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SELECTION OF ALPHA-AMYLASE PRODUCING BACTERIAL ISOLATES FROM AMAZONIAN AQUATIC ECOSYSTEM

SELEÇÃO DE ISOLADOS BACTERIANOS PRODUTORES DE ALFA-AMILASE DE ECOSSISTEMAS AQUÁTICOS AMAZÔNICOS

SELECCIÓN DE AISLADOS BACTERIANOS PRODUCTORES DE ALFA-AMILASA DE ECOSISTEMAS ACUÁTICOS AMAZÓNICOS

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ABSTRACT

The Amazonian biodiversity represents a significant source of biomolecules with potential for biotechnological application. This biodiversity can be harnessed for the development of inputs applicable to the industrial sector. In this context, bacteria possess the ability to secrete enzymes that can serve various purposes, and enzyme prospecting is considered fundamental for Brazil's socioeconomic development. Given this, the objective of this project was to prospect for alpha-amylase from the bacterial microbiota of an Amazonian aquatic ecosystem. To achieve this, water samples were collected from Lake Mamiá in the mid-Solimões region (Coari-AM) between 2022 and 2023. Subsequently, bacterial isolates with the capacity to hydrolyze starch were identified. Total DNA was then extracted from the amylolytic bacteria for amplification of the 16S Ribosomal RNA genes. Furthermore, the physicochemical conditions (Temperature, pH, and Electrical Conductivity) of the aquatic ecosystem were measured, alongside qualitative and quantitative analyses of alpha-amylase activity. In conclusion, the prospecting of enzymes from the bacterial microbiota of the

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Amazonian aquatic ecosystem is of fundamental importance for the search for novel biomolecules that meet industrial needs in bioprocesses, as well as for the enzyme's potential application in ethanol production from the processing and fermentation of cassava starch cultivated in the Amazon region.

Keywords: Biodiversity. Microorganisms. Biomolecules. Enzyme.

RESUMO

A biodiversidade amazônica representa uma fonte significativa de biomoléculas com potencial para aplicação biotecnológica. Essa biodiversidade pode ser aproveitada para o desenvolvimento de insumos aplicáveis ao setor industrial. Nesse contexto, as bactérias possuem a capacidade de secretar enzimas que podem servir a diversos propósitos, e a prospecção enzimática é considerada fundamental para o desenvolvimento socioeconômico do Brasil. Diante disso, o objetivo deste projeto foi prospectar alfa-amilase da microbiota bacteriana de um ecossistema aquático amazônico. Para isso, amostras de água foram coletadas no Lago Mamiá, na região do médio Solimões (Coari-AM), entre 2022 e 2023. Posteriormente, foram identificados isolados bacterianos com capacidade de hidrolisar amido. Em seguida, o DNA total foi extraído das bactérias amilolíticas para amplificação dos genes do RNA ribossomal 16S. Além disso, foram mensuradas as condições físico-químicas (Temperatura, pH e Condutividade Elétrica) do ecossistema aquático, juntamente com análises qualitativas e quantitativas da atividade de alfa-amilase. Em conclusão, a prospecção de enzimas da microbiota bacteriana do ecossistema aquático amazônico é de fundamental importância para a busca de novas biomoléculas que atendam às necessidades industriais em bioprocessos, bem como para o potencial de aplicação da enzima na produção de etanol a partir do processamento e fermentação do amido de mandioca cultivado na região amazônica.

Palavras-chave: Biodiversidade. Microrganismos. Biomoléculas. Enzima.

RESUMEN

La biodiversidad amazónica representa una fuente significativa de biomoléculas con potencial para aplicación biotecnológica. Esta biodiversidad puede aprovecharse para el desarrollo de insumos aplicables al sector industrial. En este contexto, las bacterias poseen la capacidad de secretar enzimas que pueden servir para diversos propósitos, y la prospección enzimática se considera fundamental para el desarrollo socioeconómico de Brasil. Ante esto, el objetivo de este proyecto fue prospectar alfa-amilasa a partir de la microbiota bacteriana de un ecosistema acuático amazónico. Para ello, se recolectaron muestras de agua del Lago Mamiá, en la región del medio Solimões (Coari-AM), entre 2022 y 2023. Posteriormente, se identificaron aislados bacterianos con capacidad de hidrolizar almidón. Luego, se extrajo el ADN total de las bacterias amilolíticas para la amplificación de los genes del ARN ribosomal 16S. Además, se midieron las condiciones fisicoquímicas (Temperatura, pH y Conductividad Eléctrica) del ecosistema acuático, junto con análisis cualitativos y cuantitativos de la actividad de alfa-amilasa. En conclusión, la prospección de enzimas de la microbiota bacteriana del ecosistema acuático amazónico es de fundamental importancia para la búsqueda de nuevas biomoléculas que satisfagan las necesidades industriales en bioprocesos, así como para el potencial de aplicación de la enzima en la producción de etanol a partir del procesamiento y fermentación del almidón de yuca cultivado en la región amazónica.

Palabras clave: Biodiversidad. Microorganismos. Biomoléculas. Enzima.

1 INTRODUCTION

The Amazon is the world's largest tropical rainforest, encompassing approximately 3.6% of the global surface. This biome represents roughly 40% of all tropical forests and harbors about 10% of the world's known biodiversity (Fearnside *et al*. 2018, Pereira *et al*. 2019, Tejada *et al*. 2020). The Amazon rainforest comprises diverse ecosystems with highly peculiar biotas and environmental conditions, where the interaction between vegetation and rivers creates floodplain forests (várzea - periodically flooded with white water), blackwater forests (igapó - continuously flooded with black water), terra-firme (upland forests away from rivers), and campinarana (white sand environments with unique vegetation) (Junk 1997, Wittmann *et al.* 2004, Junk *et al*. 2012, Junk *et al*. 2015a, Junk *et al*. 2015b).

Despite their distinct characteristics, the Amazonian forest ecosystems are connected by water pulses during the flood season and the intense activity of saprophytic microorganisms, potentiated by the region's warm and humid climate. These microorganisms recycle carbon by decomposing the numerous layers of organic material on the forest floor (leaf litter), returning essential nutrients to the forest for its maintenance (Malhi *et al*. 2012).

The product of this decomposition is leached by the intense rainfall and deposited in the igapó forests. These large deposits characterize the blackwater, which is rich in carbohydrates (most common), organic acids, pectins, minerals, growth hormones, alkaloids, and phenolic compounds (Pallardy 2008, Junk *et al*. 2015b, De Sousa-lobo *et al*. 2019). The connection between rivers is responsible for spreading these nutrients throughout the biome via flood pulses (Junk 1997, Junk *et al*. 2012, Junk *et al*. 2015a, Junk *et al*. 2015b, Melack *et al*. 2021).

The continuous flooding of the igapó promotes the establishment of a distinct flora with high levels of endemism, leading to species adaptations due to the environmental pressures of lack of light and oxygen, such as slow growth, low density, and trees that can live up to 1000 years (Junk *et al*. 2015a). These environments exhibit intermediate conditions between várzea and igapó areas with respect to the quantity of major cations (minerals such as calcium, magnesium, sodium, and potassium) and other ecological characteristics (e.g., soil and water fertility). This is because rock weathering in the paleovárzeas is greater than in the várzeas and less than in the igapó (Junk, 1997, Junk *et al*. 2012, Junk *et al*. 2015a).

Studies on igapó forests primarily focus on the description of plant species and freshwater biology; few studies address the context of the microbiota present in this environment. Recently, Ritter *et al*. 2021, described the microbiota of different Amazonian ecosystems using metabarcoding, including the description of the sediment microbiota of igapó. This study identified 39,350 operational taxonomic units (OTUs), of which 25%

corresponded to bacteria, 17% to fungi, 14% to protists, and the majority (26%) could not be classified. Furthermore, Acidobacteria, Proteobacteria, and Actinobacteria correspond to the most recurrent phyla of microorganisms in the Igapó forest. However, to our knowledge, large-scale studies with the cultivable microbiota of this environment have not yet been conducted.

Understanding the Amazonian biome and its composition is of great relevance for solving problems (inherent to the region) that hinder the sustainable economic development of the Amazon. In addition, this microbiota can also be used for the development of inputs applicable to the industrial sector, and in this sense, fungi and bacteria possess the ability to secrete enzymes that can serve various purposes. To illustrate, microbial enzymes are currently used to make over 70 commercial products, serving more than 40 industrial sectors, from household care and bioenergy to agriculture, animal health, and food (Arnau *et al*. 2019). Globally, enzyme production grows by about 6.3% per year based on global consumer market demand, with China and India being the largest consumers of enzymatic products, with a market value of US$ 7 billion in 2017 (Freedonia Industry Study 2017).

In 2005, Brazil's financial activity in this sector was approximately US$ 147 million, representing 3.7% of the global market The nation remains reliant on the importation of industrial enzymes; during this period, 86% of industrial enzymes were imported, while only 14% were exported (Monteiro & Silva 2009). This trade imbalance indicates a lag in the development and production of both specialized enzymes (for research, therapeutics, and diagnostics) and industrial enzymes (for biofuels, pulp and paper, textiles, human and animal food, and detergents) (Ahuja & Malkani 2023).

Finally, the objective of this study was to prospect for alpha-amylase from the bacterial microbiota of an Amazonian aquatic ecosystem, to isolate and identify amylolytic bacteria at the genus level. Additionally, the study aimed to analyze the physicochemical parameters of the water sampling sites within the aquatic ecosystem surrounding the municipality of Coari/AM, and to analyze the qualitative and quantitative enzymatic activity of the alpha-amylase.

2 MATERIALS AND METHODS

Water samples were collected from Lake Mamiá, near the city of Coari, Amazonas, Brazil, between October 2022 and November 2023. Following collection, the samples were transported to the Microbiology Laboratory of the Health and Biotechnology Institute (ISB) at the Federal University of Amazonas (UFAM) for processing.

Subsequently, 50 μL^{-1} of each water sample were inoculated onto solid Lauria-Bertani (LB) medium (Sezonov *et al*. 2007) modified to contain NaCl at 0.25%, yeast extract at 0.125%, and peptone at 0.25% (w v^{-1}) (LB 1/4), supplemented with 1% starch (cassava starch). The Petri dishes were then incubated at 30 °C for 24 hours. Following growth, amylolytic isolates were identified by flooding the plates with sublimed iodine within a fume hood with air flow.

The transparent halos, resulting from the activity of secreted alpha-amylase in the solid culture medium, were measured using a caliper. Bacterial colony diameters and the enzymatic index (EI) of each isolate were also determined (Effio *et al*. 2000), and the data were presented as percentage frequency. Based on the data from each collection site within the different Amazonian aquatic ecosystems, the best alpha-amylase producing isolates were selected for molecular identification at the species level.

Following the selection of amylolytic bacteria, the BIOSPIN BACTERIA GENOMIC DNA EXTRACT kit (BIOER) was used to extract total DNA from the selected bacterial isolates, following the manufacturer's instructions. Subsequently, an electrophoresis system was assembled using a 0.8% agarose gel, which was stained with ethidium bromide (Sambrook & Russell 2001). The concentration of total DNA (ng μL^{-1}) was quantified by spectrophotometry.

For the amplification of the 16S ribosomal DNA gene by PCR, the degenerate primers F16S (690 pb) and R16S (1400 pb) were used for the initial step of molecular characterization at the species level of the selected bacterial isolates. For sequencing, the PCR amplicons were sequenced using the Sanger method on a 3500 Genetic Analyzer (Thermo Fisher), and species-level identification was performed using the BLAST tool (www.ncbi.nlm.nih.gov), and the sequences were deposited in the NCBI GenBank, submission: 15530025, process: PX123011 – PX123016, link https://submit.ncbi.nlm.nih.gov/subs/genbank/ (08/14/2025 – 11:11 h).

Physicochemical analysis of the collection sites was conducted using an Akso model AK88 multiparameter meter. Probes for pH, temperature, and electrical conductivity were used for measurements, and the probes were calibrated according to the manufacturer's guidelines.

Following the selection and molecular identification of the most efficient bacteria in the genetic expression of extracellular alpha-amylase, they were inoculated into liquid LB ¼ medium with 1% starch and maintained at 30°C for 72 hours at 150 rpm. Immediately after obtaining the crude enzyme extract, the reaction system was assembled to analyze alpha-amylase activity with starch at (10 mg mL^{-1}) and 50 Mm^{-1} sodium phosphate buffer (pH 6).

Subsequently, the reaction mixtures were incubated at 40 °C with a time variation of 0, 5, 10, 20, 30, and 60 minutes. The reactions were then stopped by the addition of 1 M^{-1} acetic acid, followed by the addition of FUWA reagent (I_2 – KI solution). The reactions were homogenized by inversion, and the absorbance was read at 660 nm using a spectrophotometer. A calibration curve and its linear equation were also determined.

Therefore, the results of this study were analyzed using the Prisma program GraphPad Prism 10.3.1 (509) (October 10, 2024) by Two-way ANOVA (or mixed model) statistical analysis with Plot – mean with SD, and the data were presented in graphical format.

3 RESULTS

Bacterial microbiota sampling was conducted across different seasonal periods, with six sampling events at Lake Mamiá. The collection dates at Lake Mamiá were as follows: October 20, 2022 (1st), February 18, 2023 (2nd), May 18, 2023 (3rd), September 1, 2023 (4th), October 13, 2023 (5th), and April 11, 2023 (6th). Collection sites were identified by geographic coordinates. Within the Lake Mamiá aquatic ecosystem, four collection points were selected: two on the right bank and two on the left bank. The coordinates for the collection points are: 04°07.418”S, 063°00.393”W (1st); 04°07.338”S, 063°00.054”W (2nd); 04°06.242”S, 063°00.675”W (3rd); and 04°06.843”S, 063°01.524”W (4th).

Analysis of total bacterial isolation on LB ¼ culture medium supplemented with 1% starch yielded 8112 CFU mL^{-1} from Lake Mamiá across the six sampling events. For the selection of alpha-amylase producing bacteria, an enzymatic index (EI) of 2 or higher was used as the classification criterion. The bacterial isolates from the Lake Mamiá aquatic ecosystem were evaluated, yielding the following EI percentages: 68.75% for the collection on October 20, 2022; 84.61% for February 18, 2023; 92% for May 18, 2023; and 100% for October 13, 2023 (Figure 1). The results for isolates from September and November 2023 were not included in the enzymatic index analysis of amylolytic bacteria due to an insufficient relative percentage frequency for the analyzed periods.

The dataset from the six collections was analyzed using a Two-way ANOVA statistical method. The Lake Mamiá aquatic ecosystem showed a significant variability for the Row factor (P value = 0.0085) and non-significant variability for the Column factor (P value > 0.9999).

Among the identified bacteria through qualitative methods and amylolytic activity assays, two isolates belonged to the species *Exiguobacterium indicum*, with 99.33% and 99.47% identity, one isolate to *Bacillus wiedmannii* (99.49% identity), one to *Bacillus paramycoides* (99.59% identity), end one to *Paenibacillus polysaccharolyticus* (98.57%

identity), one to *Paenibacillus intestini* (98.59% identity), and one to *Fictibacillus rigui* (99.42% similarity).

The analysis of the physicochemical conditions of the biological sample collection sites, including temperature, pH, and electrical conductivity of the aquatic ecosystems, is presented in Figure 02. The data were obtained throughout the years 2022 to 2023, which were the most adverse in recent years, marked by low hydrological levels in the Amazon basin, high ambient temperatures, and reduced rainfall in the region.

The quantitative analysis of alpha-amylase activity began with the construction of a calibration curve, and subsequently, the equation of the line was determined as y = 0.0452X - 0.1078 (Figure 03). Two distinct isolates from the Lake Mamiá aquatic ecosystem were analyzed: Iso01 (*Bacillus paramycoides)* and Iso02 (*Bacillus wiedmannii*). The remaining isolates were not analyzed due to the absence of activity in starch hydrolysis at a concentration of 10 mg mL^{-1} in the reaction medium, with pH 6 and a temperature of 40°C, across an incubation time range of 0 to 60 minutes.

Finally, the results of the enzymatic activity analysis of the unpurified alpha-amylase from isolate Iso01 showed 6.5 mg mL^{-1} of product (dextrins), equivalent to 65% dextrinizing activity. For isolate Iso02, the result was 7.3 mg mL^{-1} of product (dextrins), corresponding to 73% dextrinizing activity (Figure 04). It is important to note that both enzymes were present in the crude enzyme extract.

4 DISCUSSION

For the isolation of amylolytic bacteria in this study, a modified LB culture medium supplemented with 1% starch was used, serving as the sole predominant and available carbon source for the isolation and prospecting of alpha-amylase producing microorganisms. This stage of the project allowed for the identification of bacteria capable of hydrolyzing starch and utilizing monosaccharides as a carbon source.

Supporting the results of isolation and selection of amylolytic bacteria in this work, Effio et al. 2000, also isolated *Bacillus* among other microorganisms from soil samples with the capacity to utilize 2% starch as a carbon source, in addition to 1% beef extract, 2% peptone, and 0.6% NaCl, components of their culture medium. Awan *et al*. 2018, isolated thermophilic and amylolytic bacteria identified as *Bacillus licheniformis* from hot water samples near an oil well, using a culture medium composed of potato peel starch, yeast extract, and agar.

Hossain et al. 2006, cultivated *Bacillus stearothermophilus* GRE1 in a culture medium with 5 g L^{-1} starch, 3 g L^{-1} beef extract, 5 g L^{-1} peptone, and 8 g L^{-1} NaCl, isolated from thermal waters in Ethiopia, for the optimization of production and characterization of alpha-amylase

and beta-amylase enzymes in a bioreactor with the addition of other salts to the culture medium composition for fermentation.

Pascon *et al*. 2011, isolated microorganisms from composting samples at the São Paulo Zoological Park Foundation and used starch as a carbon source. Muriithi *et al.* 2021, collected samples from various aquatic and terrestrial ecosystems in Kenya to prospect for amylolytic microorganisms and used corn starch and cassava starch as carbon sources for isolation, determining that cassava was the best carbon source.

Similarly, analyzing the isolation data according to the collection period and the microorganisms' ability to form transparent halos around the colonies, the best results were obtained during the periods of February 18, 2023, May 18, 2023, and October 13, 2023, which showed high enzymatic indices (EI) compared to other periods. In particular, the period of October 13, 2023, stood out with an EI greater than 2 in 100% of the isolates. It is noteworthy that during this period, the Solimões River hydrological basin was at the end of its low water season, with estimated rainfall decreasing from 70 to 50 mm in the hydrological basins of the Solimões River's main course: the Coari, Purus, Tefé, Jutai, and Beni river basins, SGB 2023 - Bulletin No. 40 – October 6, 2023; https://www.sgb.gov.br/sace/boletins/Amazonas/20231006_17-20231006%20%20175800.pdf accessed Jan 20, 2025). Furthermore, the electrical conductivity readings for this period were the highest, and significant variations were observed between the collection points in the Amazonian aquatic ecosystem, specifically in Lake Mamiá.

Corroborating these results, Bonnet *et al*. 2017, studied the physicochemical conditions of the Solimões River basin in Janauacá, near the city of Manacapurú/AM, and collected electrical conductivity data between 2009 and 2011. The authors observed values around 80 µS/cm during the low water period, with variations upwards, and approximately 55 $\mu S/cm^{-1}$ during the high water period ($spCond_A$).

However, the temperature and pH of the Amazonian aquatic ecosystem did not show disproportionate variations, according to the physicochemical profile results of Lake Mamiá. During this period, the hydrological level of the Itapéua - Coari/Solimões basin was negative, which altered the conditions of the aquatic ecosystem, especially concerning the concentration of ions in the bed of Lake Mamiá.

This is because the water pulses from the Solimões River do not supply salts (ions) and dissolved or particulate organic matter due to the difference in level between the Solimões River and Lake Mamiá during the low water season. Supporting this study, Kurek *et al*. 2021, observed changes in the concentration of Na, Mg, K, and Ca in the Amazon

basin near Óbidos-PA and described that November is characterized by high concentrations of inorganic ions and low concentrations of dissolved organic compounds. For August, which marks the end of the high water season and the beginning of the low water season, the concentration of inorganic ions is low, while the concentration of dissolved organic compounds is high. Drake *et al*. 2021, studied the physicochemical conditions of the Amazonian hydrological basin in Óbidos-PA from July 2011 to November 2013 and reported that for this period, the average temperature was 29.4 °C, the average electrical conductivity was 54 μS cm^{-1}, and the average pH was 7.1, with the highest concentration of inorganic ions occurring before the peak of the low water season.

Devol *et al*. 1995, investigated the physicochemical characteristics of the Solimões River in Manacapuru-AM and described that in January and April (beginning of the rainy season - high water), there was a high concentration of K, while the concentration was low from June to November (end of the rainy season - low water). According to the variation regime of ion concentration in the Solimões River basin, they observed a high concentration of sodium (Na) from August to December (low water) and a low concentration of Na from May to July (high water), in addition to a high concentration of Ca from February to April and a low concentration from May to October.

Among the isolated, selected, and identified bacteria by 16S ribosomal DNA gene sequencing, the genus *Bacillus* stood out as predominant. Among the six identified bacteria, *Bacillus wiedmannii* and *Bacillus paramycoides* were the best alpha-amylase producers compared to others. Corroborating the results of this work, Liu *et al*. 2022, identified the dominance of the genus *Bacillus* by 16S rDNA gene sequencing, representing 67.72% of the sampling of amylase-producing bacterial isolates, followed by *Exiguobacterium* sp. (2.60 %) and *Paenibacillus* sp. (1.57%). Mojallali *et al*. 2013, isolated *Exiguobacterium* from soil samples using the same technique and used primers 27F and 1492R for the best alpha-amylase producers.

It is relevant to note that the alpha-amylases from the selected bacteria, among the other enzymes isolated and studied in this work, showed activities of 65% and 73%. In summary, the high specificity of the enzyme in the crude extract under unfavorable *in vitro* conditions is due to the presence of cellular fragments, primary and secondary metabolites in the crude enzymatic extract.

Therefore, the molecules of the Amazonian aquatic system are strongly influenced by the seasonal oscillation of the hydrological level of the Solimões/Amazon River basin, as well as climatic changes that cause an imbalance in the rainfall regime in the Amazon, prolonging the dry season. These environmental factors directly impact the biological diversity of the

region. Consequently, they are influencing the modification of the structure and function of biomolecules, promoting adaptation to extreme adverse conditions of pH, temperature, and salts. Furthermore, these molecular conditions are important for the improvement of industrial processes. It is undeniable that future studies are necessary to investigate the structure and function of biomolecules adapted to these adverse conditions.

5 CONCLUSION

The prospecting of enzymes within the biodiversity of Amazonian aquatic ecosystems, coupled with the variation in climatic conditions and the alteration of hydrological levels in the Amazon basin, suggests a promising future for the identification of biomolecules selected by the drastic environmental variations that the Amazon basin has faced and is facing in the period from 2023 to 2024 with severe low hydrological levels. Therefore, the present study isolated two bacteria capable of genetically expressing alpha-amylase, which, even in non-purified conditions, exhibited high amylolytic activity under mild thermal energy and near-neutral pH. It is relevant to emphasize that the use of this enzyme in bioprocesses may reduce production costs and contribute to the reduction of toxic gas emissions generated by the burning of petroleum for the production and supply of electrical energy necessary for the transformation of raw materials into products.

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APPENDIX

Figure 1

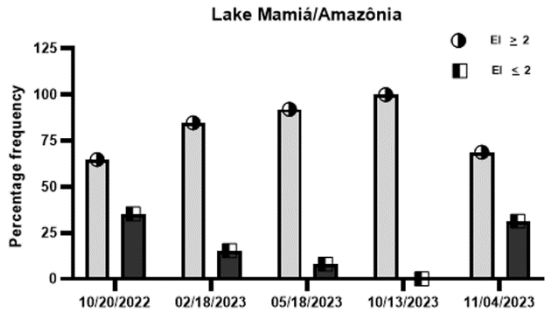

Figure 01: Enzymatic Index (EI) profile of bacterial isolates. Statistical analysis was performed using two-way ANOVA. EI values greater than or equal to 2 (EI ≥ 2) and less than or equal to 2 (EI ≤ 2) are shown.

Figure 2

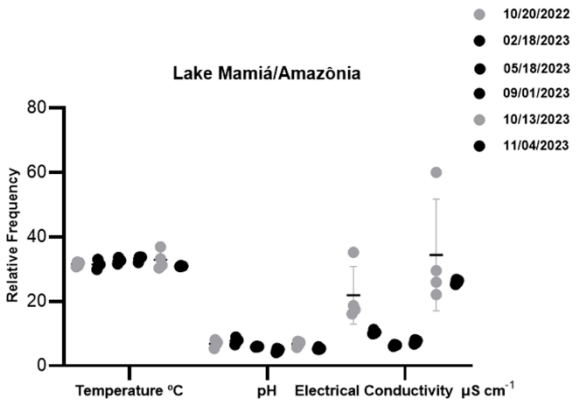

Figure 02: Physicochemical profile (temperature, pH, and electrical conductivity) of sampling sites in Lake Mamiá, Amazon. Statistical analysis was performed using two-way ANOVA. Data are presented as mean and standard deviation (SD).

Figure 3

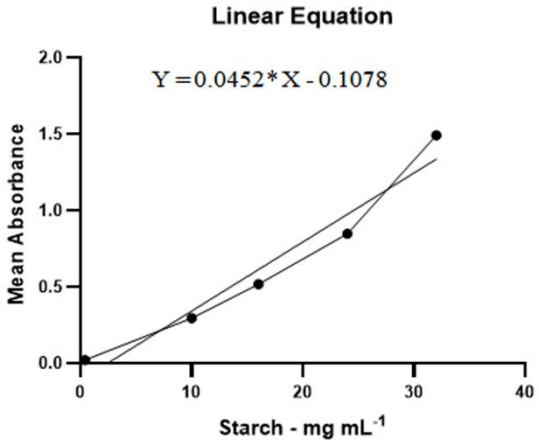

Figure 03: Determination of the linear equation using the Fuwa reagent (iodine) and the starch. ANOVA analysis.

Figure 4

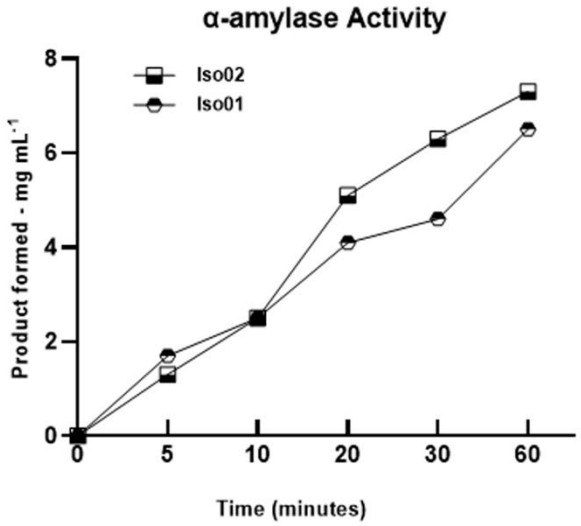

Figure 04: Profile of alpha-amylase (crude extract) from Lake Mamiá (Amazon), strains Iso01 (*Bacillus paramycoides*) and Iso02 (*Bacillus wiedmannii*). Statistical analysis method: Two-way ANOVA (mixed mode). Data are shown as mean and standard deviation-SD. Time minutes.