversity of air in two communities, Akpuoha and Ishiagu of Ebonyi State, Nigeria. The physicochemical parameters of the air were determined in situ using hand-held environmental sensor meters (Roca Raton, England), while the diversity of bacteria was determined using the settle plate technique. The results showed that temperature ranged from 28.5-33.4°C, Particulate Matter ($PM_{2.5}$) ranged from 3.0-9.4 µg/m^3, Particulate Matter (PM_{10}) ranged from 3.0-9.6 µg/m^3 and Carbon Oxides (CO_X) ranged from 3.2-8.9 µ/mol, in Ishiagu during the dry season. The same parameters were assessed in Akpuoha during the dry season with values of temperature ranging from 28.8-31.3°C, $PM_{2.5}$ ranged from 4.2-8.7 µg/m^3, PM_{10} ranged from 4.4-9.3 µg/m^3 while CO_X ranged from 3.2-7.4 µ/mol. Values were observed to be highest nearest the quarries but decreased with distances away. The same pattern was observed for the parameters during the rainy seasons in both communities; however, lower values were obtained during the rainy season, with the quarry effects being limited to 100-200 m from the quarry edge. The dry season results indicated a more gradual slope extending to 200-300 m. The bacterial diversity of the air was determined and the bacterial physiological groups determined were Total Heterotrophic Bacterial Count (THBC), Total Pathogenic Bacterial Count (TPBC), Total Coliform Count (TCC), and Total Fecal Coliform (TFC). THBC at Ishiagu and Akpuoha in both seasons had the highest counts, followed by TCC and TPBC, while TFC was the least at each sampling distance. The counts decreased with distance from the Quarry site and increased with plate exposure time. Therefore, all quarry industries should be located away from the immediate vicinity of human settlements.

Keywords: Air quality, bacteria diversity, pathogenic bacteria, physicochemical, quarry

INTRODUCTION

In the simplest terms, rock quarrying is used for making small sizes of stone from bigger rocks. The processes involved in rock quarrying are uniform in most areas, although some quarrying sites may present unique peculiarities. These processes include identifying the site where a large quantity of rock is available below the earth's surface, clearing the rocky land, mining of the rock, drilling, blasting, and then crushing it into smaller particles or desired sizes (Wanjiku 2015). Trucks move back and forth between the processing plants and the pit while transporting rocks to the crushers. The crushers break the stones into various particle sizes as may be determined by the output engineers (Wanjiku 2015).

These quarrying activities along with various other factors like human activities from domestic use of energy and operations of the industries cause alterations in the atmospheric gases, leading to air pollution and its attendant effects on the environment and public health have been reported (Peter et al. 2018; Ezekwe 2019). Air pollution poses serious environmental problems and has been a major concern for developed and developing countries. The effects vary greatly due to varying sources of air pollution (Peter et al. 2018). The dust generated during quarry activities and truck movements falls on land around the sites, plants, and water surfaces (Okafor and Njoku 2021). These dust particles cause air pollution and affect the biodiversity, animals (livestock and wildlife), and humans. Anand (2020) reported that damage is caused to biodiversity due to environmental quarrying. Plants play a significant role in maintaining the oxygen and carbon dioxide balance of the atmosphere through photosynthesis which may be altered due to the settling of dust on the leaves of plants (Enyinnaya et al. 2020; Wang 2020; Okafor et al. 2023).

Generally, the effects of dust emissions from quarries have both micro and regional dimensions. Air pollution and ground vibration from blasting, crushing, and emission of toxic gases negatively impact human health and well-being. The quarrying activities products demand is increasing for agriculture, industries, domestic purposes, and other uses (Owens et al. 1988; Wanjiku 2015). However, the dust generated during quarrying operations is usually suspended in the air over a long period, leading to environmental and health problems (Osuide 1990). Moreover, particulate

matter can be transported from the mining point to faraway areas. Once particles of varying chemical compositions are inhaled, they lodge in human lungs, causing lung damage and respiratory problems (Okafor et al. 2023). Salim (2016) stated that dust generated from granite quarrying contains 71% silica; inhaling such dust results in silicosis, which can disable an exposed person and, subsequently, lead to death. According to epidemiological studies, a close-response relationship between exposure to PM_{10} and respiration morbidity and mortality has been established (Maji et al. 2023).

Microorganisms like bacteria and fungi contribute to nutrient cycling, waste bioremediations, and soil pollutants. Reports have indicated that the community of microorganisms in the soil occupies the majority of biodiversity below the ground (Zhou et al. 2023). These bacterial communities significantly contribute to the ecosystem biomass, nutrient-cycling biodiversity, and energy flows (Nielsen et al. 2015; Jiao et al. 2019). Moreover, the activities of these bacterial communities are also affected by climate change (global warming), greenhouse gases (Dove et al. 2021; Zhou et al. 2023), and other particles released during mining activities, including rock mining and crushing. Hence, the composition of the soil bacteria can also be altered during mining and quarry activities which could affect their performance and beneficial roles in an environment. Pathogenic bacteria can be exposed to humans, the environment, and air by rock mining activities, which could be of public health importance. Therefore, this study aimed to evaluate the effect of stone quarrying activities on the physicochemical and bacterial diversity of Ishiagu and Akpuoha in Ebonyi State, Nigeria.

MATERIALS AND METHODS

Description of study areas

Ishiagu is located in the Ivo Local Government Area in Ebonyi State, Nigeria, at latitudes 50 52' to 50 60' N and longitudes 70 30' to 70 37'' E. The area is situated in the tropical rainforest zone of Nigeria, but due to intensive human activities, it is rapidly becoming a Guinea savanna. Geologically, Ishiagu is part of the Cross River Basin and lies in the well-known Calabar Trough and it's a well-known quarry mining area. Ishiagu is a semi-urban area endowed with several mineral resources, especially lead and zinc ores, including granite. Akpoha is a typical rural community located in Afikpo South LGA and also situated at the southern elbow of Ebonyi State; unlike Ishiagu, Akpoha is located in typically Guinea savanna and has more rainfall, as it lies nearly at the bank of the Calabar River. It is also in the Calabar trough zone and endowed with the same resources as Ishiagu (Peter et al. 2018; Okafor et al. 2023). It is a new mining area. Both study sites have two main seasons – rainy and dry seasons.

Collection of samples

Air samples were collected from the Crushed Rock quarry site (Ishiagu) at various distances: 0 (edge of company premises), 100, 200, and 300 meters away from the company site. Similar sampling distances were used at Akpuoha, where Julius Berger Nigeria Limited operates. Passive air sampling was conducted using the settle plate method for microbial analysis. Petri dishes containing solidified media were exposed to the air (one meter above the ground) at various time intervals ranging from 10, 20, and 30 minutes at the distances mentioned earlier (0, 100, 200, and 300 meters).

Determination of the physicochemical parameters of the air

The air quality was determined in situ using hand-held environmental sensor meters (Roca Raton and England). The meter was switched on at the various sampling points and allowed to stabilize within 1-5 minutes before taking the readings at eye level. Three readings were taken at each sampling point, and the mean was recorded as the value for that particular sampling point. Parameters measured include NO_X, SO_X, CO_X, and CH_4 (Nitrogen Oxides, Sulphur Oxides, Carbon Oxides, and Methane). Others were hydrogen sulphide (H_2S), Ammonia (NH_4), and particulate matter ($PM_{2.5}$ and PM_{10}), including Wind Direction (WD) and temperature. These readings were collected during the rainy and dry seasons according to the various sampling points and communities.

Determination of bacteria diversity/air quality around the quarry sites

The media used for the microbiological analysis were Nutrient Agar, Eosin Methylene Blue Agar, McConkey Agar, and Blood Agar. The various media were for the various bacteriological physiological groups (bacterial community profile) tested. These were prepared according to the manufacturer's instructions and used for air sampling. Passive air sampling was carried out using the settle plate method. Petri dishes containing solidified media were exposed to the air at various time intervals ranging from 10, 20, and 30 minutes at the distances mentioned earlier (0, 100, 200, and 300 meters). The plates were kept about 1-2 m above the ground level and at least 10 m away from obstructions (Agwaranze et al. 2020). This was incubated at 37°C for 24 h, and the number of colonies observed on each culture plate was counted to ascertain the Total Heterotrophic Bacterial Count (THBC), Total Pathogenic Bacterial Count (TPBC), Total Coliform Count (TCC) and Total Fecal Coliform (TFC).

RESULTS AND DISCUSSION

Figure 1 shows that the temperature values ranged from 27.4-31.7°C in Akpuoha and 27.8-32.7°C in Ishiagu (Figure 2) during the rainy season; there were slight differences in dry, but were not statistically significant. However, there were significant variations according to sampling distance from each quarry site, as the temperature values decreased away from the site edge. The highest values were the nearest site, and the lowest was the farthest from the site. The effects were up to 100 m as the values at

200 m and 300 m were statistically different from the edge and the 100 m from the site ($P < 0.05$). Additionally, the wind direction was observed northeast at the study site.

Figures 3 and 4 show the air's wind speed and H_2S values around the two study sites. At Akpuoha, the wind speed values ranged from 1.6 to 2.4 m/s, while at Ishiagu, they ranged between 1.8 to 2.6 m/s. Ishiagu values were non-statistically higher than those from Akpuoha ($P>0.05$). However, the sites had similar values; the highest ones were nearest the site and decreasing away from it. H_2S values ranged from 0.5 to 1.9% in Akpuoha and 0.4 to 3.7% in Ishiagu, with the highest values obtained nearest to the site and lowest farthest away. The effects were within 0 to 100 m but decreased significantly after 200 to 300 m. Values from 0 to 100 m of Akpuoha were significantly lower than those from similar spots at Ishiagu ($P<0.05$).

The results showed the values of $PM_{2.5}$, PM_{10}, NH_4, CH_4 and CO_X in the two study sites: $PM_{2.5}$ values ranged 2.5 to 4.2 µg/m^3 (Akpuoha) and 2.7 to 4.7 µg/m^3 (Ishiagu), PM_{10}, ranged from 2.2 to 7.55 µg/m^3 (Akpuoha) and 2.3 to 8.3 µg/m^3 (Ishiagu). The decrease in the value of PM_{10} was steeper than $PM_{2.5}$ in both sites as $PM_{2.5}$ was more gradual; the other parameter values are shown in those figures. In all the parameters, values obtained at the 0 m (edge) were not significantly different from those at 100 m ($P<0.05$) but differed from those at 200 and 300 m away, indicating that distance from the sites influenced those values.

Values obtained for SO_X and NO_X are shown in Figures 7 and 8 for Akpuoha and Ishiagu, respectively. Values for SO_X at Akpuoha ranged from 0.02 to 0.04 at 0 m (edge) and 100 m, while SO_X was not detected at 200 and 300 m; this was quite similar to 0.2 to 0.5 observed at Ishiagu. SO_X was not detected at 200 and 300 m; there was no statistical difference between values at Ishiagu and Akpuoha. Values of NO_X showed that of 200 and 300 m; there were no statistically significant differences both in distances and sites, but values of 0.09-0.06 in Ishiagu at 0 and 100 m were significantly higher than 0.07 and 0.04 observed at Akpuoha ($P>0.05$). This still showed the influence of distance on the results (Figures 5 and 6).

Temperature values during dry seasons for Akpuoha ranged from 28.5 to 31.3°C, while that of Ishiagu was 28.8 to 33.4°C (Figures 9 and 10). The values gradually decreased at each site, with the effects shown at 200 m away from the site. Only values at 300 m were significantly different from the rest ($P < 0.05$).

Wind speed ranged from 1.2 to 1.9 m/s (Akpuoha) and 1.5 to 2.0 m/s (Ishiagu). Values obtained for temperature were statistically higher in the dry season than rainy season, while the wind speed showed the reverse being higher during the rainy season than dry season. H_2S values are shown in Figures 11 and 12, which indicate higher values nearest the site and lowest away. Values obtained in the dry season were slightly lower than those in the rainy season.

Figures 13 and 14 show the values of $PM_{2.5}$, PM_{10}, NH_4, CH_4, and CO_X; the values of $PM_{2.5}$ and PM_{10} were higher than those of the rainy season and extended to 200 m sampling spot at both sites. On the other hand, values of NH_4 and CH_4 were lower than in three rainy seasons at each sampling point and site. Values obtained for CO_X during the dry season were higher than those of the rainy season, except at the control (300 m). The decrease in values of site edge (0 m) to 200 m was quite gradual, unlike the rainy season.

Results obtained for SO_X and NO_X are shown in Figures 15 and 16; SO_X values did not change significantly on the sites and pattern seasons. Values remained fairly the same, only reported at edge (0 m) and 100 m. Values for NO_X were statistically higher during rainy than during dry seasons, especially at 0 m and 100 m ($P < 0.05$) (Figures 15 and 16).

The air bacterial diversity is shown in Figures 17 and 18 (rainy season) and 19 and 20 (dry season). The bacterial physiological groups determined were Total Heterotrophic Bacterial Count (THBC), Total Pathogenic Bacterial Count (TPBC), Total Coliform Count (TCC), and Total Fecal Coliform (TFC). THBC at Ishiagu ranged from 3.0 to 21.5 cfu/10 minutes, 50 to 31.5 cfu/20 minutes, and 9.0 to 38.0 cfu/30 minutes. The TPBC had a range of 2.0 to 5.5 cfu/10 minutes, 2.0 to 8.5 cfu/20 minutes, and 3.5 to 10.0 cfu/30 minutes. For TCC, the ranges were 2.0 to 9 cfu/10 minutes, 2.5 to 10.5 cfu/20 minutes, and 3.0 to 13.0 cfu/30 minutes. The results for TFC show 2.0 to 5.5 cfu/10 minutes, 2.5 to 7.5 cfu/20 minutes, and 2.5 to 9.0 cfu/30 minutes. THBC had the highest counts, followed by TCC and TPBC, while TFC was the least at each sampling distance.

Figure 18 shows the bacterial colony count and diversity at Akpuoha. The bacterial dynamics in counts, distance, and exposure time followed the same pattern as those observed at Ishiagu. THBC had the highest counts at each sampling point, the highest at edge (0 m), and the least at 300 m. The same pattern was observed in TPBC, TCC, and TFC, which had the lowest counts. In all cases, plates exposed for 10 minutes had the lowest counts, followed by those exposed for 20 minutes, while those exposed for 30 minutes had the highest counts. Observations showed that the counts decreased extensively after 100 m. Spots indicate that the effects of the quarry did not spread out much beyond 100 m.

Figures 19 and 20 show the bacterial dynamics during the dry season. The same trends observed during the rainy season regarding plate exposure time and distance from the quarry sites on bacterial physiological groups were repeated during the dry season. However, observations showed higher bacterial counts and spread to 200 m sampling points, with only the counts at 300 m being statistically significantly low.

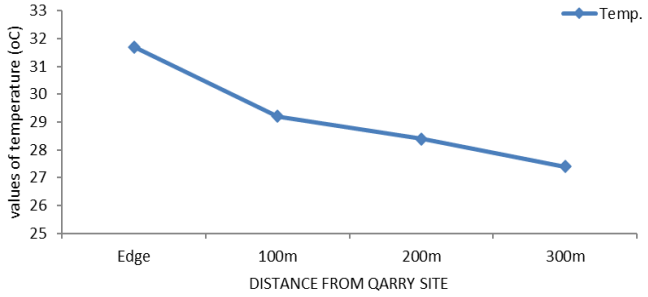

Figure 1. Temperature (°C) variations of air at the Akpuoha Quarry site, Nigeria during the rainy season

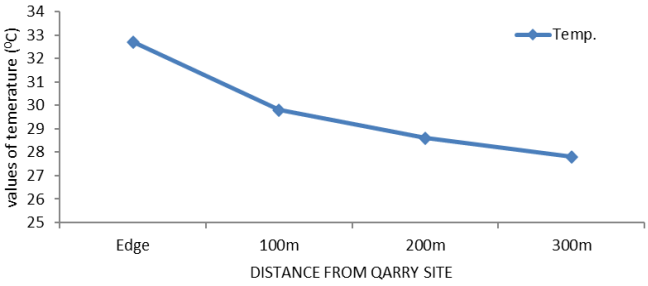

Figure 2. Temperature (°C) variations of air at Ishiagu Quarry site, Nigeria during the rainy season

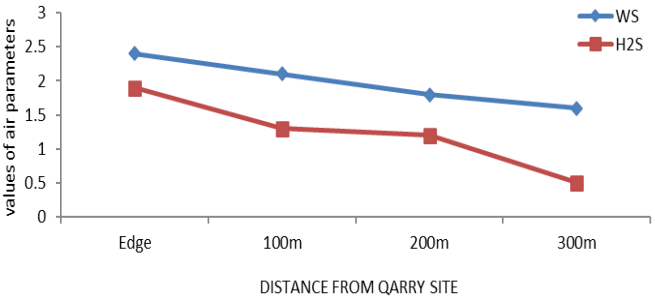

Figure 3. Wind Speed (m/s) and Hydrogen Sulfide concentrations (%) of air at Akpuoha, Nigeria during the rainy season. WS: Wind Speed, H_2S: Hydrogen Sulphide

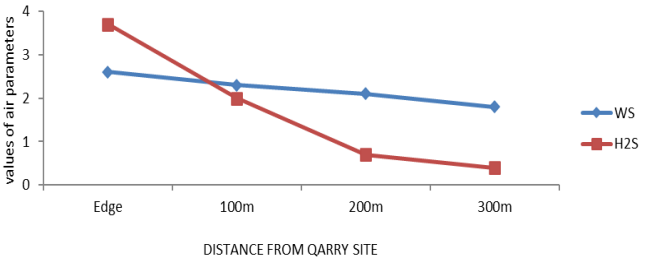

Figure 4. Wind Speed (m/s) and Hydrogen Sulfide (%) of air at Ishiagu, Nigeria during the rainy season

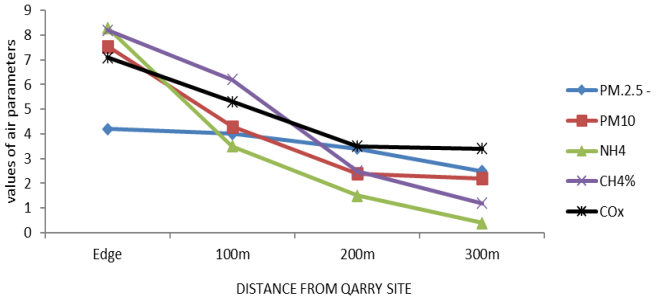

Figure 5. Particulate Matter $PM_{2.5}$, Particulate Matter PM_{10} (µg/m^3) Ammonium, Methane (%) and Carbon Oxides of air at Akpuoha, Nigeria during the rainy season

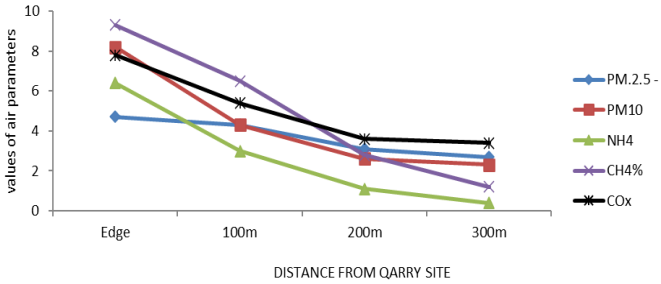

Figure 6. Particulate Matter $PM_{2.5}$ Particulate Matter PM_{10} Ammonium, Methane (%), and Carbon Oxides of air at Ishiagu, Nigeria during the rainy season

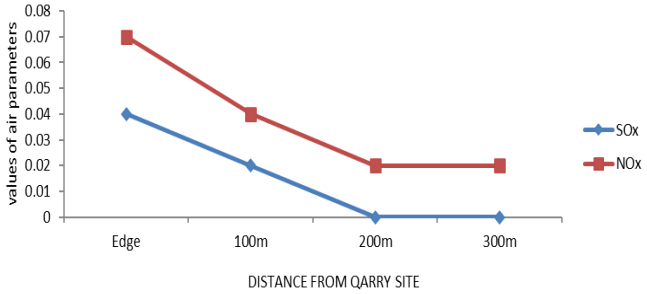

Figure 7. Sulphur Oxides and Nitrogen Oxides (µg/m^3) of air at Akpuoha, Nigeria during the rainy season

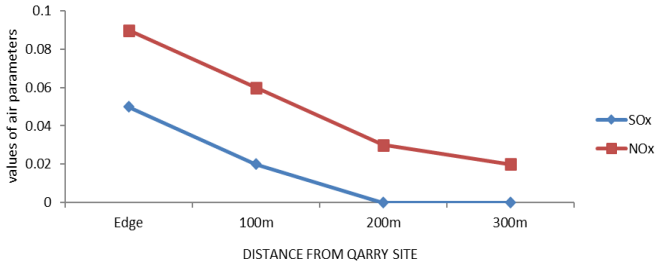

Figure 8. Sulphur Oxides and Nitrogen Oxides (µg/m^3) of air at Ishiagu, Nigeria sample during the rainy season

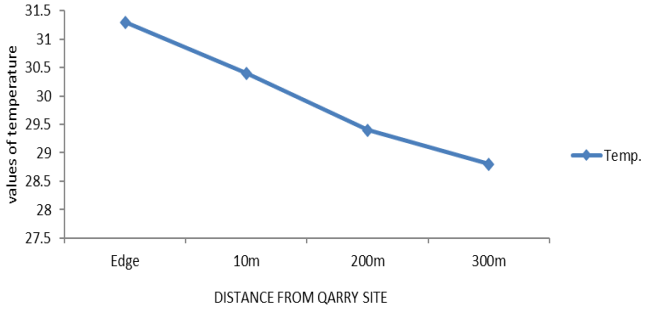

Figure 9. Temperature (°C) variations of air at Akpuoha, Nigeria during the dry season

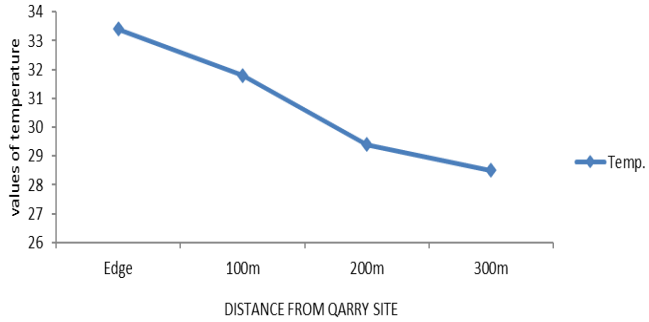

Figure 10. Temperature (°C) of air at Ishiagu, Nigeria during the dry season

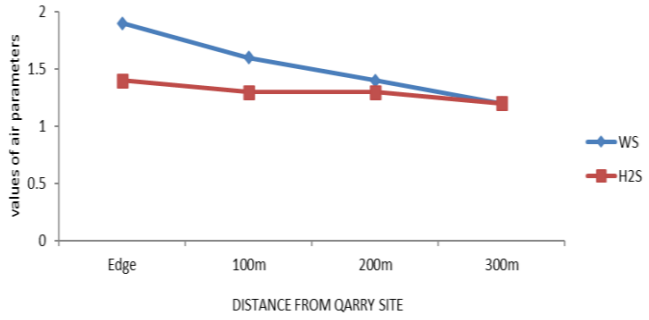

Figure 11. Wind Speed (m/s) and Hydrogen Sulfide (%) of air at Akpuoha, Nigeria during the dry season

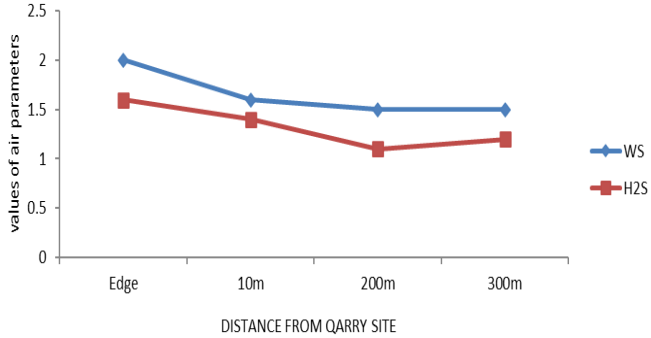

Figure 12. Wind Speed (m/s) and Hydrogen Sulfide during the dry season (%) according to the distance at Ishiagu, Nigeria

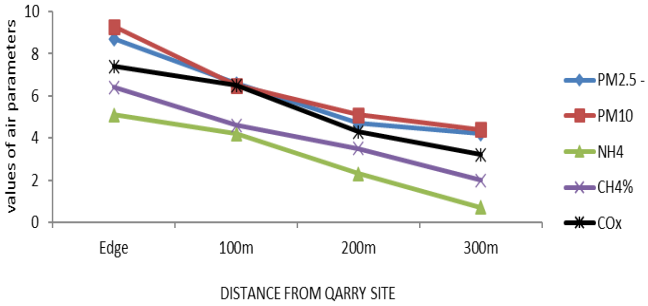

Figure 13. Particulate Matter $PM_{2.5}$, Particulate Matter PM_{10} ($\mu g/m^3$) Ammonium, Methane (%), and Carbon Oxides of air at Akpuoha, Nigeria during the dry season

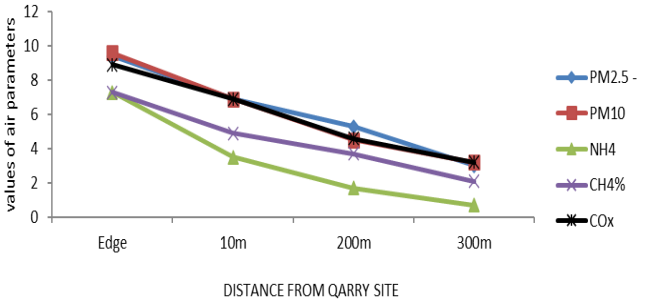

Figure 14. Particulate Matter $PM_{2.5}$, Particulate Matter PM_{10}, Ammonium, Methane (%), and Carbon Oxides of air sample during the dry season at Ishiagu, Nigeria

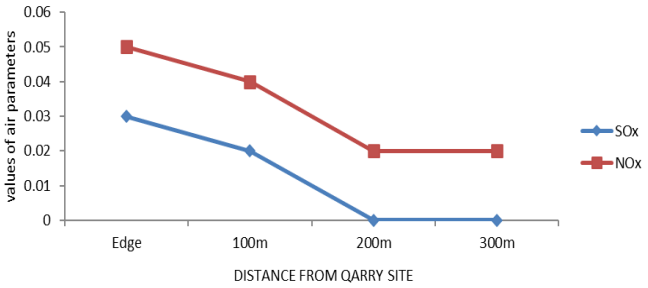

Figure 15. Sulphur Oxides and Nitrogen Oxides of air sample during the dry season (%) at Akpuoha, Nigeria

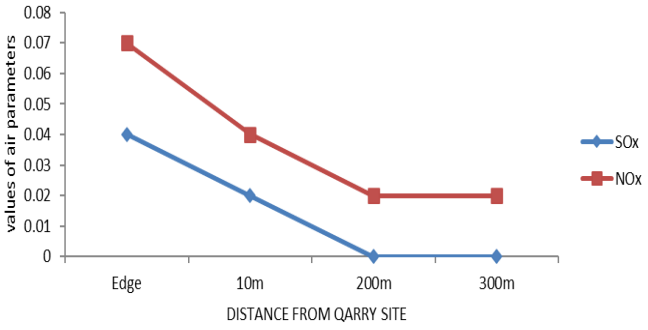

Figure 16. Sulphur Oxides and Nitrogen Oxides ($\mu g/m^3$) of air sample dry during the rainy season (%) at Ishiagu, Nigeria

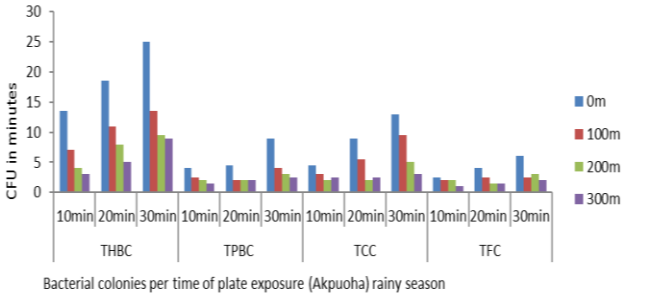

Figure 17. Total Heterotrophic Bacterial Count, Total Potential Pathogenic Bacterial Count, Total Coliform Count, and Total Fecal Count during the rainy season at Akpuoha, Nigeria

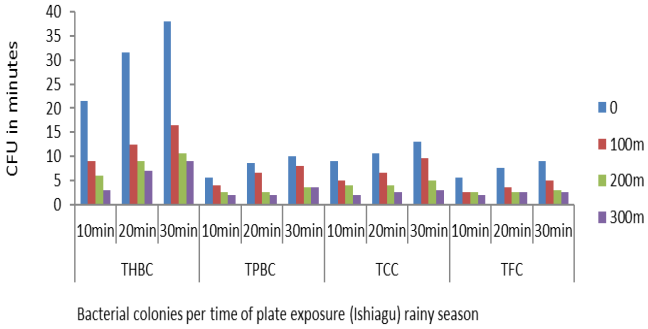

Figure 18. Total Heterotrophic Bacterial Count, Total Potential Pathogenic Bacterial Count, Total Coliform Count, and Total Fecal Count during the rainy season at Ishiagu, Nigeria

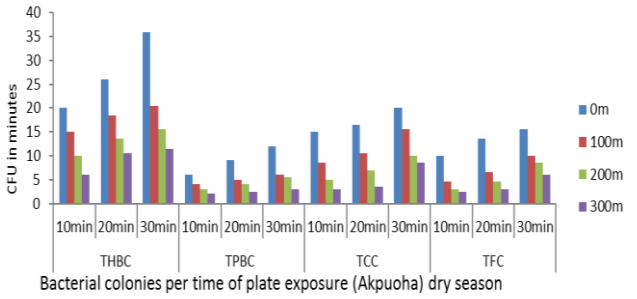

Figure 19. Total Heterotrophic Bacterial Count, Total Potential Pathogenic Bacterial Count, Total Coliform Count, and Total Fecal Count during the dry season at Akpuoha, Nigeria

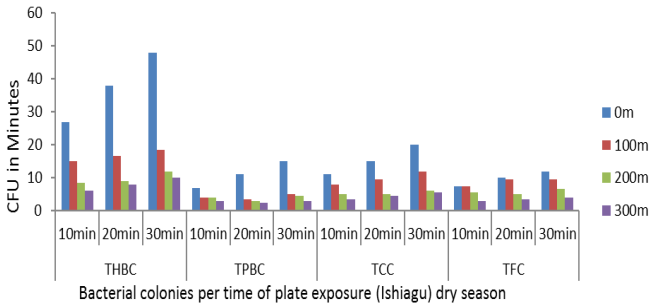

Figure 20. Total Heterotrophic Bacterial Count, Total Potential Pathogenic Bacterial Count, Total Coliform Count, and Total Fecal Count during the dry season at Ishiagu, Nigeria

Discussion

The impact of rock quarry activities on air quality was analyzed in this study. The quality of air inhaled by an individual within his environment determines, to a greater degree, the well-being of that individual. From the air quality of the two communities. It was observed that there was no significant change in the temperature of the two sites sampled (Akpuoha and Ishiagu), as the temperature did not vary significantly at various distances during the rainy season. Again, the wind direction was also the same - South-westerly in both sites. However, particulate matter $PM_{2.5}$ and particulate matter PM_{10} showed remarkable variations based on distance from the quarry but did not vary based on sites; the values decreased with distance from the edge of each quarry. This agreed with Nwaugo et al. (2006) and Abdulkarim et al. (2007) who revealed that particles settle out of the air over distance, and the highest concentration of any substance is nearest the production site. Similarly, Agwaranze et al. (2018) also reported that Ammonium (NH_4), Methane (CH_4), Sulfate (SO_4), and Nitrate (NO_3) had the highest concentration in the production area. Earlier, Chiemeka (2011) revealed that the dust particles from substance quarries containing Phosphate (PO_4), Nitrogen Oxides (NO_x), Carbon Oxides (CO_x), and Sulphur Oxides (SO_x), which are released into the air as dust. These substances, PO_4, NO_x, CO_x, and SO_x, are integral components of the quarry dust particles; hence, their presence increases in the air around the quarry sites. Generally, observations indicate that heavier particles settle out of the air quicker and more easily than lighter ones. This agrees with the observations in this work, where PM_{10} settled out of the air within a very short distance compared to the lighter $PM_{2.5}$ particles. This study revealed that 300 m away from the quarry pits had non-significant concentrations of those substances evaluated. In addition, observations showed higher values at the old quarry pits of Ishiagu compared to Akpuoha. This could be attributed to the persistence of the produced accompanying particles, which settled close to the sampling site. Any little disturbance to those dust settlements could cause the dust to spread to another surface to become airborne again, thereby increasing the quantity of the air content; even though by time, the settled particles are released back into the air. However, not much dust had settled on the surfaces near the new rock quarry sites, which could cause old quarry dust to be higher than the new quarries.

Observations indicate higher concentrations of air contaminants during the dry compared to the rainy seasons. This could be attributed to two major issues. The waters in the rainy season washed the contaminating substances that did not happen in the dry season. Second, most construction work occurs more during the dry than the rainy seasons. Quarry stones are one of the materials required for the construction; hence, quarry activities occur more during the dry season. Therefore, increased production of quarry products results in increased accompanying wastes. The above observations show the level of air contamination around quarry areas could be influenced by season and distance from the quarry sites. Moreover, a similar observation has been recorded by

Peter et al. (2018) who studied the rainwater effect. This study reported the higher values of air contaminants during the dry than the rainy season, attributed to the rainwater effect. Also, Kalu (2018) reported higher values of air physicochemical parameters in the dry seasons than in the rainy seasons due to rainwater air cleansing.

The study revealed a very gradual decrease of air contaminants during the dry season than the rainy season. This indicates that the wind of the dry season spreads these substances over a long distance before they begin to settle. This phenomenon was unlike in the rainy season, where the rainwaters are regular and easily washed those substances before they are spread out; this results in higher significant differences between the sampling points during the rainy but less so in the dry seasons.

Though higher concentrations of the contaminating substances were reported in the old Ishiagu quarry than in the newest Akpuoha, the differences were not statistically significant. This was because the quarry activities were similar and produced the contaminating substances in similar quantities, whether new or old quarry. This indicates that the main substances are similar or could be from a similar source. Etok et al. (2010) reported that the study area in this work lies within the same plain and trough – the Calabar trough and, therefore, has rock components with a similar composition. Therefore, it is normal to state that rock quarries of similar sites have similar amounts of the substances' components assessed.

Microbial assessment of the air showed similar trends in all the sampling points, though values observed in the old Ishiagu area were higher. This further bolstered the similarity in the origin of the parent material and showed that the rock mined in both sites could come from similar sources or the same source. Nwaugo et al. (2006) and Etok et al. (2010) reported that Total Heterotrophic Bacteria Count (THBC) was, in most cases, higher than all other groups of microorganisms assessed in any given substances or sites. This was attributed to the fact that all other microorganisms of any other physiological group were equally integral to the THBC. From the studies assessed, total heterotrophic bacteria count was the most prominent, followed by the Total Coliform Count (TCC) and Total Fecal Coliform Count (TFC). At the same time, the Total Pathogenic Bacteria Count (TPBC) was the lowest in each site and distance. Generally, it could be inferred that all bacterial species could be found in the THBC while other groups are specialized. The THBC is the most heterogeneous, accounting for the highest number at each site and distance. Again, the presence of the TFC suggests fecal air contamination. Though most TFCs are not spore-formers, several anthropogenic and natural activities could blow some into the air, especially as defecation in bushes around the quarry area becomes common.

The present observations suggest that the microbial groups assessed decreased with distance away from each quarry site. This suggests that the human activities at the sites caused the increase in microbial population as this was observed in both sampling areas. These human activities decreased as the distance from the site increased. Agwaranze et al. (2020) noted a high frequency of airborne bacteria isolates in indoor environments. It could also be noted that human activities include throwing away waste from food, unhealthy sanitary practices, sneezing, and coughing, all of which could send microorganisms into the air. Abu-Allaban and Abu-Qudais (2011) stated that once organisms are airborne, they can be carried over a distance. However, the distances to which these organisms could be spread depend on the substance to which the microorganisms are attached and the prevailing environmental factors besides the microorganisms' nature. Chiemeka (2011) stated that rock dust in Ugwuele, Uturu, contained SO_4, PO_4, and less NO_3, including some trace elements. Therefore, the microbes in the air could leverage these substances for survival, especially under humid environmental conditions. However, following the seasonal effects, many microorganisms were observed to be spread further away during the dry season than in the rainy season. The same reasons that affected particulate matter also applied to the microbial loads. These microorganisms were washed down quickly during the rainy season, thereby reducing their spreading, unlike the dry season, where no rain washed down the particles carrying the microorganisms.

Furthermore, human activities were limited to areas near the mining sites during the rainy season as no people went close to being wet. Similar observations were recorded at the two sites screened, suggesting a similar occurrence, no matter the age of the quarries. This indicates that the most impacted areas were those nearest the site and that the effects waned by the distance; this observation was made during both rainy and dry seasons with similar results. The excavation and blasting processes sent dust particles into the air that quickly came back down to earth. The decline rate depends on size (weight) and environmental factors. This follows, therefore, that most of the airborne particles were found nearest the mining (quarry) sites, but the lighter particles were spread out to areas far away from the site. Further observations indicated that the concentration of various compounds in the rock dust also decreased at longer distances.

In conclusion, this study revealed that the quarry activities in the studied communities significantly affect the physicochemical parameters and bacterial diversity of the air within the surroundings. Concentrations of particulate matter and gases were observed more near the quarry site and decreased with increasing distance from the mining site, irrespectively of wet or dry seasons. Similar observations were recorded for various microbial groups, indicating higher concentrations of bacteria around the sites where human activities occur. Therefore, stone mining and quarrying should be located far from residential areas to reduce air pollution and its impact on human health.

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Phylogenetic relationship and biotechnological use potential of epiphytic Actinomycetota species isolated from seagrasses from the coast of Tanzania

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Department of Molecular Biology and Biotechnology, College of Natural and Applied Science, University of Dar es Salaam. P.O. Box 37159, Dar es Salaam, Tanzania. Tel.: +255-222410129, ♥email: tlyimo2000@yahoo.com; tjlyimo@udsm.ac.tz

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Abstract. *Mbusi LD, Lyantagaye SL, Lyimo TJ. 2024. Phylogenetic relationship and biotechnological use potential of epiphytic Actinomycetota species isolated from seagrasses from the coast of Tanzania. Asian J Trop Biotechnol 21: 41-51.* Actinomycetota, previously recognized as actinobacteria, has demonstrated considerable promise as a valuable reservoir of secondary metabolites with potential pharmaceutical applications. This study examined the diversity, antimicrobial properties, and cytotoxic effects of epiphytic Actinomycetota species isolated from the seagrasses *Thalassia hemprichii* and *Syringodium isoetifolium*. Therefore, 12 strains of Actinomycetota were isolated through a process involving assessment of their morphological and biochemical characteristics, along with phylogenetic analysis using partial sequences of the 16S rRNA gene. The identified strains were associated with eight phylogenetic genera: *Cellulosimicrobium*, *Corynebacterium*, *Microbacterium*, *Rhodococcus*, *Arthrobacter*, *Leucobacter*, *Dietzia,* and *Micrococcus*. The findings unveiled five potential new species within Actinomycetota. Seven of the 12 strains displayed antimicrobial effects against at least one human pathogen tested. Notably, the *Microbacterium* strain SIP6 exhibited extensive antimicrobial efficacy against all the pathogens under examination. Toxicity tests revealed that only two strains (*Microbacterium* (THP6) and *Micrococcus* (SIP14)) were nontoxic, with the lowest LC_{50} values of 3836.7 and 1243.4 µg/mL, respectively, while the remaining extracts were toxic. This study marks the initial discovery of epiphytic Actinomycetota strains from seagrasses in the western Indian Ocean. The isolated epiphytic Actinomycetota strains, some of which are novel, showed potential for bioactive metabolites that hold promise for biotechnological use.

Keywords: Bioactivity, epiphytic bacteria, marine Actinomycetota, phylogenetic analysis, seagrass species

INTRODUCTION

Actinomycetota is a phylum that primarily consists of Gram-positive bacteria. This phylum was reclassified from Actinobacteria to Actinomycetota due to amendments in the International Code of Nomenclature of Prokaryotes concerning phylum-level classifications (Whitman et al. 2018). The Actinomycetota phylum is categorized into six classes, specifically, Actinomycetia, Nitriliruptoria, Acidimicrobiia, Thermoleophilia, Rubrobacteria, and Coriobacteria according to Bergey's Manual of Systematic Bacteriology. Actinomycetota genomes stand out due to their elevated Guanine-Cytosine (GC) content and include genes linked to the production of antibiotics (Mast and Stegmann 2019). Their reputation stems from producing more than 20,000 natural compounds widely used in pharmaceuticals and agrochemicals (Charousová et al. 2017). With the rise of bacterial resistance to antibiotics and the emergence of various diseases like cancer, AIDS, and other severe ailments, there is a worldwide urgency to uncover new drugs (Aslam et al. 2018). Infections caused by resistant microbes lead to high illness and death rates, incur greater treatment costs, and extend hospital stays, imposing significant strains on healthcare networks (Frost et al. 2019). Furthermore, 50% of the drugs currently accessible on sale are derived from natural compounds (Cita et al. 2017), including those sourced from marine organisms (Boontanom and Chantarasiri 2020). Although most Actinomycetota secondary metabolites are powerful antibiotics, this phylum also has clinical significance in synthesizing antitumor drugs. These comprise drugs like aclarubicin, actinomycin D, neocarzinostatin, doxorubicin, bleomycin, and numerous others (Ngamcharungchit et al. 2023). Nonetheless, the quest for new medications remains a crucial objective in cancer treatment, given the swift emergence of resistance to numerous chemotherapy drugs.

Actinomycetota is mainly distributed in soil, freshwater, and marine environments (Passari et al. 2017). Nevertheless, in contrast to terrestrial settings, the marine environment remains underexplored in terms of its potential as a reservoir for antimicrobial substances (Boontanom and Chantarasiri 2020). Marine Actinomycetota had to adapt from the deepsea floor's extremely high pressure, anaerobic conditions, and temperatures just below 0-8°C to the highly acidic environments with temperatures ranging from over 8°C to 100°C near hydrothermal vents at mid-ocean ridges. Consequently, very few new metabolites synthesized by marine Actinomycetota have been harnessed for pharmaceutical purposes. Marine environments include seagrass meadows distributed in coastal areas worldwide, excluding Antarctica (Piñeiro-Juncal et al. 2022). There are 13 genera, and 72 species of these seagrasses found globally. Seven genera (*Halodule*, *Cymodocea*, *Halophila*, *Enhalus*, *Syringodium*, *Thalassia*, and *Thalassodendron*)

are distributed in tropical seas (Ravikumar et al. 2010). In Tanzania, a total of 12 seagrass species, namely, *Thalassodendron ciliatum* (Forssk.) Hartog, *Thalassia hemprichii* (Ehrenb. ex Solms) Asch., *Zostera capensis* Setch., *Halophila stipulacea* (Forssk.) Asch., *Syringodium isoetifolium* (Asch.) Dandy, *Cymodocea serrulata* (R.Br.) Asch. & Magnus, *Halophila ovalis* (R.Br.) Hook.f., *Cymodocea rotundata* Asch. & Schweinf., *Halophila minor* (Zoll.) Hartog, *Halodule uninervis* (Forssk.) Boiss., *Enhalus acoroides* (L.f.) Royle and *Halodule pinifolia* (Miki) Hartog, have been recognized (Lugendo et al. 2024). Most large seagrass meadows are between shores or cliffs and nearby fringing reefs. These seagrasses host diverse bacterial communities, including epiphytic Actinomycetota bacteria that form symbiotic relationships within their leaves and roots. Mishra and Mohanraju (2018) reported *Micrococcus* as epiphytic in the seagrasses *C. rotundata* and *Thalassia testudinum* Banks & Sol. ex K.D.Koenig. These bacterial strains differ from those found in the surrounding environment, particularly those dominant in the water column (Roth-Schulze et al. 2016). Epiphytic bacteria, as described by Tarquinio et al. (2019), are benign bacteria that reside on the surface of different plant organs. The symbiotic relationship between bacteria and plants has also been associated with the ability of bacteria to produce metabolites that protect seagrasses from pathogens, nitrogen fixation, and nutrient cycling (Mishra and Mohanraju 2018; Su et al. 2023).

Among bacteria, Actinomycetota species have been described as important colonizers of seagrasses (Siro and Pipite 2024). Currently, there is no research on the population of epiphytic Actinomycetota in seagrasses in the Western Indian Ocean region. In this research, we isolated and characterized culturable epiphytic Actinomycetota strains from the seagrasses *T. hemprichii* and *S. isoetifolium* in seagrass meadows of Mjimwema, Dar es Salaam, Tanzania. Additionally, we assessed the antimicrobial effects and cytotoxicity of raw extracts derived from isolated epiphytic Actinomycetota strains, which may contribute to antibacterial or antifungal activities.

MATERIALS AND METHODS

Study area

Seagrass samples were collected at Mjimwema Beach, located 4 km south of the Dar es Salaam harbor, Tanzania, at approximately 06°50'S and 39°21'E (Figure 1). Leaf samples of the seagrass species *T. hemprichii* (THP) and *S. isoetifolium* (SIP), which are among the dominant seagrass species in this area, were collected during low tides at approximately 10 m intervals within the seagrass-dominated by each species, resulting in 10 sampling points per species. Seagrass species were identified based on the field guidebook by Oliveira et al. (2015). The samples of each species were collected in a sterile plastic bag; they were kept in a cool box with ice cubes and transported to the Molecular Biology and Biotechnology Department Laboratory, University of Dar es Salaam, Tanzania, for further analysis. The Actinomycetota isolation procedures were conducted on the same day as the sample collection.

Isolation and identification of epiphytic Actinomycetota

The procedure for isolating marine epiphytic Actinomycetota from seagrass samples was adhered to the methodology outlined by Boontanom and Chantarasiri (2020) with minor modifications. The surfaces of the seagrass leaf samples were softly rubbed using a sterile cotton swab and then placed into 2 mL of sterilized seawater. Next, 1 mL of suspension was applied to cover the surface of a Starch Nitrate Agar (SNA), while an additional 1 mL was spread onto actinomycete isolation agar. Both media were enriched with nystatin (50 µg/mL) and nalidixic acid (20 µg/mL) to prevent non actinomycetota bacterial and fungal, respectively. The plates were incubated at 28°C for 7-14 days until colonies appeared. The cultures were monitored daily to observe the growth of Actinomycetota. The epiphytic Actinomycetota were identified through morphological assessment (macroscopic and microscopic examination) and biochemical analysis using the techniques outlined by Lema et al. (2022). Biochemical characterization included methyl red tests, catalase tests, gelatin hydrolysis tests, Voges-Proskauer tests, oxidase tests, urea hydrolysis tests, indole tests, citrate utilization tests, hydrogen sulfide production tests, and starch hydrolysis tests.

Molecular characterization and phylogenetic analysis

The genomic DNA of the isolated epiphytic Actinomycetota was extracted according to the manufacturer's instructions using the ZymoBIOMICS DNA Fungal/Bacterial MinPrep kit. The extracted DNA's quality and quantity were evaluated using a Nanodrop One device from Thermo Fisher Scientific in the USA. The primers 235F-CGCGGCCTATCAGCTTGTTG and 878R-CCGTACTCCCCAGGCGGGG were used to determine whether the isolates belonged to the Actinomycetota phylum following the procedure outlined by Kaale et al. (2022). PCR was performed in PCR tubes of 200 µL, starting with an initial denaturation at 94°C for 30 minutes. This was followed by 35 cycles at the same temperature regimen, each lasting 30 seconds, primer annealing at 60°C for 50 seconds, and primer extension at 68°C for 1 minute. The reaction was then held at 68°C for 5 minutes before cooling to 4°C. The PCR products were analyzed on a 1.5% agarose gel and visualized using a UV transilluminator (Electron, VWR International Ltd.). Gel imaging was carried out using a gel documentation system (Zenith).

The PCR products underwent sequencing with the standard pair of primers (235F' and 878R') using an Applied Biosystems instrument from the USA. This sequencing was performed at the commercial facility Macrogen Europe (www.macrogen-europe.com). The partial sequences of these 16S rDNA sequences were subjected to BLAST analysis against the GenBank sequence database on the National Centre for Biotechnology Information (NCBI) website (http://www.ncbi.nih.gov). Phylogenetic analyses were performed using MEGA version 11 (Tamura et al. 2021). The MUSCLE program in MEGA version 11 was employed to align the sequences, and the evolutionary history was deduced using the maximum likelihood method (Tamura et al. 2021). A consensus tree based on 1000

replicates using bootstrap was created to depict the evolutionary lineage of the studied taxa.

Determination of the bioactivity potential

Extraction of bioactive compounds

The culture broth (1500 mL) of Actinomycetota isolates underwent solvent extraction to collect the crude metabolites. Initially, the culture broth was filtered through cotton wool and subsequently centrifuged at 15,000 rpm for 10 minutes. Ethyl acetate was introduced into the filtrate at a 1:1 (v/v) ratio, and the blend was vigorously shaken for 24 hours to achieve thorough extraction. The extracts obtained were dried to completion using a rotary evaporator (BUCHI Labortechnik AG, Switzerland) at 45°C, following the procedure described by Lema et al. (2022).

Antimicrobial activity

The extracts were evaluated for their antimicrobial activity against four specific reference microorganisms: the Gram-positive bacterium *Staphylococcus aureus* (ATCC-25923) Rosenbach, 1884, the Gram-negative bacteria *Pseudomonas aeruginosa* (ATCC-27853) A, and *Escherichia coli* (ATCC-35218) E, and the fungus *Candida albicans* (ATCC-14053) (C.P.Robin) Berkhout. The evaluation was conducted using the agar well diffusion technique, following the protocol described by Hajizadeh et al. (2023) with slight adjustments. The pathogenic bacteria were cultured on Muller Hinton agar, whereas the fungal pathogen was cultivated on Sabouraud's dextrose agar. Once the media had solidified, wells were formed, and the bacterial and fungal cultures were introduced using a sterile micro tip (1 mL), after which 100 µL of the extracts dissolved in dimethyl sulfoxide (DMSO) was added. The bacteria were incubated at 37°C, while the fungi were kept at 28°C. After 24 hours of incubation, the diameter of the inhibition zones was measured to assess the antimicrobial effectiveness of the Actinomycetota isolates.

Minimum Inhibitory Concentration (MIC)

The Minimum Inhibitory Concentration (MIC) was determined using the micro broth dilution method, following the procedure described by Hamisi et al. (2023). The extracts were serially diluted in sterile Mueller Hinton broth (Liofilchem, Italy) and subsequently added to 96-well microtiter plates containing bacterial cultures. This resulted in final concentrations ranging from 25 mg/mL to 0.195 mg/mL. The plates were then incubated at 37°C for 24 hours, with each test performed in duplicate. After incubation, an indicator dye of p-iodonitrotetrazolium (0.02%/40 µL) was added, and the mixture was incubated for an additional hour at 37°C. A pink color change indicated microbial growth, while the indicator remained colorless without growth. The MIC was identified as the lowest concentration, causing a color change.

Cytotoxicity test

The Brine Shrimp Lethality Test (BSLT) was used to evaluate the cytotoxicity of the extracts following the method outlined by Hamisi et al. (2023). The crude extracts were dissolved in DMSO to prepare a stock solution with a 40 mg/mL concentration for each sample. Brine shrimp eggs were hatched by placing one teaspoonful of 300 mL of filtered seawater in a container, which was then illuminated using an electric bulb for 48 hours. Next, 10 shrimp larvae were carefully chosen and transferred into individual sample vials using 100 µL pipettes. The volume in each vial was then adjusted to 5 mL using artificial seawater, which was made by dissolving 3.8 g of sea salt in 1 liter of distilled water. Different amounts of the extract from the stock solution were introduced into the wells, achieving concentrations of 240, 120, 80, 40, 24, and 10 µg/mL. Each concentration of the extract was evaluated in duplicate. The negative control sample was DMSO (0.6%) without extract treatment. Following 24 hours, the surviving larvae were tallied, and the percentage mortality was computed for each dosage. The concentration causing 50% mortality of the brine shrimp (LC_{50} in µg/mL with 95% confidence intervals) was calculated using the Microsoft Excel computer program. The brine-shrimp percentage mortality rates were graphed against the logarithm of the concentration using the Microsoft Excel program; furthermore, the regression equation was determined. The LC_{50} (µg/mL) was determined from the logarithmic plot. The following formula calculated the percentage mortality:

$$\% \text{ Mortality} = \frac{\text{Number of dead brine shrimp larvae}}{\text{Total number of brine shrimp larvae}} \times 100$$

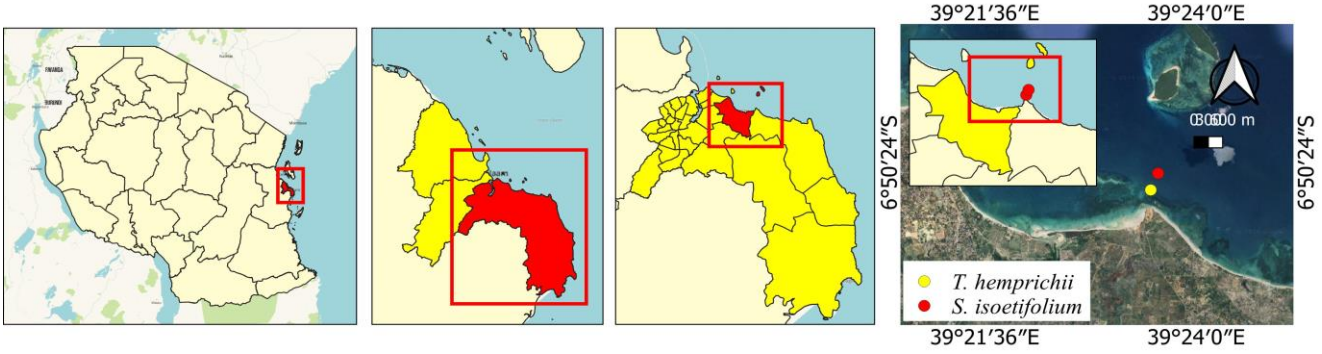


Figure 1. Map of Mjimwema, Dar es Salaam, Tanzania, illustrating sampling points for each seagrass species

The toxicity criteria for the fractions were established based on the guidelines by Hamidi et al. (2014): LC_{50} values greater than 1000 µg/mL were deemed nontoxic, LC_{50} values between 500 and 1000 µg/mL were considered weakly toxic, and LC_{50} values below 500 µg/mL were classified as toxic.

RESULTS AND DISCUSSION

Morphological and biochemical identification of isolated Actinomycetota strains

Moreover, 12 isolates (8 from *T. hemprichii* and 4 from *S. isoetifolium* seagrasses) were identified as Actinomycetota based on their phenotypic characteristics. All the isolates were Gram-positive and rod, cocci, or rod-filamentous in shape with different colony colors, including yellow, cream, and orange (Figure 2, Table 1). Morphological traits such as the color and texture of colonies have traditionally served as key criteria for categorizing Actinomycetota (Hasani et al. 2014; Lema et al. 2022). According to these observations, we inferred that our isolates could be assigned to eight distinct Actinomycetota genera, as detailed in Table 1. *Microbacterium* was the most dominant among the genera identified, followed by *Micrococcus*. Biochemical analysis revealed that all 12 isolates were Voges-Proskauer negative and could produce catalase, oligo-1,6-glucosidase (starch hydrolysis), gelatinase, and a-amylase enzymes. Most isolates were Indole- and citrate-negative, and other biochemical test results are explained in Table 2.

Phylogenetic analysis of the isolated epiphytic Actinomycetota strains

Genotyping revealed that the marine epiphytic strains isolated from the seagrass *T. hemprichii* belonged to six genera of the phylum Actinomycetota, namely, *Cellulosimicrobium*, *Corynebacterium*, *Microbacterium*, *Rhodococcus*, *Arthrobacter* and *Micrococcus*, while the strains from the seagrass *S. isoetifolium* belonged to four genera, namely, *Leucobacter*, *Microbacterium*, *Dietzia* and *Micrococcus*. Seven Actinomycetota isolates in this study exhibited sequence similarities ranging from 98.96% to 100% with type strains documented in the NCBI database. According to Kim et al. (2014), differentiating species with higher GC content is possible when their 16S rRNA gene sequence similarity decreases to less than 98.65%. Therefore, five of the acquired Actinomycetota strains in this study were classified as potentially new species (Table 3).

Table 1. Morphological characteristics of epiphytic Actinomycetota strains obtained from Mjimwema seagrasses, Tanzania

Isolates code	Aerial mass color	Reserve color	Texture	Elevation	Cell shapes (microscopic)	Probable genera	Reference
THP1	Cream yellow	Cream yellow	Smooth	Flat	Rod	*Cellulosimicrobium*	Zhang et al. (2020)
THP5	Whitish grey	Whitish grey	Rough	Raised	Rod	*Corynebacterium*	Sunbul (2000)
THP6	Cream	Cream	Smooth	Raised	Rod	*Microbacterium*	Meddeb-Mouelhi et al. (2016)
THP7	Pale yellow	Pale yellow	Rough	Flat	Rod	*Rhodococcus*	Ward et al. (2018)
THP9	Cream	Cream	Smooth	Flat	Rod, filamentous	*Arthrobacter*	Siddiqi et al. (2023)
THP10	Pale yellow	Pale yellow	Smooth	Raised	Cocci	*Microbacterium*	Cheng et al. (2019)
THP12	Orange	Orange	Rough	Flat	Rod	*Microbacterium*	Suzuki and Moriyuki (2015)
THP13	Cream	Cream	Rough	Flat	Rod, filamentous	*Micrococcus*	David et al. (2017)
SIP3	Yellow	Yellow	Smooth	Raised	Cocci	*Leucobacter*	Clark and Hodgkin (2015)
SIP6	Yellow	Yellow	Smooth	Flat	Cocci	*Microbacterium*	Cheng et al. (2019)
SIP12	Pale yellow	Pale yellow	Smooth	Flat	Cocci	*Dietzia*	Hirvonen et al. (2012)
SIP14	Creamy yellow	Creamy yellow	Rough	Raised	Cocci	*Micrococcus*	David et al. (2017)

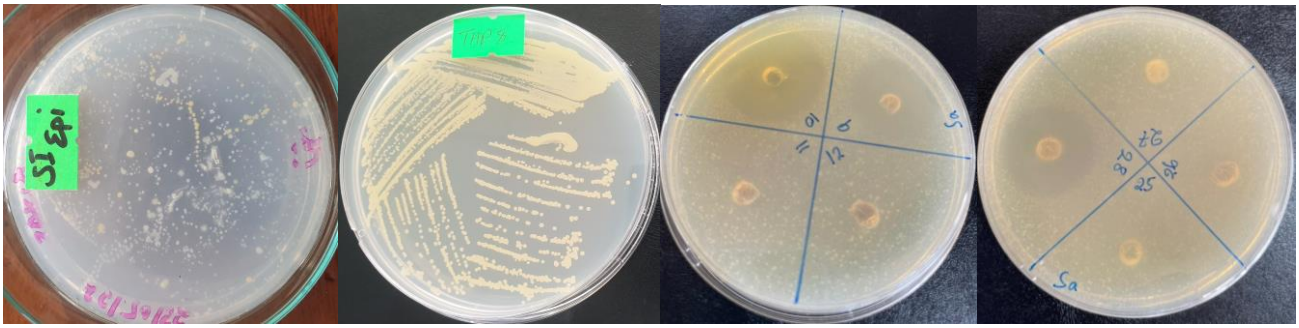
Table 2. Biochemical characterization of epiphytic Actinomycetota from Mjimwema seagrasses, Tanzania

Isolates code	Oxidase	MR test	Urea hydrolysis	Indole test	Citrate	Casein
THP1	-	+	+	-	-	+
THP5	-	+	-	-	-	-
THP6	-	+	+	-	-	-
THP7	-	+	+	-	-	-
THP9	+	-	+	-	-	+
THP10	+	-	-	-	-	+
THP12	+	-	+	-	-	-
THP13	+	+	-	-	+	-
SIP3	+	+	+	+	+	+
SIP6	-	+	+	+	+	-
SIP12	+	+	+	-	+	+
SIP14	+	-	+	-	+	+

Table 3. BLAST results for epiphytic Actinomycetota Isolated from seagrasses with their closest match from the GENBANK database

Cluster	Isolate (Accession number)	Size (bp)	Closest match (accession number)	%	Source	Reference microorganism
1	THP1 (OR936744)	590	*Cellulosimicrobium funkei* (ON678198)	100	Lake	Glekas et al. (2022)
2	THP5 (OR936745)	591	*Corynebacterium casei* (DQ361013)	100	Cheese	Monnet et al. (2006)
3	THP6 (OR936746)*	585	*Microbacterium paraoxydans* (MK403853)	97.09	Desert Soil	Belov et al. (2019)
4	THP7 (OR936747)*	585	*Rhodococcus rhodnii* (X80622)	96.36	Culture	Rainey et al. (1995)
5	THP9 (OR936748)*	580	*Arthrobacter* sp. M44 (AF235113)	81.36	North sea	Eilers et al. (2000)
6	THP10 (OR936749)*	597	*Microbacterium indicum* (NR_042459)	84.35	Sea sediment	Shivaji et al. (2007)
7	THP12 (OR939738)*	601	*Microbacterium indicum* (NR_042459)	85.64	Sea sediment	Shivaji et al. (2007)
8	THP13 (OR936750)	605	*Micrococcus luteus* (OP986538)	100	Soybean plant	Twizeyimana et al. (2023)
9	SIP3(OR936740)	600	*Leucobacter chromiireducens* (NR_042287)	100	sludge of a treatment plant	Morais et al. (2004)
10	SIP6 (OR936741)	599	*Microbacterium* sp. U50 (KU598706)	99.15	Juncus acutus	Syranidou et al. (2017)
11	SIP12 (OR936742)	604	*Dietzia timorensis* (NR_112775)	99.65	Soil	Yamamura et al. (2010)
12	SIP14 (OR936743)	597	*Micrococcus luteus* (MG603678)	98.96	Soft coral	Saranya et al. (2018)

Note: *: Putative novel isolate, OR: Accession number, % sequence similarity: percentage sequence



A **B** **C** **D**

Figure 2. Representative plates showing A. Mixed of Actinomycetota colonies from *Syringodium isoetifolium* epiphytes (SI epi) and B. Pure culture colonies of Actinomycetota from *Thalassia hemprichii* (THP8) as well as C and D showing antimicrobial activities of different strains. Numbers in the plates represent different Actinomycetota crude extracts and controls, i.e., in Figure 2.C, 9: SIP6, 10: Positive Control, 11: Negative control, 12: THP6, in Figure 2.D, 25: THP9, 26: THP13, 27: THP12, 28: THP3

These potential new strains were *Microbacterium* THP6 (OR936746), *Microbacterium* THP7 (OR936747), *Microbacterium* THP9 (OR936748), *Arthrobacter* THP10 (OR936749), and *Rhodococcus* THP12 (OR939738). Most isolated strains showed similarities to type strains isolated from various sources, such as soils and lakes, with few from marine environments (i.e., from soft coral and sea sediment), and none of them have been reported before in seagrasses (Table 3). Figure 2 displays the phylogenetic rebuilding of partial 16S rRNA gene sequences of the isolated epiphytic Actinomycetota strains alongside their closely related matches from the database. The phylogenetic tree illustrates the relationships and placement of 7 known Actinomycetota species, and the 5 newly discovered strains were shown to form unique clusters within their respective taxa, as depicted in Figure 3.

Antimicrobial activity of the isolated epiphytic Actinomycetota strains

Twelve strains of epiphytic Actinomycetota were examined to determine their potential to produce antimicrobial substances against four test microorganisms. The findings revealed that a significant proportion of isolates (7 out of 12, accounting for 58%) exhibited antibacterial activity against at least one of the test organisms, with inhibition zones varying from 8 to 30 mm (Table 4). Among these strains, only one (*Microbacterium* SIP6) displayed antifungal activity against *C. albicans*, with an inhibition zone measuring 15 mm. Seven strains showed antagonistic effects against the Gram-positive bacterium *S. aureus*. However, fewer isolates demonstrated antibacterial activity against the Gram-negative bacteria *P. aeruginosa* (3) and *E. coli* (2) (as delineated in Table 4). Remarkably, the antimicrobial agent *Microbacterium* SIP6 exhibited the most substantial inhibitory impact on *E. coli* (26 mm) and *S. aureus* (30 mm). In comparison, the inhibition zones for the positive controls (ciprofloxacin at 4 mg/mL) were 25 mm, 26 mm, and 22 mm for *S. aureus*, *P. aeruginosa*, and *E. coli*, respectively, while the antifungal agent fluconazole at 64 µg/mL resulted in a 15 mm inhibition zone.

Minimum Inhibitory Concentration (MIC)

Of the 12 extracts from the isolated epiphytic Actinomycetota strains, only four demonstrated antimicrobial activities characterized by an estimated

minimum inhibitory concentration (MICs) at or below the defined cut-off value of 3.13 mg/mL. The strain *Dietzia* (SIP12) displayed inhibition to all three tested organisms, followed by *Microbacterium* (THP10) and *Microbacterium* (SIP6) (Figure 4). Only two strains, *Micrococcus* (THP13) and *Dietzia* (SIP12), exhibited MICs of 1.56 mg/mL and 3.13 mg/mL against *C. albicans*, respectively. The MICs of the positive control (ciprofloxacin) were 0.015, 0.5, and 0.25 µg/mL for *E. coli*, *S. aureus*, and *P. aeruginosa*, respectively, while those of the negative control were ≥25 mg/mL.

The Brine Shrimp Lethality Assay

The Brine Shrimp Lethality Test (BSLT) findings revealed that most of the extracts from the isolates were toxic, with LC_{50} values ranging from 134.40 to 399.24 µg/mL (Table 5). Extracts from the two strains THP6 (*Microbacterium* sp.) and SIP14 (*Micrococcus* sp.) exhibited low cytotoxicity and were classified as nontoxic, with LC_{50} values of ≥ 3836.7 µg/mL and 1243.4 µg/mL, respectively. Cyclophosphamide was used as a toxic standard drug and exhibited an LC_{50} value of 16.367 µg/mL, showing that all our strains had lower toxicity.

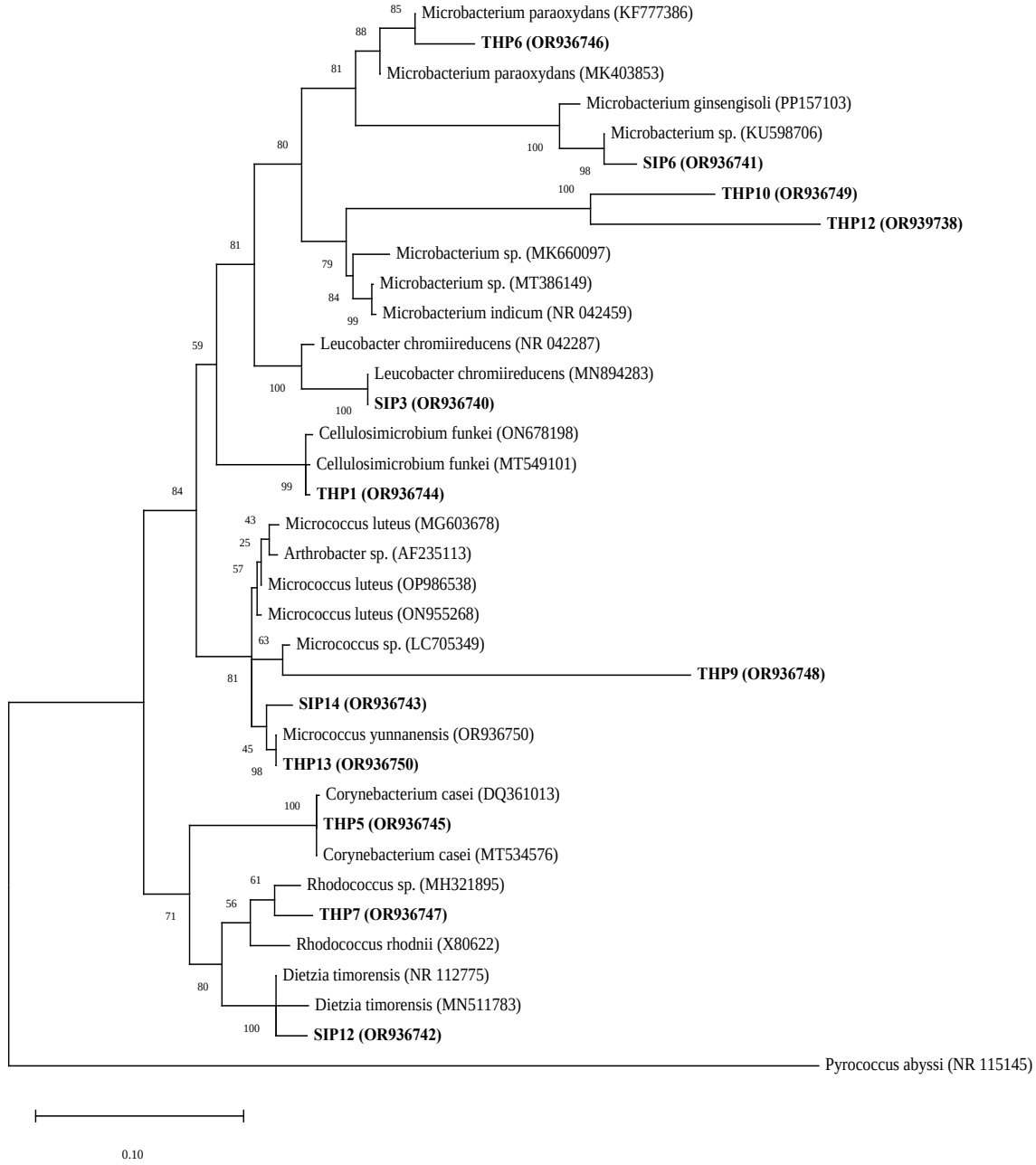


Figure 3. The construction of the phylogenetic tree involved using partial 16S rRNA gene sequences derived from epiphytic Actinomycetota found on the seagrasses *T. hemprichii* (THP) and *S. isoetifolium* (SIP) from Mjimwema, Dar es Salaam, Tanzania. This analysis also included type strains of closely related genera. The maximum likelihood heuristic method was applied for tree generation using nearest neighbor interchange. Bootstrap values were calculated through 1,000 data resamplings, with branch points showing bootstrap values exceeding 22%. The scale indicated on the tree represents a rate of 0.10 substitutions per nucleotide position

Table 4. Diameters of the zone of inhibition of the crude extracts of epiphytic Actinomycetota

Isolates	Zone of Inhibition (mm) at 100 mg/mL			
	E.coli	***S. aureus***	***P. aeruginosa***	***C. albicans***
Cellulosimicrobium (THP1)	NA	10	NA	NA
Corynebacterium (THP5)	17	12	NA	NA
Microbacterium (THP6)	NA	NA	NA	NA
Rhodococcus (THP7)	NA	10	NA	NA
Arthrobacter (THP9)	NA	NA	NA	NA
Microbacterium (THP10)	20	8	NA	NA
Microbacterium (THP12)	NA	NA	NA	NA
Micrococcus (THP13)	NA	25	16	NA
Leucobacter (SIP3)	NA	NA	NA	NA
Microbacterium (SIP6)	26	30	21	15
Dietzia (SIP12)	NA	NA	NA	NA
Micrococcus (SIP14)	NA	20	NA	NA
Ciprofloxacin	22	25	26	NA
Fluconazole	NA	NA	NA	15

Note: NA: Not active

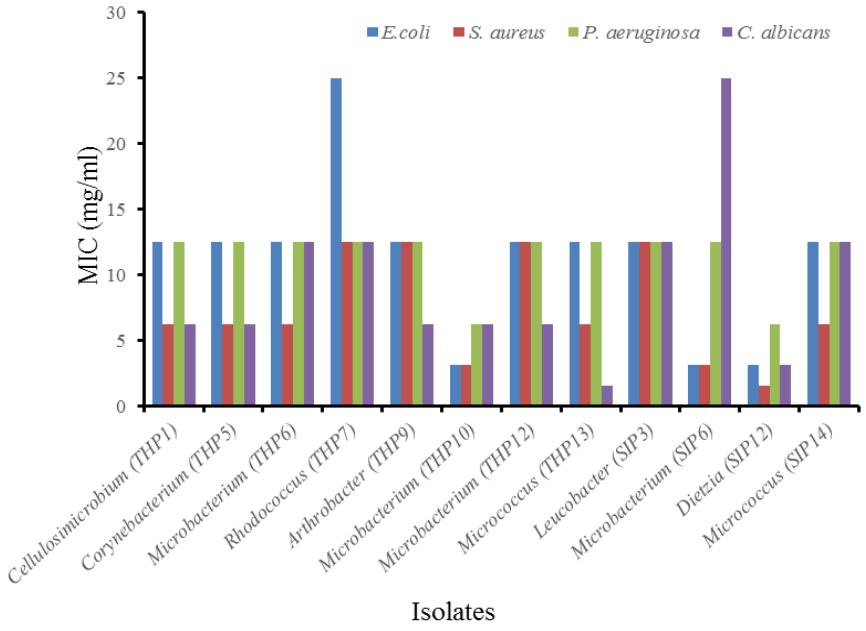


Figure 4. Minimum inhibitory concentration for marine epiphytic Actinomycetota

Table 5. Brine shrimp activity of epiphytic Actinomycetota extracts from *T. hemprichii* and *S. isoetifolium* seagrasses

Isolate extracts	LC_{50} (µg/mL)	95% Confidence interval (CI)	Regression equation	Regression coefficient (r)
Cellulosimicrobium (THP1)	165.50	150.89 -181.53	y=65.715logx-95.808	0.9248
Corynebacterium (THP5)	235.44	175.87 -315.19	y=49.693logx-67.866	0.9519
Microbacterium (THP6)	3836.7	830.11 -17733.6	y=33.223logx-69.07	1
Rhodococcus (THP7)	369.56	342.25 -399.06	y=70.838logx-131.89	0.9469
Arthrobacter (THP9)	168.64	134.23 -211.89	y=74.168logx-115.17	0.9589
Microbacterium (THP10)	399.24	62.789 -703.17	y=42.323logx-60.092	0.9864
Microbacterium (THP12)	134.40	91.048 -194.40	y=100.01logx-162.86	0.9734
Micrococcus (THP13)	323.11	294.05 -355.08	y=72.628logx-132.35	0.9954
Leucobacter (SIP3)	139.32	100.65 -192.85	y=81.665logx-125.09	0.9326
Microbacterium (SIP6)	201.88	134.03 -304.08	y=126.55logx-241.71	0.9984
Dietzia (SIP12)	164.55	89.329 -303.15	y=139.48logx-259.13	0.9773
Micrococcus (SIP14)	1243.4	557.76 -2771.7	y=42.781logx-82.39	0.983
Cyclophosphamide	16.367	12.006 - 22.305	y=69.968logx-34.93	0.995

Discussion

Epiphytic microbial communities in seagrasses have previously been reported in *C. rotundata*, *T. testudinum*, and *Zostera marina* L. (Mishra and Mohanraju 2018; Tasdemir et al. 2024). This study reports for the first time epiphytic Actinomycetota species diversity within seagrass *T. hemprichii* and *S. isoetifolium* meadows of the coast of Tanzania. All the isolated strains in this study were found to belong to rare Actinomycetota genera. The term "rare Actinomycetota" generally denotes less frequently isolated strains than *Streptomyces* strains (Martin-Pozas et al. 2023). Research conducted by Parra et al. (2023) has demonstrated that most marine ecosystems are abundant in rare Actinomycetota species. These species have been studied relatively less than the genus *Streptomyces*, often due to their slower growth rates under laboratory conditions (Parra et al. 2023).

Most Actinomycetota isolates in this study exhibited sequence similarities ranging from 98.96% to 100% with type strains documented in the NCBI database, showing that they were already known from other ecosystems. According to Kim et al. (2014), species with higher GC content can be differentiated when their 16S rRNA gene sequence similarity drops below 98.65%. The predominant genus encountered was *Microbacterium* spp., with two strains (THP6 (OR936746) and THP10 (OR936749)) out of four that were notably distinct from type strains, with percentage similarities ranging from 84.36-97.06%. *Microbacterium* species have been found in the seagrass species *T. hemprichii* (Siro and Pipite 2024) and *Z. marina* (Tasdemir et al. 2024) and various other marine environments (Xie et al. 2021; Zhu et al. 2021a). However, our findings mark the first report showing the occurrence of *Microbacterium* species as epiphytes of the seagrass *S. isoetifolium*. *Microbacterium* spp. are recognized for their enzymatic production (Li et al. 2019), capacity for bioremediation, and biomolecule transport (e.g., glucose, fructose, and galactose) (Avramov et al. 2016). Other researchers documented the antimicrobial properties of *Microbacterium* species, e.g., Graça et al. (2013). For the first time, our findings demonstrated the strong antimicrobial activity of *Microbacterium* (SIP6) and the antibacterial effects of a novel strain, *Microbacterium* (THP10). This new strain exhibits potential for use in pharmaceutical applications. Three of the four *Microbacterium* strains demonstrated toxicity in the brine shrimp test, indicating their potential as anticancer agents (Yusriadi et al. 2019).

The genus *Micrococcus* emerged in both seagrass species, with our isolated strains showing greater than 98.96% similarity to known type strains in GenBank, meaning that they were all already isolated in other studies (Saranya et al. 2018; Twizeyimana et al. 2023). They have been reported in various seagrass species, such as *H. uninervis* (Wahbeh et al. 1984), *H. stipulacea* (Pereg et al. 1994), *C. rotundata* and *T. testudinum* (Mishra and Mohanraju 2018). Nevertheless, their occurrence in seagrass meadows of *T. hemprichii* and *S. isoetifolium* has never been reported. We have demonstrated that these strains exhibit antibacterial properties, consistent with the findings of Tizabi and Hill (2023), who documented the antibacterial effects of *Micrococcus luteus* (Schroeter, 1872) Cohn, 1872 against various other bacteria. Furthermore, in our study, one strain (*Micrococcus* THP13) was found to be nontoxic, whereas the other exhibited toxicity in brine shrimp, making it a potential candidate for further investigation regarding its anticancer or cytotoxic properties (Omeke et al. 2018; Yusriadi et al. 2019). In another study, a strain of *M. luteus* was reported to be capable of causing infections such as bacteremia, hepatic and brain abscesses, and septic arthritis in immunocompromised patients (Zhu et al. 2021b). This also calls for additional investigations to evaluate the clinical potential of our isolate as an opportunistic pathogen in clinical settings.

The other two isolated strains (*Rhodococcus* THP7 and *Arthrobacter* THP9) appeared distantly related to *Rhodococcus rhodnii* Goodfellow & Alderson, 1979 (X80622) and *Arthrobacter* sp. M44 (AF235113) species might be novel species, showing similarity identities of 96.36% and 81.36%, respectively (Kim et al. 2014). *Rhodococcus* species have been successfully isolated from seagrasses such as *Z. marina* (Ettinger and Eisen 2021), *Halodule wrightii* Asch., *T. testudinum*, and *H. stipulacea* (Aires et al. 2021). However, these species have not yet been isolated from the seagrasses *T. hemprichii* and *S. isoetifolium*. We demonstrated the antibacterial effects of the isolated novel strain of *Rhodococcus* (THP7) against *S. aureus*, similar to the findings of other studies (e.g., Naragani et al. 2014) documenting the antimicrobial properties of *Rhodococcus* species. In addition, some species of *Rhodococcus* sp. can break down various compounds, produce bioactive steroids, and play a role in the biodesulfurization of fossil fuels (Zappaterra et al. 2021). However, a few species within this genus are pathogenic (Savory et al. 2020). Species of the genus *Arthrobacter* have been reported from different seagrass species, such as *H. uninervis*, *H. ovalis*, *H. stipulacea*, *Z. marina* and *Zostera japonica* Asch. & Graebn. (Bibi et al. 2018: Siro and Pipite 2024). Nonetheless, we are documenting their presence in the seagrasses *T. hemprichii* and *S. isoetifolium* for the first time. Our new strain, *Arthrobacter* (THP9), did not exhibit any antimicrobial activity against the tested human pathogens, unlike some other reports, such as that of Ramlawi et al. (2021), who documented the antimicrobial effects of *Arthrobacter* species isolated from disease-suppressive compost. Additional research has indicated that Arthrobacter species can break down polymeric compounds, contributing significantly to the biodegradation of agrochemicals and pollutants (Gobbetti and Rizzello 2014). Our strains were also identified as toxic, but further investigations of their antitumor, cytotoxic, and pesticide activities are warranted (Omeke et al. 2018).

Other species identified in this study, including *Cellulosimicrobium*, *Corynebacterium*, *Leucobacter*, and *Dietzia*, showed similarity scores ranging from 99.65% to 100% with type strains from GenBank. These species have previously been isolated from various seagrasses, for example, *Cellulosimicrobium* in *T. testudinum* (Couto-

Rodríguez 2009) and *Corynebacterium* in *H. uninervis* (Bibi et al. 2018). However, our study marks the first report of their presence in the seagrasses *T. hemprichii* and *S. isoetifolium*. Notably, this research marks the initial documentation of the *Leucobacter* and *Dietzia* genera within seagrass species. Tanveer et al. (2021) discovered the antioxidant properties of marine *Cellulosimicrobium funkei* Brown et al. 2006. In contrast, our study marks the first report of its antimicrobial activity. Researchers have predominantly focused on reporting the antimicrobial activities of *Corynebacterium* species apart from *Corynebacterium casei* Brennan et al. 2001 (Gowramma et al. 2015; Menberu et al. 2021). Our study, however, presents the antimicrobial activity of Actinomycetota *C. casei* for the first time. Our study is the first to report on the antimicrobial activities of strains *Leucobacter chromiireducens* A and *Dietzia timorensis* Yamamura et al., 2010. None of the strains exhibited any activity against the tested human pathogens. The isolated strains of the genera *Cellulosimicrobium*, *Corynebacterium*, *Leucobacter*, and *Dietzia* exhibited toxicity, indicating their potential use in anticancer applications.

In conclusion, this investigation aimed to assess the diversity and biotechnological applications of epiphytic Actinomycetota species obtained from seagrasses along the coastline of Dar es Salaam. The research identifies and describes 12 rare Actinomycetota species, 5 of which show promise as potentially new species. The isolated Actinomycetota can produce extracellular enzymes that may be used in drug development to target diseases involving enzyme dysregulation. Most isolated Actinomycetota species showed antibacterial activities against at least one tested human pathogen. Moreover, the *Microbacterium* (SIP6) strain displayed potent antimicrobial effects against all examined human pathogens, suggesting its possible pharmaceutical value. *Micrococcus* (THP13) and *Dietzia* (SIP12) exhibited activity against *C. albicans*, indicating their potential utility in the production of antifungal agents. Furthermore, most isolated strains in this study are toxic, making them potential candidates for further exploration of their anticancer and cytotoxic activities. These findings highlight the diverse biotechnological potential of marine epiphytic Actinomycetota strains and warrant further investigation into their medicinal and therapeutic applications. It is necessary to conduct additional studies, possibly involving in vitro and in vivo experiments, to evaluate the isolated strains' potential clinical applications, safety, and effectiveness in a clinical context. Additionally, comprehensive toxicity studies and risk assessments should be carried out to assess the safety profile of the extracts for potential biotechnological and pharmaceutical applications. Furthermore, future research should investigate how these findings can be extended to other marine environments, enhancing the overall generalizability of the results.

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Purification and characterization of cellulase from May beetle (*Phyllophaga errans*) gut

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Abstract. *Ezima EN, Adeqbesan BO, Osonuqa IO, Adefuye AO, Adepoju AR, Bello TH, Olalekan SO. 2024. Purification and characterization of cellulase from May beetle (*Phyllophaga errans*) gut. Asian J Trop Biotechnol 21: 52-58.* Cellulose constitutes a significant portion of plants' primary and secondary cell walls. Insects, traditionally believed to lack the ability to digest cellulose, have evolved distinctive strategies for cellulose degradation. May beetle (*Phyllophaga errans* LeConte, 1859) is herbivorous insects that feed on numerous plant species, including vegetables, maize plants, and other fresh food crops, causing a lot of damage to farm products. The ability of these beetles to thrive on plant diet may be due to the presence of highly effective cellulose digesting enzyme (cellulase) in their gut which aids the breaking down of cellulose into glucose. Therefore, this study focused on the extraction and purification of cellulase from the *P. errans* gut sourced from a farm in Ijebu-Ode, Ogun State, Nigeria. Following the dissection and homogenization of the beetle's guts, cellulase isolation and purification were carried out utilizing techniques such as ammonium sulfate precipitation, ion-exchange chromatography on CM-Sephadex G-200 and gel filtration using Sephacryl S-200 gel. The resulting pure cellulase from the gut of the *P. errans* exhibited a purification fold of 43.71 and a yield of 21.61% with a specific activity of 70.38 units/mg of protein. Characterization revealed the enzyme's native molecular weight of 24.6 kDa, Km (0.67 mM), and Vmax (192.31 mg/mL/min), with optimal activity observed at pH 9 and 70°C. The cellulase from the *P. errans* gut showcased distinctive characteristics that, if properly harnessed, could pave the way for practical applications in various industries, particularly in developing an efficient pesticide for pest management and environmental conservation.

Keywords: Cellulase, gut, May beetle, pest control, *Phyllophaga errans*, purification

INTRODUCTION

Polysaccharides are major constituents of cell walls and serve as energy reserves within plant and animal cells. In plants, starch acts as the primary energy reserve, while animals store energy in the form of glycogen. Phytophagous insects disrupt plant cell walls to access the storage polymers in cell content. This disruption can result from mastication, but it more commonly involves the action of digestive enzymes (Rehman et al. 2009; Barcoto and Rodrigues 2022). Cellulose, a linear polymer of β-1,4-linked D-glucose units, is the most abundant biopolymer on Earth and is a key structural component in plant cell walls, providing rigidity and strength to plant cells (Li 2021; Hemati et al. 2022). Efficient biodegradation of cellulose requires the coordinated effort of a sophisticated enzymatic system called cellulases due to its resistant characteristics (Chatterjee et al. 2015; Jayasekara and Ratnayake 2019). These enzymes catalyze the breakdown of β-1,4-glycosidic linkages in cellulose, converting it into smaller sugar units that can be further utilized in various applications.

Cellulases have received significant attention due to their potential applications in industries such as biofuel production, textile manufacturing, food processing, and waste management (Atousa et al. 2017; Sreeramulu et al. 2023). The demand for sustainable and eco-friendly processes has driven the search for novel and efficient cellulase sources. Although cellulases are produced by a variety of microbes such as bacteria, fungi, and protozoa, as well as in herbivorous invertebrates such as arthropods, nematodes, and mollusks (Barzkar and Sohail 2020), recent research has highlighted the potential of insect-derived cellulases as promising alternatives (Fagbohunka et al. 2017; Soeka and Ilyas 2020). The nutritional significance of cellulase for leaf-feeder organisms was previously deemed negligible due to its resistance to digestion in most animal species. The discovery of GH9 cellulases in facultative leaf-consuming grasshoppers (Ademolu and Idowu 2011) and GH45 cellulases, as well as GH11 xylanases in herbivorous beetles (Pauchet and Heckel 2013), implied that the function of cellulases in other plant-eating insects requires reassessment.

Presently, the existence of cellulases in invertebrates and insects has been documented across various insect orders such as Isoptera, Blattodea, Coleoptera, Orthoptera, Diptera, and Lepidoptera (Chatterjee et al. 2015; Tokuda 2019; Uddin et al. 2021). The presence of cellulase has also been documented in the intestinal fluids of many insect species of different orders (Imran et al. 2016; Fagbohunka et al. 2017; Soeka and Ilyas 2020). It is commonly believed

that the cellulase activity found in these insects is due to symbiotic gut microorganisms. Herbivorous insects, particularly, depend on their gut microbes to break down plant materials like cellulose and lignin (Li 2021; Rajeswari et al. 2021). Cellulases of microbial origin play a vital role in the bioconversion of cellulose, acting as phytopathogens that produce a range of enzymes (Zhang et al. 2016). Insects host many microbes in their gut and thus serve as a reservoir of microbial diversity (Rajeswari et al. 2021; Hemati et al. 2022). Cellulolytic enzymes have been well-studied in wood-feeding insects, which depend on endogenous or symbiotic microbial enzymes to break down lignocellulose into assimilative sugars or nutrients (Afzal et al. 2019; Hemati et al. 2022). However, the role of cellulases in monophagous leaf-feeding (phytivorous) insects is less explored.

May beetle belong to a large family called scarabs (Scarabaeidae). Several Scarabaeidae beetles have been identified as capable of degrading cellulose; these include *Protaetia brevitarsis* Lewis, 1879 larvae (Wang et al. 2022a), *Trypoxylus dichotomus* Linnaeus, 1771 (Wang et al. 2022b), *Gastrophysa viridula* De Geer, 1775 beetle (Busch et al. 2018). Adult May beetle (*Phyllophaga errans* LeConte, 1859) is leaf feeders, feeding on foliage of trees and shrubs, causing significant damage, especially in large numbers. The ability of these beetles to thrive on this diet may be due to the presence of a highly effective cellulose-digesting enzyme (cellulase) in their gut, which helps break cellulose into glucose that is readily absorbed and used as their energy source. Therefore, understanding the nature and characteristics of *P. errans* cellulolytic enzymes will help design effective pesticides to control the beetle and contribute to understanding insect-derived cellulases.

MATERIALS AND METHODS

Materials

The reagents and chemicals used in this work were analytical, sourced from BDH Chemicals Limited (Poole, England), Sigma Aldrich Chemicals Company (St. Louis. Mo. USA), Pharmacia Fine Chemical (Uppsala, Sweden), Eastman Kodak (Rochester, N. USA) and Pierce Chemical Company (Rockie, Illinois USA)

Methods

Collection of beetles and sample preparation

Phyllophaga errans samples were collected nightly from maize and vegetable farms in Ijebu-Ode, Ogun State, Nigeria. They were stored in a well-ventilated container and taken to the laboratory. The beetles were washed in normal saline and stored at 4^{o}C until use. Before dissection and excision of the beetle's gut, the beetles were thawed and rinsed in normal saline. All the collected gut samples were weighed (25.6 g) and homogenized in 3 volumes of 0.1 M acetate buffer pH 5.5. The resulting homogeneous solution was centrifuged at 4,000 rpm for 15 minutes, and the supernatant obtained was used as the crude enzyme. The beetles were identified at the Zoology Department of Olabisi Onabanjo University, Ago-Iwoye, Ogun State.

Enzyme assay

The assessment of cellulase activity was conducted using an enzymatic assay method initially described by Afzal et al. (2019) with minor adjustments. This process involves quantifying the quantity of reducing sugar liberated from the reaction between the enzyme and substrate (CMC) using the DNSA (3, 5-dinitrosalicylic acid) technique. A standardized glucose curve was employed to determine the quantity of glucose liberated. The assay mixture comprised 0.5 mL of the substrate (CMC) solution (1%, w/v in 0.1 M acetate buffer of pH 5.5) mixed with 0.1 mL of the enzyme sample. Subsequently, the mixture was incubated for 30 minutes at 37^{o}C with gentle agitation. Following the incubation period, 0.4 mL of DNSA reagent was introduced to the mixture and incubated in a boiling water bath for 10 minutes, then cooled in an ice bath before adding 3 mL of distilled water to dilute the solution. The absorbance at 540 nm was measured after the incubation. Therefore, to ensure reliability, all experiments were repeated thrice. Cellulase activity was defined as the quantity of enzyme necessary to release 1 mg of glucose under the specified assay parameters.

Estimation of protein concentration

The Bradford (1976) method was used to determine the enzyme's protein concentration using Bovine Serum Albumin (BSA) as a standard.

Purification cellulase

Ammonium sulfate precipitation

The crude enzyme obtained from the gut of *P. errans* was brought to 70% ammonium sulfate saturation. The calculated amount of solid ammonium sulfate was added to the crude enzyme mixture and stirred gently until well dissolved. The mixture was refrigerated for 12 hours and then centrifuged at 6,000 rpm for 30 minutes; the precipitate was suspended in an aliquot amount of 0.1 M acetate buffer of pH 5.5 and stored at 4^{o}C for further use. The enzyme precipitate was dialyzed against several changes of 0.1 M acetate buffer pH 5.5 to remove the ammonium sulfate salt for the ion exchange chromatography.

Ion exchange chromatography on CM-Sephadex G-200

The salt-free enzyme obtained after dialysis from the previous step was administered onto a chromatographic column (2.5x40 cm) containing CM-Sephadex G-200 resin, equilibrated with 0.1 M acetate buffer at pH 5.5. The enzyme was separated using the same buffer with a flow rate of 30 mLh^{-1}. Next, 5 mL portions were collected, and the enzyme's protein and cellulase activity were assessed. The fractions exhibiting high activity levels were combined and concentrated in 50% glycerol.

Gel filtration on Sephacryl S-200

The recovered enzyme solution from glycerol concentration was then applied to the Sephacryl S-200 column already equilibrated with 0.1M acetate buffer of pH 5.5. The column was washed with the same buffer at a flow

rate of 30 mLh^{-1}. Next, 5 mL fractions were collected, and the enzyme's protein concentration and cellulase activity were determined. The fractions were pooled where high activity was observed.

SDS-PAGE

The purity and molecular weight of the enzyme were ascertained on SDS-PAGE (Laemmli 1970) with resolving and stacking gel compositions of 12 and 5%, respectively. The molecular weight of the enzyme was calculated with the help of a protein ladder marker, 10-220 kDa. Next, 30 µm of sample buffer was added to 90 µml of active *P. errans* gut cellulase fraction from Sephacryl S-200, and the mixture was boiled for 5 minutes to denature the enzyme. The same procedure was performed on the protein marker, ammonium precipitate, and crude enzyme. After gel polymerization, denatured enzymes and protein markers were stacked at 100 Volts, and the process was run in a separating gel at 150 Volts for 2 hours in a Bio-Rad electrophoresis. The electrophoresis was stopped when the dye fronts were approximately 1 cm from the end of the separation gel. The gel was then fixed for one hour in a 10% (w/v) acetic acid solution and 40% (w/v) methanol. It was then stained overnight with shaking to ensure uniform mixing and Coomassie brilliant blue R-250 adsorption onto the protein bands. A solution of 5% methanol and 7.5% glacial acetic acid in distilled water was then used to destain the gel completely.

Effect of pH on Phyllophaga errans *gut cellulase*

The impact of pH on enzyme activity was investigated by assessing enzyme activity within the pH range of 4 to 11 at a temperature of 37ºC, employing diverse buffers with varying pH levels: citrate (pH 3-5), phosphate (pH 6-8), and borate (pH 9-11). The cellulase activity was assayed as previously described.

Effect of temperature on Phyllophaga errans *gut cellulase*

Cellulase activity was evaluated within a temperature range of 20 to 100ºC at pH 5. The assay mixture was first incubated at the respective temperature for 10 min before the commencement of the reaction by introducing 500 µL of the purified enzyme that had also been equilibrated at the same temperature. The assessment of cellulase activity followed the standard procedure as previously outlined.

The study of the kinetic properties of Phyllophaga errans *gut cellulase*

The kinetic parameters, Km and Vmax, were determined for the purified *P. errans* gut cellulase using 1% (w/v) CMC as substrate. The concentration was varied from a final concentration of 1 to 10 mg/mL CMC and the assay was conducted in triplicate measurements. The kinetic parameters were determined from the Lineweaver-Burk plot of the reciprocal of the initial velocity (1/V) against the reciprocal of the substrate concentrations [1/S].

RESULTS AND DISCUSSION

The investigation presented in this study offers valuable insights regarding the purification and characterization process of cellulase extracted from the gut of *P. errans*. The outcomes of the purification techniques are briefly outlined in Table 1. The cellulase obtained from the gut of the *P. errans* exhibited a specific activity of 70.38 U/mg of protein with a yield of 21.61% and was purified to homogeneity. The results of the purification processes are shown in Figures 1 and 2, which illustrate the use of ion-exchange chromatography on Sephadex G-200 and Sephacryl S-200. Verification of the enzyme's purity was conducted through SDS-PAGE, displaying a single band for the purified enzyme as depicted in Figure 3. The molecular weight was determined to be 24.6 kDa, also using SDS-PAGE as shown in Figure 4.

Table 1. Summary of the results of the purification procedures of cellulase from the gut of *Phyllophaga errans*

Procedures	Volume (mL)	Activity (units/mL)	Protein (mg/mL)	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Yield (%)	Purification fold
Crude enzyme	30.0	1.14	0.709	21.27	34.20	1.61	100	1.00
70% Ammonium sulfate $(NH_4)_2SO_4$	10.5	3.38	1.750	18.36	35.46	1.93	103.7	1.20
Ion-exchange Chromatography CM-Sephadex G-200	55.0	0.09	0.025	1.38	4.29	3.12	12.54	1.94
Gel Filtration Sephacryl S-200	35.0	0.21	0.003	0.11	7.39	70.38	21.61	43.71

Values for activity and protein are the mean of the triplicate assay for *Phyllophaga errans* gut cellulase activity and protein concentration. Cellulase activity was defined as the amount of enzyme needed to release one milligram of glucose under the specified assay conditions

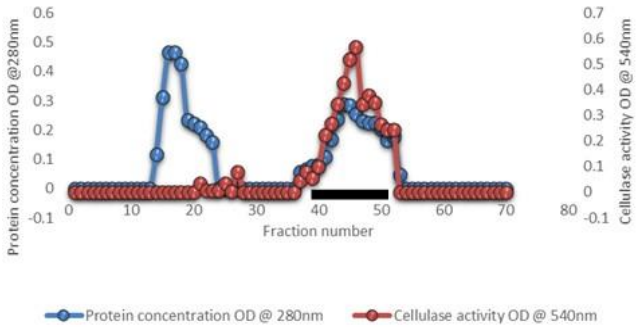

Figure 1. Sephadex G-200 elution profile of the ion-exchange chromatography of cellulase from the gut of *Phyllophaga errans*. Fractions with very high cellulase activity (40-50) were pooled for subsequent analysis

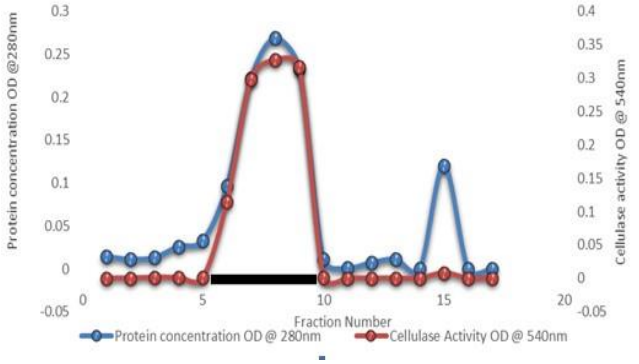

Figure 2. Sephacryl S-200 elution profile of the cellulase ion-exchange chromatography from the *Phyllophaga errans*' gut. Fractions with very high cellulase activity (5-11) were pooled for subsequent analysis

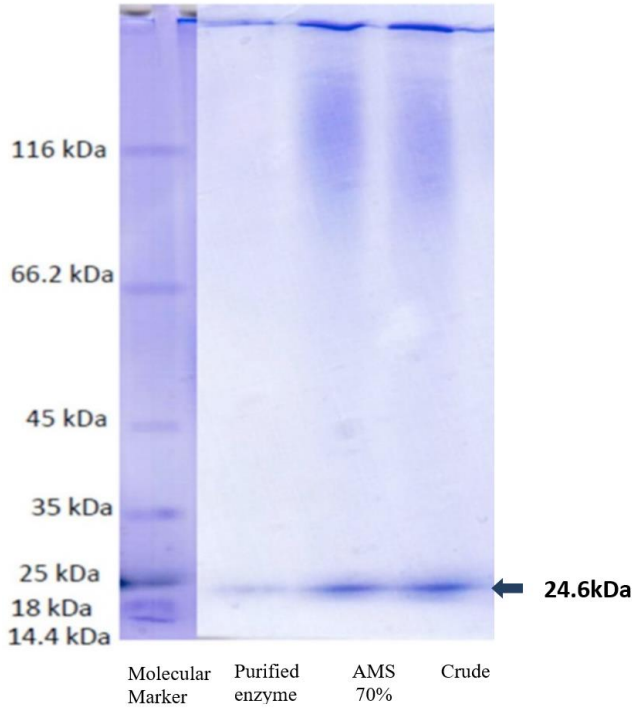

Figure 3. SDS-Polyacrylamide slab gel electrophoresis of *Phyllophaga errans*' gut cellulase (Lane 1 protein marker, Lane 2 purified enzyme, Lane 3 ammonium sulfate precipitate, and Lane 4 crude enzyme)

The specific activity (70.38 U/mg) obtained in this study surpassed the findings from a previous study on cellulase sourced from worker termites *Amitermes eveuncifer* Silvestri, 1901, which recorded a specific activity of 0.25 U/mg (Ezima et al. 2014). In a separate study, Haloi et al. (2012) reported a lower specific activity of 0.687 U/mg for Grasshopper *Hieroglyphus banian* Fabricius, 1798 (Orthoptera: Acrididae), while Fagbohunka et al. (2017) gave an account of 5.04 U/mg for soldier termite *A. eveuncifer* cellulase. Furthermore, Pachauri et al. (2020) observed a specific activity of 30 U/mg for cellulase sourced from a novel isolate of fungi *Trichoderma longibrachiatum* Rifai, while Goswami et al. (2022) reported a specific activity of 31.4 U/mg for bacteria *Novosphingobium* sp. Cm1. In contrast, Islam and Roy (2018) and Sreeramulu et al. (2023) reported significantly higher specific activities of 2655 U/mg and 2858 U/mg, respectively, for cellulase-producing bacteria found in molasses and larvae of the banana pseudostem weevil, *Odoiporus longicollis* G.A.K.Marshall, 1930 (Coleoptera: Curculionidae).

Various literature sources have reported different molecular weights for cellulases from various microbes and insects, ranging from 35 to 184 kDa (Atousa et al. 2017; Banerjee et al. 2020; Malik and Javed 2021). A recent study by Fouda et al. (2024) revealed a molecular weight of 436 kDa for Thermotolerant *Bacillus subtilis* F3. So far, reports of cellulase with low molecular weight are few. The molecular weight of *P. errans* cellulase aligns closely with the findings of Rahman et al. (2014), who identified an enzyme called AkEG21, from the common sea hare *Aplysia kurodai* Baba, 1937 with a molecular weight of 21 kDa. Furthermore, Listyaningrum et al. (2018) documented a molecular weight of 18 kDa for *Bacillus* strains isolated from carrageenan solid waste. It has been noted that cellulases with low molecular weights exhibit thermal stability and offer advantages in diverse applications such as heterogeneous expression and protein-engineering studies. According to Cowan and Fernandez-Lafuente (2011), enzymes with lower molecular weights are more amenable to modification, facilitating processes like immobilization or genetic manipulation and ultimately enhancing enzyme specificity.

Environmental conditions such as pH and temperatures have great effects on enzyme performance and stability; the characterization of the optimal pH and temperature of *P. errans* gut cellulase revealed optimal activities at pH 9 and 70°C respectively (Figures 4 and 5), indicating a moderately thermostable enzyme that works well in alkaline medium. The enzyme possessed a unique thermostability, with activity stability over a broad range of

temperatures, from 50°C to 75°C. Similar temperature optima have been reported (Padilha et al. 2015; Maswati 2022). Other thermostable cellulases have been reported from different sources with optimum temperatures between 50°C to 60°C (Atousa et al. 2017; Kim and Ku 2018; Shyaula et al. 2023; Sreeramulu et al. 2023). Lower optimum temperatures of 30-45°C were also reported (El-Sersy et al. 2010; Rahman et al. 2014; Banerjee et al. 2020; Fagbohunka et al. 2021). However, Zhang et al. (2016) reported an optimum temperature as low as 28°C for cellulase from *Pseudomonas mendocina.*

Many documented insects and microbial cellulases have neutral pH optima (Lee et al. 2008; Fagbohunka et al. 2017; Kim and Ku 2018), few acidic optima (from 3 to 5) had also been reported (Rahman et al. 2014; Atousa et al. 2017; Shyaula et al. 2023). The optimum pH of 9 obtained for the *P. errans* gut cellulase is one of the highest so far reported. Listyaningrum et al. (2018) reported an optimum pH of 8 for *Bacillus* strains isolated from the Carrageenan solid waste. The alkaline nature of the *P. errans* cellulase could be of great importance for the solubilization of plant biomass and efficient cellulose degradation in the insect, as suggested by Wang et al. (2022a). Our study also revealed that although cellulase enzymes from *P. errans*' gut can work well in an alkaline medium (8-9) higher pH values have a detrimental effect on the enzyme.

The cellulase enzyme from the gut of the *P. errans* exhibited promising characteristics for various industrial applications, especially due to its optimal activity within the temperature range of 50-70°C (Figure 5). The enzyme shows high efficacy in environments with elevated pH levels and temperatures, making it particularly suitable for applications in textile treatments, paper and pulp processing, and specific stages of biofuel production and other industrial processes requiring enzymatic activity in harsh environments.

The insights into an enzyme's catalytic efficiency and substrate affinity are mostly provided by the Kinetic parameters of the enzyme, specifically the maximum velocity (V_{max}) and the Michaelis-Menten constant (K_m). Understanding these parameters is crucial to monitoring enzyme behavior and optimizing its performance in various applications. The K_m and V_{max} values estimated for cellulase from the *P. errans* gut were 0.67 mg/mL and 192.31 units/mL/min (Figure 6). Though the K_m and V_{max} values documented for cellulases vary between different species, the K_m for the *P. errans* cellulase is lower than that documented by Sreeramulu et al. (2023) with K_m and V_{max} of 1.03 mg/mL and 343 units/mL/min for the gut cellulase of the larvae of banana pseudostem weevil, *O. longicollis*, and Afzal et al. (2019) reported K_m and V_{max} of 2.24 mg/mL and 454.05 µg/mL/min for cellulase isolated from *Bacillus licheniformis* HI-08 from the hindgut of wood-feeding termite. Likewise, Shyaula et al. (2023) reported K_m and V_{max} values were 1.8 mg/mL and 10.92 µg/mL/min for cellulase of *B. licheniformis* PANG L isolated from Himalayan soil, while Pachauri et al. (2020) gave an account of K_m and V_{max} values for cellulase isolated from *T. longibrachiatum* of 0.121 mg/mL and 0.421 µmol/min.

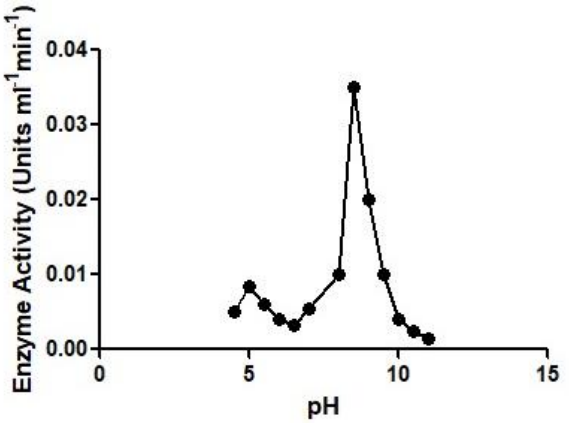

Figure 4. Effect of pH on cellulase activity from *Phyllophaga errans*. Enzyme activity was assayed within the pH range of 4 to 11. The values shown represent the average of triplicate experiments

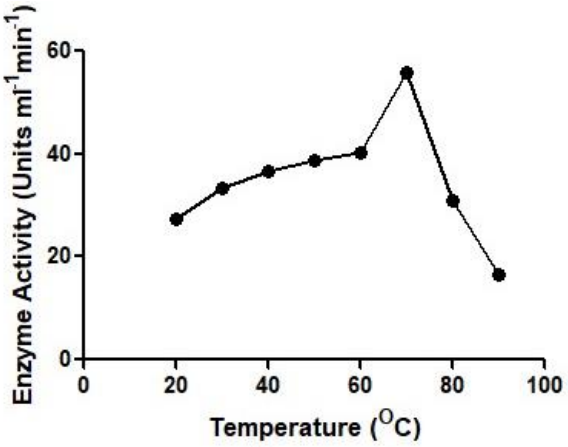

Figure 5. Effect of temperature of *Phyllophaga errans* cellulase. The cellulase activity was evaluated at temperatures between 20 and 100ºC at pH 5. The values shown represent the average of triplicate experiments

The variations in K_m values reflect the different enzymatic efficiencies and substrate affinities of cellulases from various species, highlighting the diverse adaptations in utilizing cellulose as an energy source. Since adult *P. errans* feed mainly on the foliage of trees and shrubs high in cellulose content, the beetle requires an effective cellulase for effective nutrition. Therefore, the Km of 0.67 mg/mL showed that cellulase from the *P. errans*' gut has an effective cellulase with a high affinity for cellulose.

Insects from diverse taxonomic orders have been discovered to exude β-glucosidase and endo-β-1,4-glucanas enzymes that exhibit significant attraction towards cellulose and cellobiose, respectively, thereby emphasizing their cellulolytic potential (Watanabe and Tokuda 2010; Shelomi et al. 2014; Linton 2020). The cellulose in the gut of the *P. errans* is presumed to be Indigenous owing to its strong affinity towards substrates, optimal temperature conditions, and capacity to operate efficiently under high pH environments.

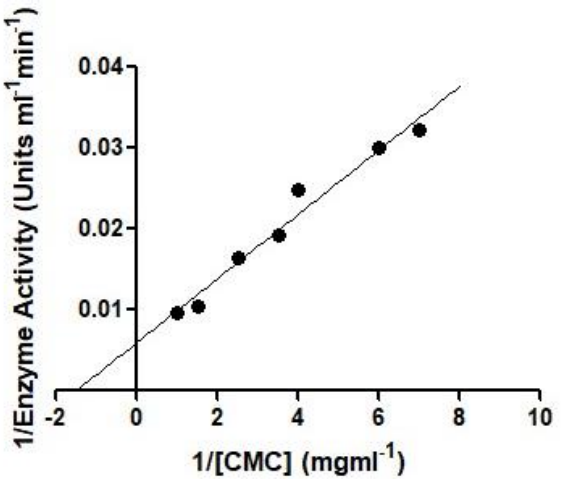

Figure 6. Lineweaver-Burk plot of the purified cellulase from the gut of *Phyllophaga errans*. The concentration of the substrate (CMC) was varied between 1 to 10 mg/mL, and the plot of the reciprocal of initial velocity (1/V) against the reciprocal of the substrate concentrations [1/S] was used to estimate the values of the enzyme Km and Vmax

In conclusion, the cellulase extracted and identified from *P. errans* guts was effectively purified and characterized. The enzyme demonstrates features that establish it as a viable option for various industrial processes, especially for waste treatment and the production of biofuels that demand strong enzyme efficacy in harsh conditions. Furthermore, the efficient cellulolytic properties of cellulases obtained from the gut of *P. errans* could be harnessed for developing pesticides aimed at managing beetle populations in farming settings, where they present significant risks. This objective can be accomplished through targeted approaches to either suppress or hinder the activity of cellulases, disrupting their digestion and depleting their source of nourishment. It is strongly advised that additional research be carried out, particularly focusing on optimizing the extraction and purification processes through techniques like enzyme stabilization, enhanced purification methods, or the application of advanced biotechnologies such as genetic engineering, which substantially increases the enzyme yield. Also, further work is recommended to confirm the cellulase's origin from the *P. errans*'s gut, whether it is truly endogenous or from the symbiotic microbes. Again, more research is advised to validate the cellulase source derived from the *P. errans*'s digestive system, determining whether it is endogenous or originating from the symbiotic microorganisms residing in the gut.

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