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Temporal Dynamics of Extracellular Oxidoreductase and Hydrolase Activities in a Microbial Consortium Exposed to Petroleum Hydrocarbons

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ABSTRACT

Hydrocarbon pollution from petroleum is one of the main sources of contamination in the marine environment, causing impacts on biodiversity and ecosystem integrity. Certain types of microbial consortia have demonstrated the capacity to bioremediate environments by metabolizing carbonaceous compounds present in petroleum and its derivatives through the production of specific enzymes, such as oxygenases and hydrolases. The experiment was conducted in Bushnell–Haas medium supplemented with 1% (v/v) petroleum and diesel in each experimental group, in addition to a control condition. Cultures were incubated for 60 days under constant agitation, with periodic sampling for the determination of enzymatic activity by spectrophotometric assays in the extracellular fraction. Activities associated with the enzymes laccase, manganese peroxidase, alkane hydroxylase, catechol 2,3-dioxygenase, and lipase were analyzed. Temporal variation in the detectable activity of these enzymes was observed, with specific differences between the hydrocarbon-containing treatments and the control. The results indicate that the consortium exhibits a measurable enzymatic response under hydrocarbon exposure. Therefore, this study aims to evaluate the detectable extracellular enzymatic activity of oxidoreductases and hydrolases produced by a mixed microbial consortium exposed to crude oil and diesel.

Keywords: Microorganisms; Bioremediation; Hydrocarbons; Enzymes.

INTRODUCTION

Enzymes are proteins that catalyze complex compounds and produce simpler products; their use in petroleum degradation, in conjunction with microbial consortia, represents an alternative for improving bioremediation approaches, a strategy that has been widely employed (Xu A. et al., 2023).

Fungal and bacterial enzymes exhibit important differences related to their origin, biochemical characteristics, and applications. In a mixed consortium, however, it is possible to express the properties of both, encompassing, in a cooperative manner, attributes such as multifunctionality, resilience, robustness, and stress resistance (El-Gendi H. et al., 2021; Ghattavi, S.; Homaei, A., 2023).

Oxidoreductases are enzymes capable of catalyzing oxidation–reduction reactions by transferring electrons between molecules. Hydrolases, in turn, incorporate water molecules, promoting the cleavage of chemical bonds in compounds. Both enzyme groups are capable of degrading a wide range of substrates (Kumar, A.; Chandra, R., 2020); when associated, their effects are enhanced.

Manganese peroxidase and laccase are effective in the degradation of complex aromatic compounds (Wong, D., 2009); lipase assists in the breakdown of fats and oils (Nimkande V., Bafana A., 2022); alkane hydroxylase is crucial for alkane degradation (Fenido E.O. et al., 2023); and catechol 2,3-dioxygenase plays a central role in aromatic ring cleavage, facilitating the complete degradation of aromatic compounds (Huang, Z. et al., 2024).

These enzymes are described as having a broad capacity for molecular cleavage, and their application in the degradation of complex hydrocarbon chains has been a subject of continuous study. Concomitantly, as a crucial energy source, petroleum poses a significant environmental challenge due to its recalcitrance to degradation, being classified as a persistent organic pollutant (POP) (Mishra P. et al., 2023).

Therefore, the application and study of enzymatic bioprospecting of these compounds by microbial consortia are relevant in order to explore the synergistic action of different microorganisms and their bioremediation potential. Thus, this study aimed to evaluate the activity of enzymes belonging to the oxidoreductase and hydrolase classes produced by a mixed microbial consortium in the presence of crude oil and diesel, through spectrophotometric assays performed on the extracellular fraction, with the objective of characterizing detectable enzymatic activity under different hydrocarbon exposure conditions.

2. MATERIALS AND METHODS

2.1 Experimental Setup

The experiment was conducted using a mixed microbial consortium in the presence of degradative substrates, namely crude oil provided by the independent onshore oil and gas exploration and production operator PetroRecôncavo to LEPETRO/IGEO/UFBA for academic research purposes. The oil, originating from the Recôncavo Basin, is classified as light crude (API gravity > 29.5) of a paraffinic type (predominance of *n*-alkanes), in addition to diesel, defined as a petroleum-derived product; both are composed mainly of longer and heavier hydrocarbon chains (Lima et al., 2017).

The consortium in its preserved state, stored at −80 °C in the Microbiology Unit of LEPETRO, was reactivated, and its pre-inoculum was prepared in triplicate by adding 200 mL of distilled water, 4 g of LB (Luria–Bertani) medium, and 3.25 mL of the consortium (18.9×10^8 CFU/mL), and maintained for seven days in a shaker incubator (Lab Companion) at 180 rpm and 30 °C.

The initial inoculation of the consortium was performed at 9% in Bushnell–Haas (BH) medium, specifically formulated for the isolation of hydrocarbon-degrading bacteria with a minimal nutrient source, thereby promoting the metabolization of these compounds by microorganisms. The pH of the medium was adjusted to 6, considered optimal for the growth of the mixed microbial consortium composed of 36 organisms, isolated from Todos os Santos Bay and protected under the patent by Lima et al. (2021), with deposit number at the National Institute of Industrial Property (INPI) BR 10 2021 002341 4. The sequences are available in the NCBI under the following accession numbers: MW881196–MW881204, MW865711–MW865718, MW855898–MW855903, MW865719–MW865729.

The degradative substrates were individually added to the medium at a concentration of 1%, forming different experimental groups. Each condition was prepared in triplicate, including diesel, petroleum, and a negative control (blank), containing only the microbial consortium without the addition of organic compounds.

The cultures (100 mL each) in Erlenmeyer flasks were incubated under continuous agitation at 180 rpm and 30 °C to ensure adequate aeration and homogenization of the medium. Sample collection was carried out at time points T1 (0 days), T2 (3 days), T3 (8 days), T4 (15 days), T5 (30 days), T6 (45 days), and T7 (60 days) of incubation, and enzymatic activities were monitored throughout this period.

The production of the enzymes of interest was determined by absorbance measurements using spectrophotometry with a LMR-96 Loccus microplate reader, according to specific protocols for each enzyme studied. In addition, optical density (OD) was recorded as an indicative parameter of system turbidity and was not interpreted as a direct measure of cellular biomass, considering the biphasic nature of the medium (aqueous phase and hydrocarbon phase) and the mixed composition of the microbial consortium.

2.2 Enzymatic Activity Assays

The enzymatic extract was prepared by withdrawing a 2 mL aliquot from each Erlenmeyer flask and transferring it to microtubes, which were centrifuged for 30 minutes at 25 °C and 7500 rpm to separate the pellet, using only the extracellular supernatant fraction. No cell lysis or intracellular fraction extraction was performed; therefore, the activities determined in this study refer exclusively to the enzymatic activity detectable in the extracellular fraction under the adopted experimental conditions.

2.2.1 Alkane Hydroxylase

Alkane hydroxylase activity was analyzed using the nicotinamide adenine dinucleotide (NADH) depletion method (Lee et al., 1996). The assay consisted of 40 µL of enzymatic extract, 356 µL of 27 mM tris(hydroxymethyl)aminomethane hydrochloride (TRIS-HCl) buffer (pH 7.4), 40 µL of 1 mM NADH, 4 µL of 1% iso-octane, and 20 µL of 0.15% CHAPS. Enzyme activity was measured at 0 and 10 minutes of reaction at a wavelength of 340 nm. The molar absorption coefficient used for calculations was 6220 $L \cdot mol^{-1} \cdot cm^{-1}$.

2.2.2 Catechol 2,3-Dioxygenase

Catechol 2,3-dioxygenase activity was measured based on catechol oxidation and the formation of 2-hydroxymuconic semialdehyde (Baggi et al., 1987). The reaction mixture consisted of 134 µL of enzymatic extract, 132 µL of 50 mM phosphate buffer (pH 7.0), and 134 µL of 0.3 mM catechol. The reaction was evaluated at 0 and 10 minutes of incubation by measuring absorbance at 375 nm. The molar absorption coefficient used in the calculations was 36000 $L \cdot mol^{-1} \cdot cm^{-1}$.

2.2.3 Laccase

Laccase activity was determined through the oxidation of a syringaldazine-based substrate with quinone formation (Szklarz et al., 1989). The reaction mixture contained 200 µL of enzymatic extract, 160 µL of 0.05 M citrate–phosphate buffer (pH 7), and 40 µL of 0.1% syringaldazine solution. The reaction was monitored at 525 nm at 0 and 10 minutes. The molar absorption coefficient used for calculations was 65000 $L \cdot mol^{-1} \cdot cm^{-1}$.

2.2.4 Manganese Peroxidase

Manganese peroxidase activity was determined through the oxidation of phenol red (Kuwahara et al., 1984). The assay mixture consisted of 200 µL of enzymatic extract, 10 µL of 2 mM manganese sulfate, 20 µL of hydrogen peroxide in 20 mM sodium succinate buffer (pH 4.5), 40 µL of 0.01% phenol red, 40 µL of 0.25 M sodium lactate, and 80 µL of 0.5% bovine serum albumin. Measurements were taken at 0 and 10 minutes; subsequently, 40 µL of 2 M sodium hydroxide solution was added to stop the reaction, and absorbance was measured at 610 nm. The molar absorption coefficient used for calculations was 22000 $L \cdot mol^{-1} \cdot cm^{-1}$.

2.2.5 Lipase

Lipase activity was determined using a method based on the hydrolysis of the chromogenic substrate *p*-nitrophenyl palmitate (pNPP), resulting in the release of the yellow-colored compound *p*-nitrophenol (Nolasco-Soria, H., 2024). The reaction mixture consisted of 100 µL of enzymatic extract and 900 µL of substrate emulsion composed of two solutions: pNPP at 3 mg/mL in 0.05 M TRIS-HCl buffer (pH 8.0), and a mixture of gum arabic with Triton X-100. Both the emulsion and the sample were pre-equilibrated at 37 °C for 5 minutes prior to mixing to avoid variations. Absorbance was measured at 405 nm at 0 and 30 minutes of incubation at 37 °C.

2.3 Data Processing and Enzymatic Activity Calculation

Calibration curves relate the measured absorbance values to the known concentrations of the compound under study, in this case, the enzymes. Thus, with previously defined concentrations of enzymes and substrate, it is possible to establish a relationship with the quantified absorbance.

Based on the construction of the calibration curve and the determination of the trendline, the derived equation was then used to calculate the enzymatic rates in the experiment. From the calibration curves and the absorbance values measured for each reaction, enzymatic activity is expressed in units per liter (U/L), reflecting the enzyme reaction rate.

To calculate catalytic capacity, an arithmetic mean was obtained from the triplicates of each sample, resulting in a single absorbance value for calculation. Additionally, after calculating the mean, outliers were discarded to ensure the accuracy of the calculations, thereby improving data representation and the robustness of the analyses. Data organization, as well as the generation of descriptive graphs and the heatmap, were performed using the Google Colaboratory (Colab) platform, employing computational tools for data visualization and exploratory analysis.

Data interpretation was based on measures of central tendency and dispersion, such as mean and standard deviation, complemented by direct comparisons between treatments through graphical representations, including bar charts and heatmaps, which facilitated the visualization of relative variations between the P and CP samples.

3. RESULTS AND DISCUSSION

The composition of the microbial consortium is consistent with the observed enzymatic profile, since several identified genera are well recognized as producers of the aforementioned enzymes and have already been described as involved in hydrocarbon degradation. Species of the genus *Bacillus* are reported as producers of hydrolytic enzymes, such as lipases and esterases, and are also involved in the biodegradation of hydrophobic compounds (Dalmaso, Ferreira, Vermelho, 2015). *Brevibacillus* exhibits enzymatic potential associated with the production of extracellular enzymes and the degradation of complex organic compounds (Siddikey et al., 2025).

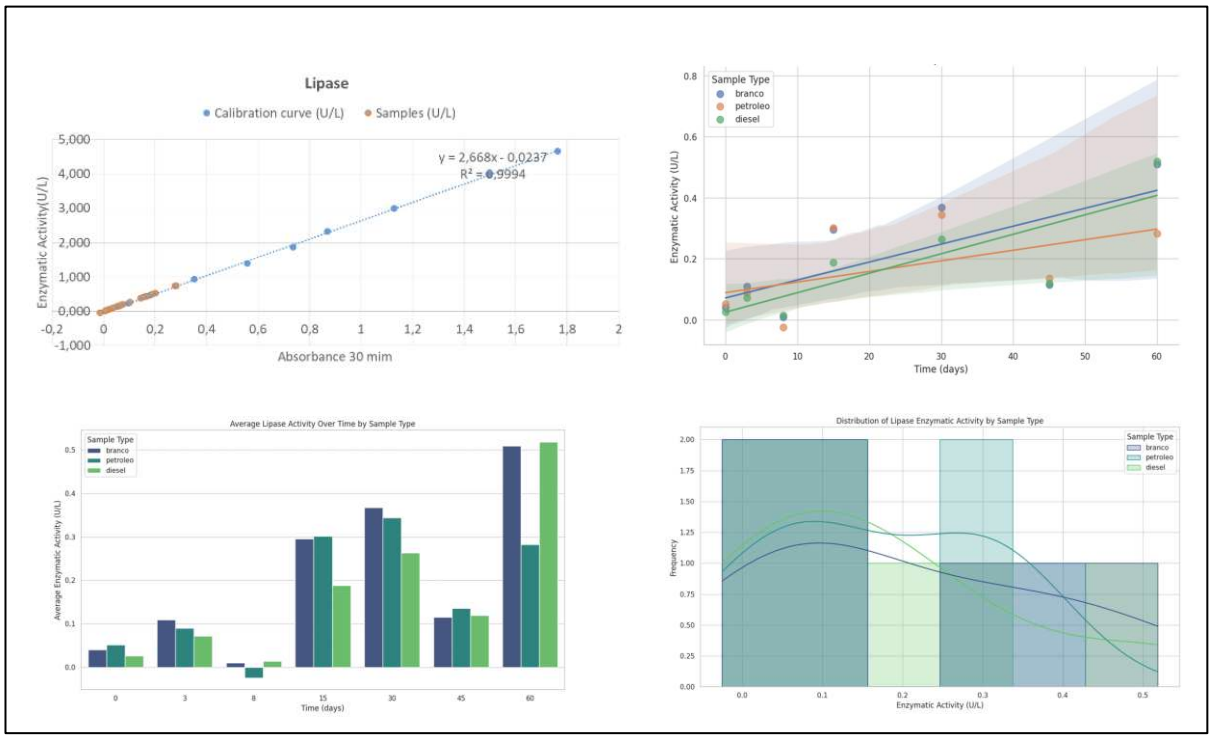
The genus *Pseudomonas* is one of the most frequently reported in hydrocarbon biodegradation, being capable of producing enzymes such as alkane hydroxylases, in addition to oxidoreductases involved in the degradation of aromatic compounds (El-Gendi H. et al., 2021). *Stenotrophomonas*, in turn, has been associated with the production of oxidative enzymes and adaptation to contaminated environments, contributing to bioremediation processes (Maurya et al., 2023).

Among fungi, the genera *Aspergillus* and *Penicillium* stand out due to their production of extracellular enzymes, including laccases, peroxidases, and lipases, which play a fundamental role in the degradation of recalcitrant compounds, such as polycyclic aromatic hydrocarbons (Kumar, A.; Chandra, R., 2020).

3.1 Lipase

The calibration curve helps to assess the sensitivity of the analytical method, allowing the determination of detection and quantification limits. Thus, in Figure 1a, it is possible to observe that the experimental results corroborate the calibration curve, indicating the suitability of the method under the experimental conditions employed.

Figure 1: (a) Comparison of calibration curve vs. sample results for Lipase; (b) Relationship between incubation time and enzymatic activity for different sample types, with linear regression and 95% confidence interval; (c) Lipase enzymatic activity results; (d) Histogram and density curves of lipase enzymatic activity.



The lipase graph (Figure 1b) shows that, in all three samples, there was an increase in enzymatic activity, with both the control and diesel samples reaching their maximum values at T7, and the diesel sample being slightly

higher than the control. At T5, the activity was already significant; however, a decrease is observed at T6, indicating a cycle of degradation and renewed enzymatic synthesis approximately every 30 days. This decline in enzymatic activity may be attributed to lipase degradation due to reduced enzymatic stability over time (Figure 1c) (Silva et al., 2024; Wang et al., 2023).

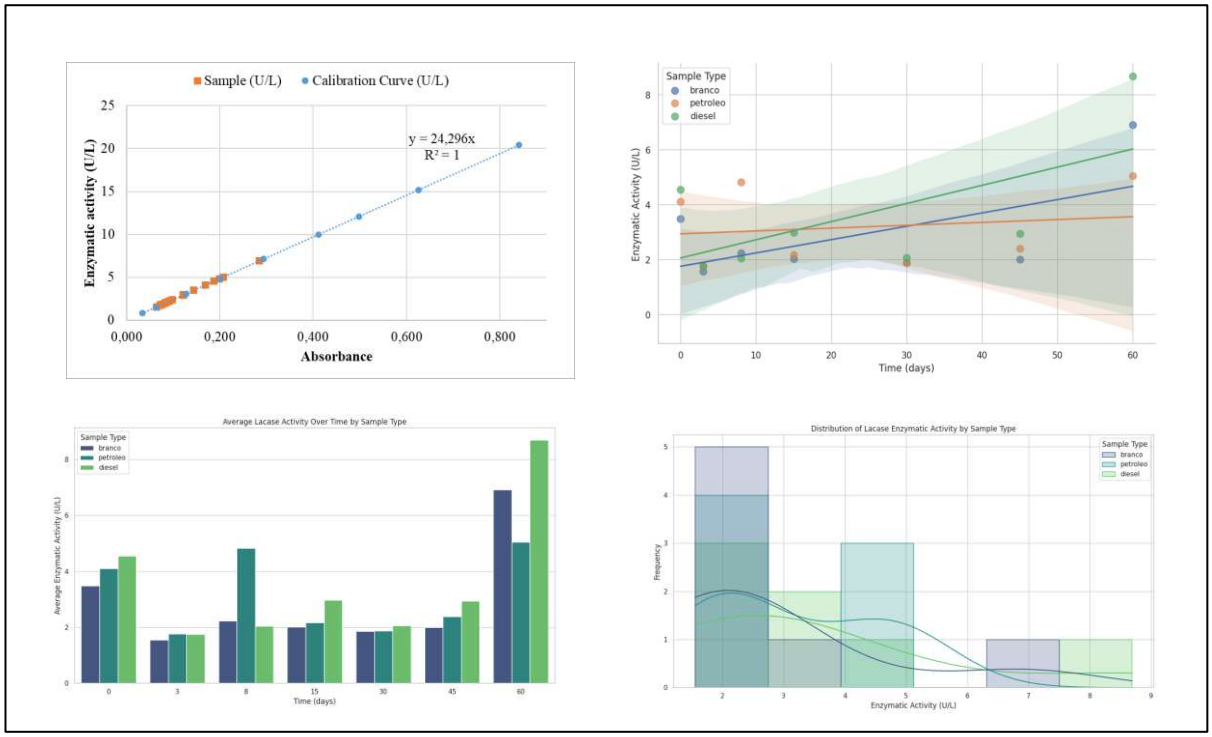
Despite these fluctuations, the trendline indicates a significant increase in enzymatic rates and, consequently, in lipase production. It is also observed that there is no variation among the interactions with the media, suggesting that the presence of hydrocarbon compounds does not interfere with the metabolism of this enzyme (Figure 1d) (Ławniczak et al., 2020).

It is important to emphasize that the results refer exclusively to the activity measured in the extracellular fraction and do not allow direct inference regarding gene expression or intracellular synthesis. Therefore, the data should be interpreted as indicative of detectable enzymatic response under the experimental conditions adopted.

3.2 Laccase

Comparatively, for laccase, Figure 2a shows that the data exhibit significant agreement with the previously constructed calibration curve. This correspondence indicates that the data more accurately reflect the expected trend, demonstrating a more precise representation aligned with the theoretical model.

Figure 2: (a) Comparison of calibration curve vs. sample results for laccase; (b) Relationship between incubation time and enzymatic activity for different sample types, with linear regression and 95% confidence interval; (c) Laccase enzymatic activity results; (d) Histogram and density curves of laccase enzymatic activity.



At T1 in Figure 2b, high laccase enzymatic activity rates are observed, indicating strong immediate induction in the production of this enzyme, followed by degradation occurring at T2. The sample in the experimental medium containing petroleum shows a marked peak in enzymatic activity at T3, while the other two conditions (control and diesel) maintain lower and similar activity levels.

At T4, an increase in enzymatic activity is observed in the diesel sample; however, activity decreases significantly in the petroleum sample, suggesting catalysis of the available substrate and consequent laccase degradation.

Enzymatic activity decreases again at T5 in all three conditions, with very similar values, indicating stabilization of laccase production.

On the penultimate day of analysis, the diesel and petroleum samples show a slight increase in enzymatic activity, with the diesel sample reaching the highest activity. A significant variation is also observed, with notable fluctuations from T3 to T6, as shown in Figure 2c.

Petroleum induces a peak in activity at T3, whereas diesel shows higher activity from T4 to T7. These fluctuations suggest a variable response of the microbial consortium to the presence of different compounds, with petroleum and diesel modulating laccase production at distinct stages of cultivation, showing an overall increasing trend (Figure 2d) (Janusz et al., 2020).

3.3 Manganese Peroxidase

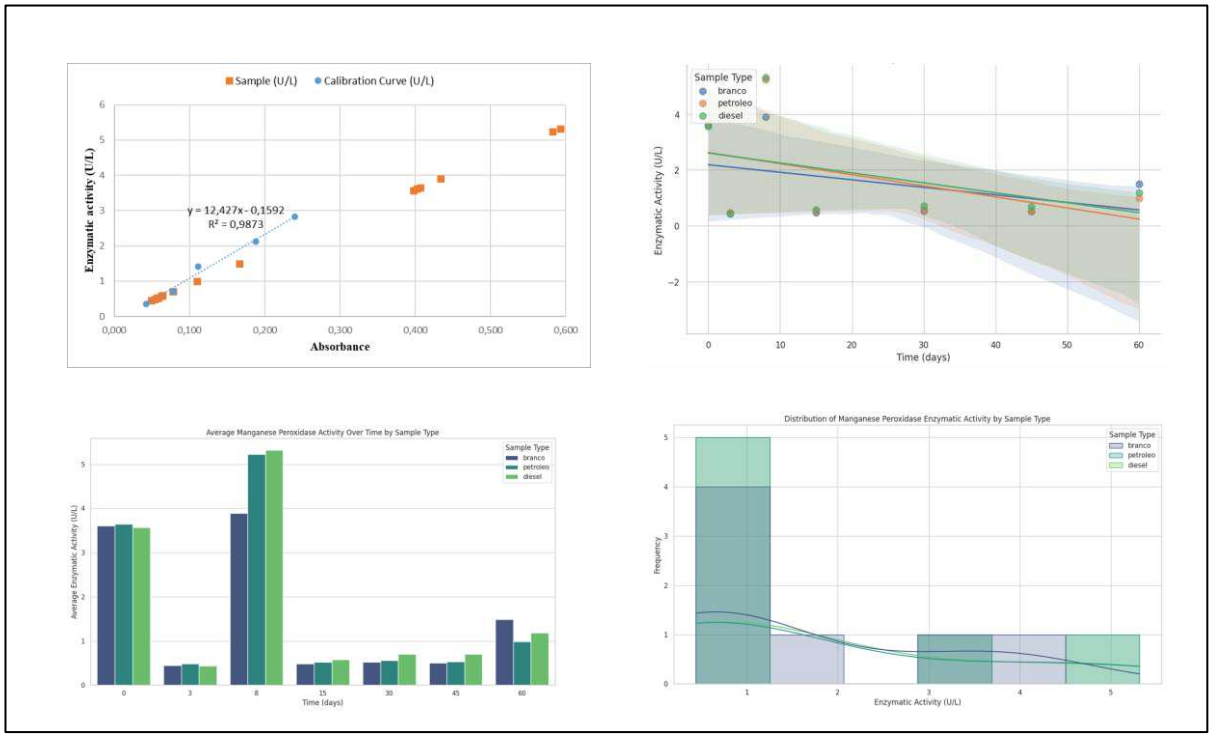
Some of the results obtained for manganese peroxidase, when compared to the calibration curve presented in Figure 3a, showed significant agreement, while other values exceeded the limits of the curve, suggesting the need for the development of a more extensive and comprehensive calibration curve.

Similarly to laccase, high enzymatic activity rates of manganese peroxidase are observed (Figure 3b) in all compositions at T1, representing a strong immediate induction in the initial production of this enzyme (Hammel; Cullen, 2008). However, the activities decrease rapidly, and at T2 they reach minimal values, suggesting a possible inhibition of the enzyme after the beginning of the experiment, with all three compositions maintaining a similar pattern.

At T3, the highest peak of enzymatic activity occurs under the diesel and petroleum conditions, where activity exceeds 5,000 U/L, while the control shows slightly lower activity, indicating the positive influence of these compounds on manganese peroxidase metabolism by the microbial consortium.

The activity values become low and remain relatively constant over the following days in all conditions, indicating stabilization of manganese peroxidase production. The graph (Figure 3c) reveals a sharp decrease followed by recovery on the eighth day, especially under conditions with diesel and petroleum addition. After this second peak, enzymatic activity remains stable, suggesting that the consortium exhibits a rapid and intense response in manganese peroxidase production during the first 8 days, possibly in response to the presence of hydrocarbons, followed by stabilization at low levels.

Figure 3: (a) Comparison of calibration curve vs. sample results for MnP; (b) Relationship between incubation time and enzymatic activity for different sample types, with linear regression and a 95% confidence interval; (c) MnP enzymatic activity results; (d) Histogram and density curves of MnP enzymatic activity.



The similar behavior across the three experimental conditions in the final days indicates that, after the initial peaks, enzyme production is not significantly influenced by the presence of petroleum or diesel and gradually declines over the course of the experiment (Figure 3d).

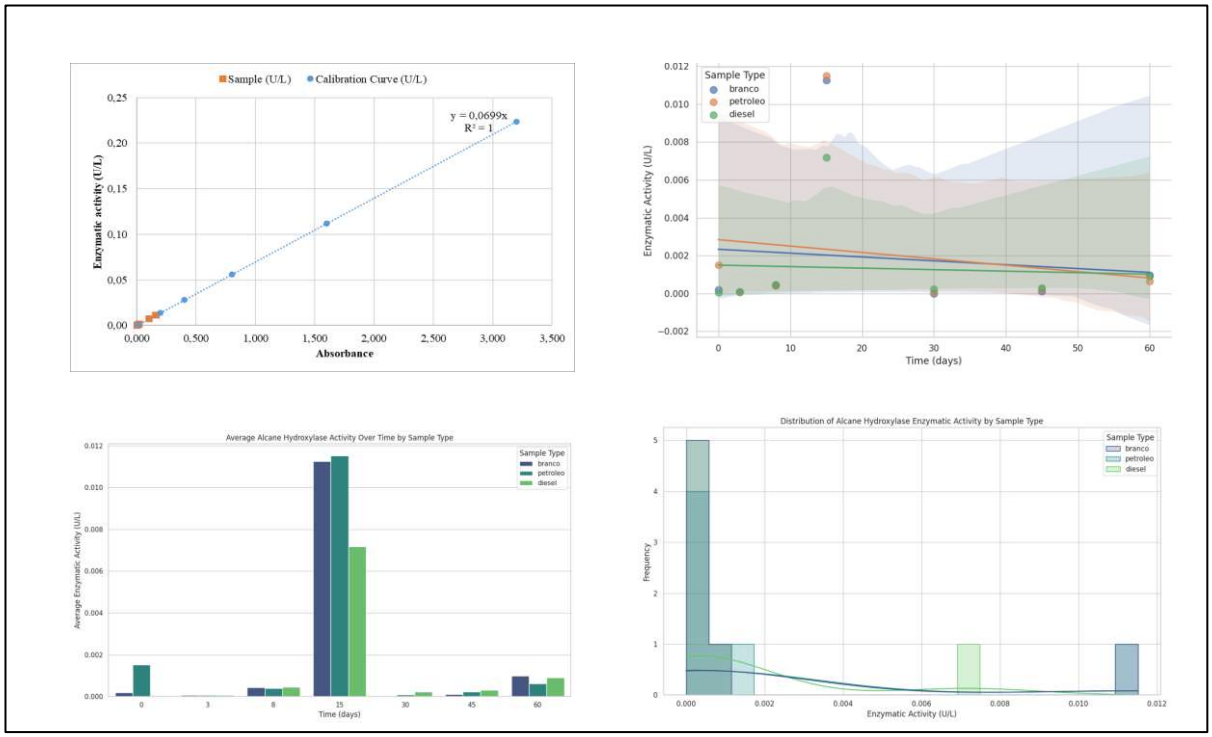
3.4 Alkane Hydroxylase

The results obtained for the alkane hydroxylase enzyme showed remarkable agreement with the calibration curve presented in Figure 4a. The close alignment between the experimental data and the calibration curve indicates the accuracy of the calculations performed to determine enzymatic activity.

At T1, the highest enzymatic activity of alkane hydroxylase (Figure 4b) was observed in the condition with crude oil, followed by the control and diesel, suggesting that the presence of oil induces a rapid enzymatic response in the cells. At T2, enzymatic activity decreased considerably under all conditions, which may indicate an initial adaptation to the environment or a period of enzymatic latency.

T4 marked the point of highest enzymatic activity across all conditions. Diesel showed the highest activity, followed by crude oil and the control, with a gradual increase also observed at T3. This peak suggests effective enzymatic induction, possibly related to the degradation of compounds present in crude oil and diesel (Van Beilen; Funhoff, 2007). From T5 onward, all enzymatic activities decreased again. On the final day, activity showed a slight increase (Figure 4c).

Figure 4: (a) Comparison of calibration curve vs. sample results for Alkane Hydroxylase; (b) Relationship between incubation time and enzymatic activity for different sample types, with linear regression and a 95% confidence interval; (c) Alkane Hydroxylase enzymatic activity results; (d) Histogram and density curves of Alkane Hydroxylase enzymatic activity.



The fluctuation in enzymatic activity over time suggests an adaptive cellular response to the environment and available substrates. The sample with crude oil showed a significant difference at T4. This peak in the hydrocarbon-enriched sample may be associated with a cycle of enzymatic degradation, indicating that alkane hydroxylase is influenced by the presence of crude oil (Figure 4d) (Van Beilen; Funhoff, 2007).

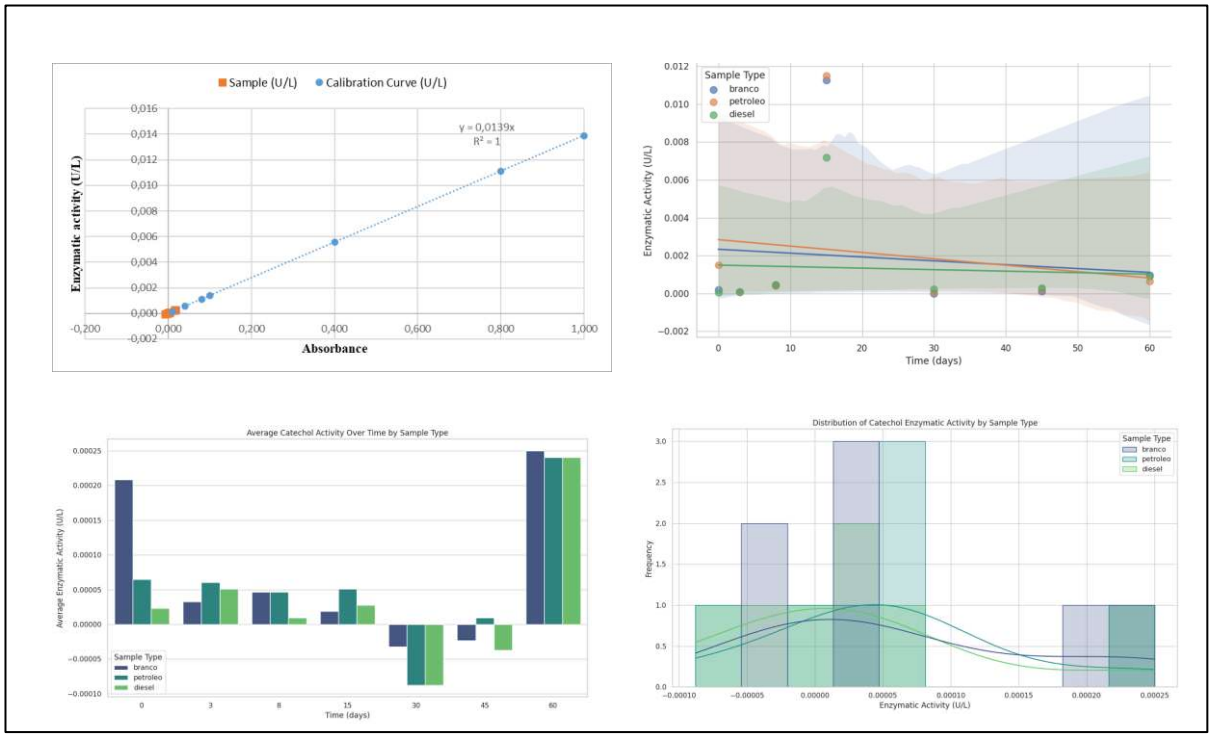
3.5 Catechol

The calibration curve for catechol 2,3-dioxygenase (Figure 5a) showed similarity with the data obtained during the experiment, indicating that the calculated conditions were properly adjusted, thus allowing an accurate assessment of enzymatic activity.

On the first day of enzyme analysis (Figure 5b), although it suggests an immediate induction in the production of catechol 2,3-dioxygenase by the consortium—reflected by the high enzymatic rate at day 0—it becomes lower than the control, which exhibits extremely high activity. At days 30 and 45, the enzymatic activity is negative or very low, suggesting that at these points there was no significant enzyme production or that the activity was insufficient to be detected (Figure 5c).

Enzymatic activity in all samples shows a peak at 60 days; this uniform behavior may indicate that the increase was not induced by hydrocarbons, but rather may represent a delayed response of the microbial consortium to prolonged incubation periods or a reaction to another experimental factor (Olukunle; Babajide; Boboye, 2015). The condition with petroleum, in comparison to diesel, shows a more consistent increase in enzymatic activity over time, without abrupt fluctuations (Figure 5d).

Figure 5: (a) Comparison of calibration curve vs. sample results for Catechol 2,3-Dioxygenase; (b) Relationship between incubation time and enzymatic activity for different sample types, with linear regression and a 95% confidence interval; (c) Catechol 2,3-Dioxygenase enzymatic activity results; (d) Histogram and density curves of Catechol 2,3-Dioxygenase enzymatic activity.



3.5. Bacterial Concentration

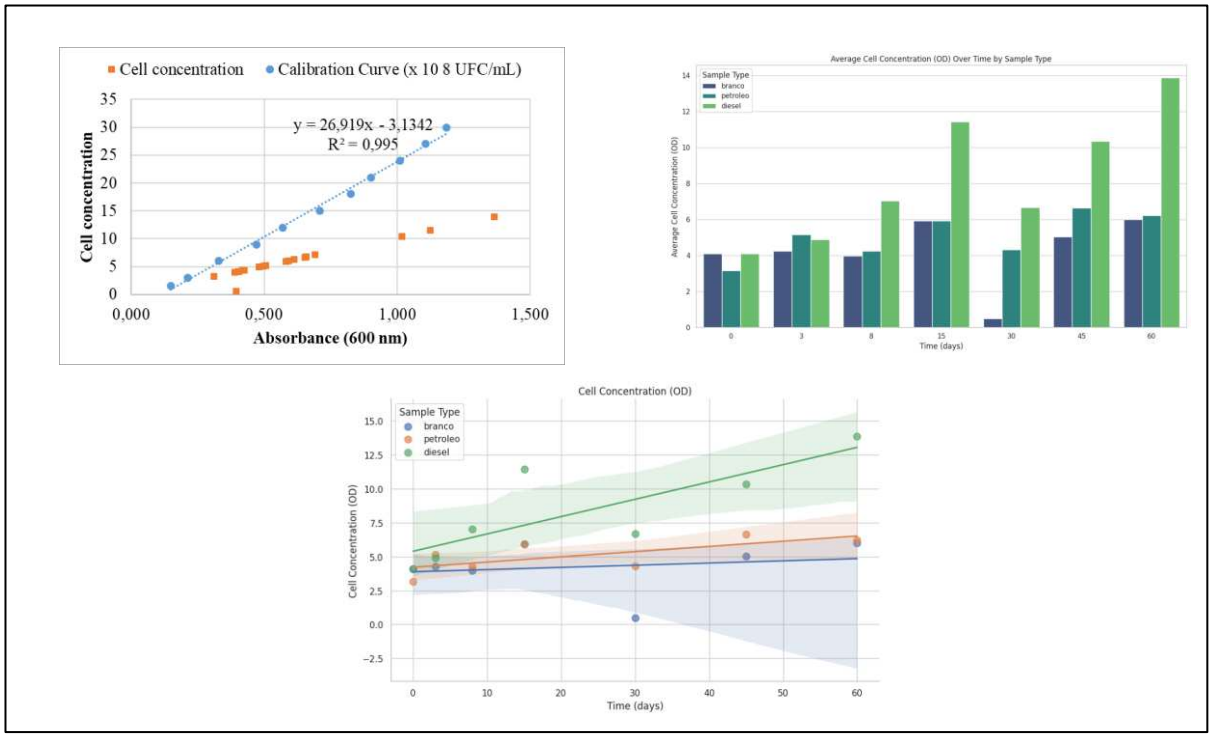
Optical density (Figure 6a) represents the turbidity or opacity of the liquid and reflects the concentration of cells in the medium, thereby indicating cellular growth.

With the graphical representation (Figure 6b), it is observed that on the first day, cell concentrations remain similar across the three conditions, with the petroleum sample showing slightly lower values. However, by the third day of growth, this pattern changes, with a considerable increase in cell numbers in all samples, with the petroleum-enriched condition reaching the highest level.

From the eighth day onward, the diesel sample shows a significant increase compared to the other two (petroleum and control), reaching its first growth peak on the fifteenth day and its maximum on day 60.

Both the petroleum-enriched sample and the control also showed an increase in cell load on the fifteenth day, with the control reaching its peak concentration; however, both decreased slightly on the thirtieth day, followed by another increase on the forty-fifth day, at which point the petroleum sample reached its peak.

Figure 6: (a) Comparison of curve vs. sample results for cell concentration; (b) Optical density results; (c) Relationship between incubation time and enzymatic activity for different sample types, with linear regression and a 95% confidence interval.



A slight biweekly trend can be observed across all conditions; however, the diesel sample exhibited this pattern more markedly, with a progressive increase in the initial sampling points, a decrease on day 30, and then a strong increase up to day 60 (Figure 6c).

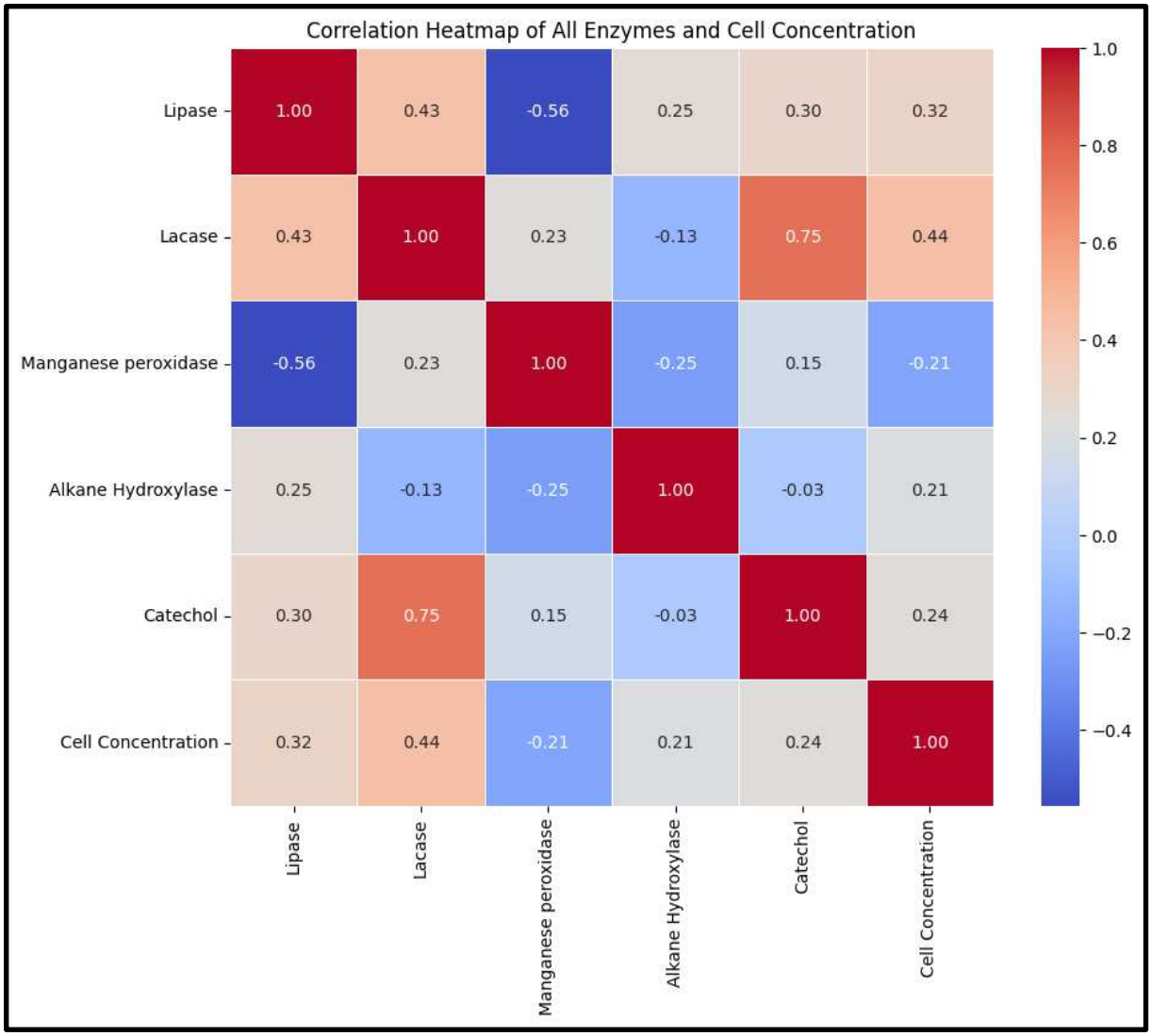
This pattern suggests a cycle of degradation and renewed enzymatic synthesis occurring every 15 days; the decrease in enzymatic activity may be attributed to a reduction in metabolic activity due to decreased stability of the consortium. Such observation of biweekly periodicity indicates a cycle of metabolic regulation in response to environmental conditions (Qi et al., 2025).

Although temporal oscillations in optical density (OD) values can be observed throughout the experiment, such variations may be associated with multiple factors, including cell aggregation, formation of dispersed biofilms, partial emulsification of hydrocarbons, and physical variations in the system. OD data should be interpreted as indicative of changes in medium turbidity over time and do not allow direct inferences regarding quantitative cell growth, metabolic cycles, or hydrocarbon degradation rates.

The correlation matrix between enzymatic activities and optical density (Figure 7) reveals different patterns of association among the analyzed variables. The heatmap highlights a clear functional division within the microbial consortium: laccase and catechol act in an integrated manner, whereas manganese peroxidase exhibits an antagonistic pattern relative to lipase, suggesting distinct ecological or temporal roles. Alkane hydroxylase appears to operate relatively independently. These correlation patterns reinforce the metabolic complexity involved in hydrocarbon biodegradation.

Furthermore, considering that the activities were determined exclusively in the extracellular fraction and that optical density represents only a turbidity parameter of the system, the results should be interpreted as indicative of concomitant temporal patterns rather than as evidence of specific biochemical interactions.

Figure 7: Heatmap of enzymes and cell concentration.



Furthermore, in order to encompass the entire study, the results obtained indicate that the mutualistic relationship between fungi and bacteria is effective in the production of enzymes involved in the degradation of polycyclic aromatic hydrocarbons (PAHs). The conclusion is aligned with previous reports, as described by Silva et al. (2024), which highlight the potential of microbial consortia in bioremediation, revealing that the strains evaluated in a petroleum-degrading medium reached the peak of enzymatic production within the first 24 hours.

The rapid production and high activity are essential for efficient bioremediation, since these enzymes catalyze the simultaneous oxidation, reduction, and hydrolysis of aromatic compounds, playing an essential role in the degradation of PAHs (Behera et al., 2021).

According to Ghorbannezhad et al. (2021), fungi act in the initial stages of degradation of high molecular weight PAHs, while bacteria remove the residues produced by fungal metabolism, assisting in the final stages of degradation. In this way, reinforcing the importance of microbial interactions in enhancing the degradation of recalcitrant compounds in the environment.

Other authors highlight that the metabolic capacity of organisms is intensified when appropriate mechanisms are used and can expand the range of compounds susceptible to microbial action (Ławniczak, Ł. et al., 2020).

CONCLUSION

The data of the enzymes laccase, manganese peroxidase, alkane hydroxylase, and catechol 2,3-dioxygenase showed significant compatibility with respect to the calibration curve, indicating that the measurements faithfully reflect the expected trend. The adherence to the curve strengthens the reliability of the presented analysis and the enzymatic activity calculations, allowing a precise evaluation of the experimental absorbance values.

The lipase enzyme curve is adequate; however, it requires expansion to encompass some practical data. In addition, the results of bacterial concentration rates also showed slight deviation from the curve, although this interval is small, it highlights the importance of developing an adjusted curve.

Concomitantly, the enzyme manganese peroxidase did not show a significant difference between the different media; however, a slightly higher enzymatic expression was observed on certain days in the petroleum and diesel composition, which may indicate some induction in microbial metabolism.

The presented results of the alkane hydroxylase enzyme indicate a complex dynamic in enzymatic activity over time under different conditions, reflecting a possible microbial adaptation to the presence of hydrocarbons such as petroleum and diesel. Analogously, the increase observed at the end of the experiment suggests that prolonged incubation time is an important factor for the production of catechol 2,3-dioxygenase, although the influence of hydrocarbons (diesel and petroleum) was not so evident, the high enzymatic activity of catechol 2,3-dioxygenase in the samples with diesel may be an indicative.

The biweekly trend observed in cell concentration confirms the existence of a natural cycle of growth and depletion of the microbial consortium. This cycle may be influenced by the need for regeneration of cells or specific enzymes, and the description of this biweekly process and specific enzymatic rates may have important implications for understanding bioremediation processes in marine environments, suggesting that bioremediation interventions could be timed to coincide with peaks of enzymatic activity to maximize efficiency.

Taken together, the results indicate that the mixed microbial consortium presents detectable extracellular enzymatic activity under exposure to hydrocarbon substrates, characterizing a measurable functional response under the adopted experimental conditions.

The understanding of organism metabolism, including the regulation of enzymatic expression and environmental factors that influence their activity, is fundamental to optimize bioremediation strategies. In this sense, investigations in this field may contribute to the development of more effective and sustainable approaches for the removal of hydrocarbons from the environment, with future studies including direct quantification of hydrocarbon chemical degradation, analysis of the intracellular fraction, and molecular evaluation of gene expression being able to deepen the understanding of the metabolic mechanisms involved and expand the applicability of the system in bioremediation strategies.

Authors' Contributions

Conflict of Interest

The author declares that the research was conducted without any commercial or financial commitments with relationships that could be construed as a potential conflict of interest.

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The authors declare that artificial intelligence tools (ChatGPT, OpenAI) were used solely to assist with language editing, grammar correction, and formatting of the manuscript. All scientific content, data, results, interpretations,

and conclusions presented in this work are the original work of the authors and have not been generated or influenced by AI. The use of AI did not affect the integrity, analysis, or originality of the research.

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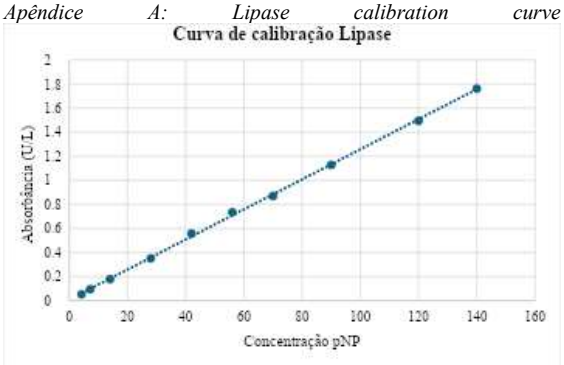
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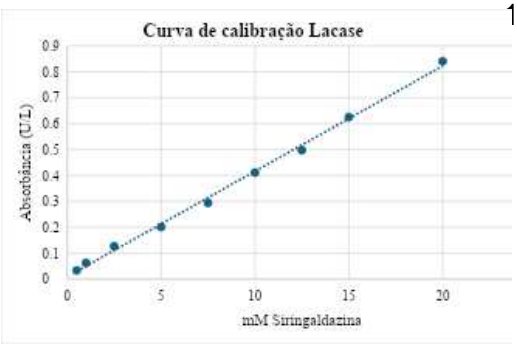
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6. APPENDICES

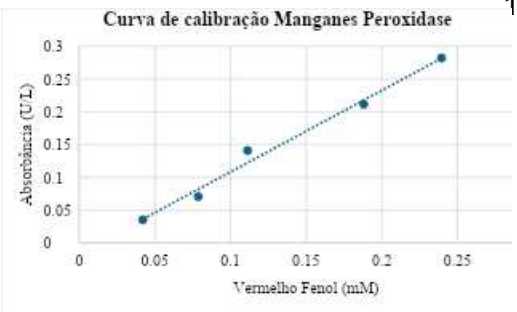
Apêndice A: Lipase calibration curve



Apêndice B: Laccase calibration curve



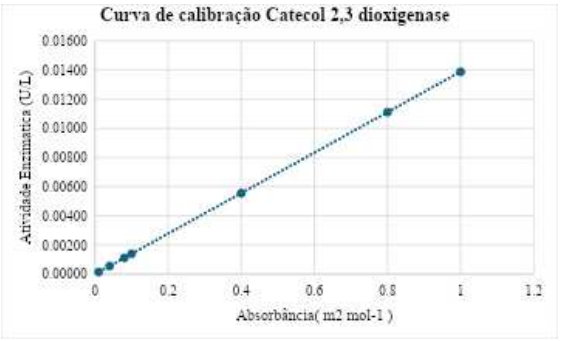
Apêndice C: Manganese peroxidase calibration curve



Apêndice D: Alkane hydroxylase calibration curve



Apêndice E: Catechol 2,3-dioxygenase calibration curve



Apêndice F: Bacterial concentration calibration curve

