

Biotechnology: identification and evaluation of the *Bacillus cereus* amylolytic activity

Biotecnologia: identificação e avaliação da atividade amilolítica de *Bacillus cereus*

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Abstract

Biotechnology is the branch of science that uses molecules, viruses, microorganisms, cells, animals, plants or part of them in technological processes to generate benefits for humans, to flora, fauna and the environment. In this context, enzymes are natural biocatalysts that present substrate specificity and extreme importance to vital processes, as they develop indispensable functions in biochemical reactions of cell metabolism, and can be used in biotechnological processes. Knowing the human needs, for a world population of about 7.7 billion people and the functional usefulness of enzymes, there is, on the one hand, a gigantic demand for the consumption of various products in the agricultural sector, processed and industrialized, such as: food, beverages, clothing in the textile sector, medicines, vaccines, cosmetics in the chemical-pharmaceutical sector, as well as in the production of paper and fuels, in which enzymes, mainly amylases, have been widely used in production processes. In this work, experiments were carried out with the wild *Bacillus cereus* bacterium to verify the production of amylases, the results obtained could demonstrate the formation of amylolysis halos around the colonies in Petri dishes containing Tryptic Soy Agar + starch (1%) medium,

pH 7.3 and grown in a biological oven at 37°C for 24 hours, when revealed in iodine vapor; the Amylolysis Index (AI) was 3.3 and the efficiency of starch substrate degradation by amylases was greater than 90% in the evaluated treatments.

Keywords: Amylase; Bacillus; Bioprocess; Biotechnology; Enzymes.

Resumo

A biotecnologia é o ramo da ciência que utiliza moléculas, vírus, microrganismos, células, animais, plantas ou parte deles em processos tecnológicos para gerar benefícios para o homem, à flora, à fauna e ao meio ambiente. Nesse contexto, as enzimas são biocatalisadores naturais que apresentam especificidade aos substratos e extrema importância aos processos vitais, pois desenvolvem funções indispensáveis nas reações bioquímicas do metabolismo celular, podendo ser utilizadas em processos biotecnológicos. Conhecendo as necessidades humanas, para uma população mundial de cerca de 7,7 bilhões de pessoas e a utilidade funcional das enzimas, existe, por um lado, uma gigantesca demanda pelo consumo de diversos produtos do setor agrícola, processados e industrializados, tais como: alimentos, bebidas, vestuário no setor têxtil, medicamentos, vacinas, cosméticos no setor químico-farmacêutico, bem como na produção de papéis e combustíveis, nos quais as enzimas, principalmente amilases, têm sido amplamente utilizadas nos processos produtivos. Neste trabalho foram realizados experimentos com a bactéria *Bacillus cereus* de origem selvagem para verificar a produção de amilases; os resultados obtidos puderam demonstrar a formação de halos de amilólise ao redor das colônias em placas de Petri, contendo o meio Ágar Triptona de Soja + amido (1%), pH 7,3 quando cultivadas em estufa biológica a 37°C por 24 horas, quando reveladas em vapor de iodo; o Índice de Amilólise (IA) foi de 3,3 e a eficiência de degradação do substrato amiláceo, pelas amilases, foi superior a 90% nos tratamentos avaliados.

Palavras-chave: Amilase; Bacilo; Bioproceto; Biotecnologia; Enzimas.

Resumen

La biotecnología es la rama de la ciencia que utiliza moléculas, virus, microorganismos, células, animales, plantas o parte de ellos en procesos tecnológicos para generar beneficios para los seres humanos, la flora, la fauna y el medio ambiente. En este contexto, las enzimas son biocatalizadores naturales que presentan especificidad a sustratos y son de suma importancia para procesos vitales, ya que desarrollan funciones esenciales en las reacciones bioquímicas del metabolismo celular, pudiendo ser utilizadas en procesos biotecnológicos. Conociendo las necesidades humanas, para una población mundial de alrededor de 7.700 millones de personas y la utilidad funcional de las enzimas, existe, por un lado, una demanda gigantesca para el consumo de diversos productos del sector agrícola, procesados e industrializados, tales como: alimentos, bebidas, confección en el sector textil, medicamentos, vacunas, cosméticos en el sector químico-farmacéutico, así como en la producción de papel y combustibles, en los que las enzimas, principalmente las amilasas, han sido ampliamente utilizadas en los procesos productivos. En este trabajo se realizaron experimentos con la bacteria *Bacillus cereus* silvestre para verificar la producción de amilasas; los resultados obtenidos permitieron demostrar la formación de halos de amilólisis alrededor de las colonias en placas Petri conteniendo medio Triptona Soy Agar + almidón (1%), pH 7.3 y cultivadas en horno biológico a 37°C durante 24 horas, cuando se desarrolló en vapor de yodo; el Índice de Amilólisis (IA) fue de 3.3 y la eficiencia de degradación del sustrato almidonado, por amilasas, fue superior al 90% en los tratamientos evaluados.

Palabras clave: Amilasa; Bacilo; Bioproceto; Biotecnología; Enzimas.

1. Introduction

Biotechnology has been a fundamental tool in the search for solutions and optimization of bioprocesses related to issues of human, plant, animal, energy and also the balance of ecosystems on our planet. According to Brazil, 2010, biotechnology can be defined as “any technological application that uses biological systems, living organisms or their derivatives, to create or modify products and processes for specific uses”. In the incessant search for solutions, biotechnology emerges as a tool with multiple functions and possibilities in the improvement and generation of new products, as well as the establishment of new markets, since it can make use of cellular systems for the development and improvement of processes and products of economic and social interest. Abreu et al., (2015), emphasizes the importance of biotechnology as an instrument in obtaining and producing several metabolic substances useful to human beings and to the environmental balance that come from the manipulation of fungi. Beattie et al., (2011), highlighted the importance of biotechnology through microbial prospecting that corresponds to the methods and tests used in the discovery of new bioactive compounds, from biological sources such as fungi and bacteria, capable of secreting substances of interest in the most diverse areas of human activity and need, such as such as: agriculture, pharmaceuticals, aquaculture, bioremediation, bioenergy and nanotechnology. In the

bioprospecting of microorganisms, enzymes have shown high biotechnological interest, and the environmental compartment of the soil has been the ideal environment for the collection and isolation of microorganisms, mainly due to the great bacterial biodiversity present in nature (Singh, et al., 2011). With regard to enzymes, these are natural substances of protein origin associated with organic and inorganic cofactors, vitamins and mineral salts, respectively, involved in all cellular biochemical processes of synthesis and catalysis, sometimes composing simple molecules into complex ones and sometimes decomposing complex molecules into smaller units (monomers), such as in polysaccharide (starch) synthesis reactions from glucose and its decomposition into simple carbohydrates (triose) until its complete oxidation into CO₂ and H₂O with the release of energy (Oliveira, et al., 2004).

The use of enzymes as biocatalysts in industrial processes has been highlighted when compared to chemical catalysts, due to factors such as: biochemical safety, non-toxicity, energy reduction in the system, production time reduction, energy cost reduction in the production of products, economic viability and the fact that enzymes do not pollute the environment (Wohlgemuth, 2010). For Gopinath et al., (2017), enzymes of microbial origin have been the most used in industries due to: ease of handling, capacity for production in large volumes, ability to release to the extracellular environment, at low cost of production, reduced reaction time, stability in extreme conditions and possibility of expression and production of recombinant enzymes in host cells.

According to data from the Ministry of Industry, Foreign Trade and Services of the Brazil (2018), the country is essentially an importer of enzymes which are used in research and in the national industry. In the 2016/2017 period, Brazil spent US\$164.41 million on enzyme imports and only US\$58.93 million on exports. According to Pandey et al. (2000), although Brazil has a high potential for the production of enzymes, as it contains a vast microbial biodiversity in its national territory and has large amounts of low-cost substrates for bioprocesses - such as agricultural residues - the country does not yet it is self-sufficient in the production of enzymes, that is, 73.61% of the enzymes used are imported and only 26.39% are of national origin.

Among the main enzymes used in bioprocesses, amylases stand out for their ability to hydrolyze starch, a polysaccharide composed of amylose and amylopectin polymers. Amylose is a linear polymer, which contains more than 6,000 glucose monomers joined by α -1,4-glycosidic bonds, while amylopectin consists of linear chains of 10-60 glucose units, joined by α -1,4- bonds. glycosidic and branched chains of 15-20 glucose units, joined by α -1,6 bonds (Souza & Magalhães, 2010). According to Pandey et al., (2006), amylases can be divided into four groups: the first is endohydrolases, such as α -amylase (EC3.2.1.1./International Biochemistry Union) 1,4- α - D-Glucan-glucon hydrolase which is responsible for the hydrolysis of α -1,4-glycosidic bonds in the internal region of starch, generating glucose and maltose as reaction products. The second group are the exohydrolases that act on the non-reducing ends of the polysaccharide chain, which can be: β -amylase that hydrolyzes the α -1,4-glycosidic bonds generating maltose dimers; α -glucosidase that hydrolyzes α -1,4-glycosidic bonds generating glucose units; amyloglucosidase (glucoamylase) that hydrolyzes α -1,4 and α -1,6 glycosidic bonds generating glucose units. The third group of amylases is composed of “debranching”, enzymes responsible for lysing the branches of the polysaccharide, such as isoamylases and pullulanases that hydrolyze α -1,6 glycosidic bonds. The fourth group corresponds to transferases that cleave α -1,4 glycosidic bonds transferring part of the molecules to an acceptor and forming a new glycosidic bond, such as cyclodextrins (Sundarram & Murthy, 2014).

Amylase enzymes can have various origins, that is, naturally produced by plants, animals or microorganisms, the latter, highlighting the microbial enzymes that have greater demand in the world market, mainly due to factors such as: easy handling in the laboratory, rapid growth of organisms, high production on a large scale and greater stability (Gupta et al., 2003). The level of production of microbial amylases varies greatly from one organism to another, depending on the origin of the microorganism, the genus, species and lineage; strains isolated from naturally starchy environments produce the highest

amounts of enzymes. In addition, factors such as pH, temperature, carbon and nitrogen sources are added, which influence the production of amylase in fermentation processes (Gopinath et al., 2017).

In the Monera kingdom, several bacterial species capable of producing amylases for industrial use are found, which have been obtained mainly from *Bacillus* genus bacteria, such as: *Bacillus subtilis*, *B. licheniformis*, *B. stearothermophilus* and *B. amyloliquefaciens*, since they are capable of producing thermostable amylases (Souza & Magalhães, 2010). Depending on their origin, amylases are also capable of reacting in different concentrations of salts, such as those from halophilic bacterial strains isolated from oceans, such as *Marinobacter sp.* (Kumar & Khare, 2015) and *Rhodothermus marinus* (Yoon et al., 2008).

There are several industrial sectors that use amylases at some stage of manufacture, such as in the production of glucose and fructose from starch conversion, for example: in the manufacture of corn glucose syrup. According to Van Der Maarel et al., 2002, amylases have been used in the paper, detergents (to enhance stain removal), starch (polysaccharide molecular weight reduction, to obtain viscosity and sweetness) industries. including being able to act in the hydrolysis process together with cellulases, proteases and lipases (enzymatic cocktail), in addition to being an organic compound that does not pollute the environment (Hmidet et al., 2009). Amylases can also be used for the production of biofuel, such as ethanol, from the enzymatic hydrolysis process of the starch substrate, which is easily found in nature or with industrial residues, achieving a low production cost (Chi et al., 2009). In the processed food industry, amylases can also be used during the baking steps, such as in the production of cakes, fruit juices and starch syrup (Couto & Sanromán, 2006), as well as in the textile industry for starch removal (Dalvi et al., 2007); it is noteworthy that part of these industrialized products have been destined for export trade (Brazil, 2018).

As already highlighted, amylases are among the most important industrial enzymes, with great biotechnological importance. Despite coming from different sources, including plants, animals and microorganisms, microbial enzymes generally meet the industrial demand satisfactorily. Currently, large amounts of microbial amylases are commercially available and have applications in the food, beverage and cleaning products sector, accounting for 14% of the total demand for industrial enzymes (Gupta et al., 2003; Pandey et al., 2006). According to Politzer et al., 2006, the food and beverage sectors accounted for more than 50% of the world demand for amylases. Amylases constitute one of the most important groups of enzymes with applications in different industries such as food, textile, chemical, pharmaceutical and detergents (Nielsen & Borchert, 2000; Gupta et al., 2003; Surmely, 2003; Demirkan et al., 2005; Soccol, 2006; Pandey et al., 2006).

Among the amylolytic enzymes, α -amylase is the most important, as it plays a fundamental role in the conversion of starch into low molecular weight glycosidic products, which can be used by other enzymes of the same group. In industrial processes, it is intended that α -amylases are thermophilic, that is, have hydrolytic activity at high temperatures, as in the gelatinization step: 70 to 90°C and liquefaction: 100 to 110°C of starch (Sidhu et al., 1997; Sarikaya et al., 2000; Peixoto et al., 2003). Among the thermophilic α -amylases, industrially, those produced by thermophilic microorganisms, such as *Bacillus* genus bacteria, stand out due to the following characteristics: stability at high temperature and pH.

Among the 48 *Bacillus* species already described, 32 produce α -amylase, but only a few of these are capable of secreting active enzymes at high temperatures. In the genus *Bacillus*, the species: *B. amyloliquefaciens*, *B. stearothermophilus*, *B. subtilis* and *B. licheniformis* are the four most used species in the industrial production of α -amylases (Sajedi et al., 2005; Souza & Magalhães, 2010). Among the main advantages of using thermostable α -amylases in industrial processes, there is a reduction in the risk of contamination, an increase in the rate of diffusion of reagents and a reduction in costs with external refrigeration (Lin et al., 1998).

According to Pandey, 2003, the bacterium *Bacillus cereus* is considered one of the main microorganisms capable of producing amylase enzymes, in addition to *B. megaterium*, *Bacillus subtilis*, *B. licheniformis*, *B. amyloliquefaciens*, as well as

the fungi *Aspergillus niger*, *Penicillium sp.*, *Cephalosporium sp.*, *Rhizopus sp.* and *Neurospora sp.* The α -amylase produced by *Bacillus* genus has an apparent molecular weight of 50kDa, is stable in a pH range from 5.5 to 9.0 and remains active even at elevated temperatures between 80 and 90°C. Its biosynthesis is controlled by both substrate induction and catabolic repression, being produced at the end of the exponential growth phase and at the beginning of the stationary phase. It presents itself as an extracellular enzyme, but small amounts of the enzyme can remain attached to the membrane (Morcel & Biedermann, 1994). *Bacillus sp.* is a gram-positive, rod-shaped bacterium, mobile by peritric and sporogenic flagella (Welker and Campbell, 1967). The species was originally isolated from soils and obtained this classification for its ability to secrete large amounts of amylases. *Bacillus amyloliquefaciens* is also capable of producing a variety of other enzymes, such as: galactanase, isoamylase, mannanase, xylanase, metal-protease, serine-protease, alkaline phosphatase and ribonuclease (Priest, 1977). The range of enzymes produced by *Bacillus* species corresponds to about 50% of commercially produced bacterial enzymes. The wide use of *Bacillus* in industry is due to the ease of growth of these species, such as the temperature range that varies from 30 to 40 °C and pH 6.0 - 7.0 for optimal growth; also for presenting, naturally, high levels of extracellular enzymes and for being considered a non-pathogenic species (Shimada et al., 1985; Vehmaanperä et al., 1991). The production of α -amylase of microbiological origin can occur in various forms of processes, such as in solid or submerged fermentations, taking into account the conditions of the bioprocess, such as the type of fermentation, the concentration of nutrients, the speed of agitation/aeration, pH and temperature, which can be modified in order to optimize microbial growth and enzyme production (Milner et al., 1996). In the production of amylases by *Bacillus*, the aeration rate must be taken care of, as the amount of oxygen dissolved in the medium is an important factor in the fermentation process. On a laboratory scale, the aeration rate needed to maintain the proper level of dissolved oxygen is on the order of one volume of air per volume of fermentation medium per minute; other studies have shown that the system's aeration can be interrupted in the stationary phase, controlling the pH, without significant losses in enzyme yield (Milner et al., 1996). Regarding the carbon source, the production of α -amylase by bacteria of the genus *Bacillus* is usually induced by the presence of the starch substrate or its hydrolytic product, maltose. Several studies demonstrate that starch and maltose act as inducers of α -amylase activity. On the other hand, the presence of sugars, such as xylose and fructose, act as repressors of enzyme synthesis, reducing its production (Gupta et al., 2003). The availability of nitrogen is essential in the culture medium for the microbial growth of *Bacillus* species and, consequently, for the production of enzymes. In synthetic culture media, the addition of organic nitrogen, such as meat extract and peptone, has been preferred for bacterial amylases production. However, several sources of inorganic nitrogen have been reported for better production of amylase by fungi, such as ammonium sulfate, ammonium nitrate (Gupta et al., 2003); pH is another factor with a strong influence on the production of amylases. Most *Bacillus* strains used commercially in the production of bacterial α -amylases, by submerged fermentation, show excellent growth in the pH range between 6 and 7, which may vary according to the genus and species of the producing bacteria (Gupta et al., 2003). Temperature is also one of the most important factors in the production of enzymes, as it acts in the environmental system of microorganism growth in a decisive way in the functions of biomolecules, in the stability and maintenance of biological structures. Thus, bacterial amylases from *Bacillus cereus*, *B. amyloliquefaciens*, *B. subtilis*, *B. licheniformis* and *B. stearothermophilus* are industrially produced at temperatures ranging from 37° to 60° degrees Celsius (Swetha et al., 2006).

Another biotechnological potential explored in microorganisms is as probiotics, as they present health benefits when ingested, such as: balance of the intestinal microbiota, chemiosmotic balance in the intestinal system, reduction of diarrhea, control of cholesterol and triglycerides, anti-inflammatory effect, antimicrobial activity, anti-tumor activity, immune system modulator, cancer reduction. Added to this are the characteristics of rapid cell reproduction, ability to produce antimicrobial substances, ability to resist the digestive process of animals, production of enzymes (phytase, cellulase, amylases, B-galactosidase) and non-pathogenicity, such as those of the genera *Lactobacillus*, *Bifidobacterium*, *Enterococcus*,

Saccharomyces, *Bacillus cereus*, *Bacillus subtilis*, *B. licheniformis*, *B. pumilus*, *B. clausii*, *B. coagulans*, *B. thuringiensis* (Ibrahim, 2000; Fao/Who, 2001; Mattila-sandholm et al, 2002; Hong et al, 2005; Malkov et al, 2005; Begley et al, 2006; Parvez et al, 2006; Rhodes, 2006; Vasiljevic & Shah, 2008; Nedelcheva et al, 2010; Abriouel et al, 2011; Nithya & Halami, 2013). *Bacillus cereus* has been used as an antimicrobial in the treatment of gastrointestinal diseases, diarrhea caused by bacteria and rotavirus, under the trade names Subtyl, Bactisubtil, Paciflor, Toyocerin (Ishibashi et al, 2001, Hong et al, 2005).

2. Material and Methods

The experiments were developed based on the works of Caraway (1959), Hankin and Anagnostakis (1975), Astolfi Filho et al. (1986), Rajkowski and Bennett (2003); Carrín et al. (2004), Morita and Assumpção (2007), Ashwini et al. (2011).

2.1 Biological Material

The experiments were carried out at the Microbiology Laboratory, block 7 of the Federal University of Santa Maria, Frederico Westphalen Campus (UFSM – FW). The microorganism was isolated from the rhizome of the plant *Edycheium choronarium*, collected in the forest of the Municipality of Frederico Westphalen-RS (Brazil), being inoculated in Muller Hinton Broth medium and selected in Petri dishes containing Muller Hinton Agar medium, both were grown under standard conditions of temperature (37°C), pH=7.0 and incubation time of 24 hours; initially in a Shaker type incubator (Luca 220 – Lucadema, regulated at 140 rpm), and later in a B.O.D. SL 200.

2.1.1 Identification of Biological Material

a) Morphological. The microorganism was identified by visualizing its morphological structure with the aid of the OPTON TIM – 30 Binocular Stereomicroscope equipment, in a Petri dish, Muller Hinton Agar medium. Bacterioscopy was used to identify the shape and arrangement of the bacteria, through microscopic observation in the Digilab Planachromatic Trinocular Biological Microscope (10x Eyepiece; Achromatic Objectives: 4x, 10x, 40x, 100x(R) oil).

b) Molecular: Taxonomy Classification. Target DNA amplification (PCR). The Consensus sequences were generated by BioEdit (HALL, 1999) from sequences obtained in Polymerase Chain Reactions by primers) 27F (5' – AGAGTTTGATCMTGGCTCAG–3') and 1492R (5'– TACGGYTACCTTGTTACGACTT – 3'), according (WEISBURG et al., 1991). The taxonomic classification of the samples was based on the comparison between the consensus sequence and those deposited in GenBank (www.ncbi.nlm.nih.gov/)-MEGABLAST. The unique sequence was aligned with similar ones available in the database and a phylogenetic distance tree was built using the MEGA 7 program (Kumar et al., 2016). Analyzes were carried out in the laboratories of the Tropical Culture Collection of the André Tosello Foundation (CCT – FAT)/ São Paulo-Brazil.

2.2 Gram Stain

The GRAM staining technique (Brasília, 2001) was used to characterize the microorganism, the same being observed in a Digilab Planachromatic Trinocular Biological Microscope.

2.3 Growth of Microorganism in Liquid Medium

The microorganism, obtained from the isolation plates (Muller Hinton Agar), was inoculated in Tryptic Soy Broth medium with pH 7.3 in 250 mL Erlenmayer. The inoculum was grown for 24 hours in a Shaker Luca 220 – Lucadema Incubator, regulated at 140 rpm, with a constant temperature of 37°C.

2.3.1 Growth in Solid Medium with Starch

The microorganism was inoculated in Petri dishes containing Tryptic Soy Agar growth medium added with starch (1%) for 24 hours in a B.O.D. SL 200 at 37°C.

2.3.2 Qualitative Analysis of Starch Substrate Consumption

Inoculums of the microorganism were grown in Petri dishes containing Tryptic Soy Agar medium added with starch (1%) for 24 hours in a B.O.D. SL 200 at temperature of 37°C and, subsequently, submitted to iodine vapors to verify the formation of amyolysis halos around the colonies, in order to characterize the amyolytic activity of the bacteria - (Hankin and Anagnostakis, 1975; Astolfi Filho et al., 1986; Ashwini et al., 2011).

2.4 Construction of the Starch Standard Curve

The standard curve (calibration) of starch was constructed using the spectrophotometry technique by the reaction of starch with Iodine/Lugol (Morita and Assumpção, 2007), modified from Carrín et al., 2004. The aliquots, containing different concentrations of the solution of starch, were added with Lugol's solution and transferred (2mL of mixture volume) in glass cuvettes (Glass) to carry out the Transmittance and Absorbance readings (λ 620 nm) in the spectrophotometer (Nanocolor Visivel – Macherey Nagel).

2.5 Microorganism Growth in Inducer Medium for Amyolytic Enzyme Production

Inoculums of the amyolytic microorganism were grown in Tryptic Soy Broth liquid medium added with a starch solution (2%) to induce the production of amylase enzymes. The medium was sterilized in an autoclave Digitale AV 30, at 121°C for 15 minutes. The final growth medium had pH 7.3 measured in a pHmeter-Akso device. The inoculation of the microorganism in Tryptic Soy Broth + starch medium took place in a Lutech laminar flow chamber. The growth of the microorganism took place in an Incubator Shaker Luca 220 – Lucadema, regulated at 140 rpm, at a temperature of 37°C for 24 hours.

2.6 Obtaining Enzymes - Transfer of Inoculum, Centrifugation and Separation of Supernatant from Cells

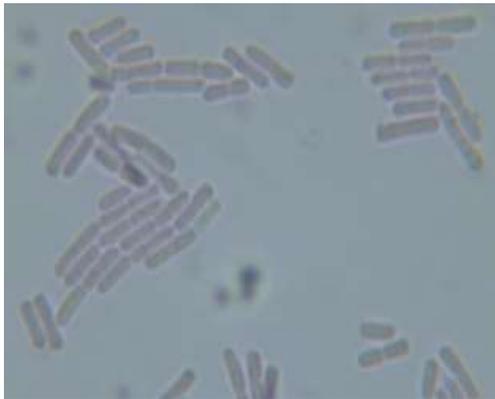
After 24 hours of growth of the microorganism in Tryptic Soy Broth + starch medium under standardized conditions, the liquid medium was divided into 10 mL volumes and placed in 10 sterile Falcon tubes. These were followed by centrifugation in a Daiki Spinplus centrifugal device under conditions of 5,000 rpm for 15 minutes. From the centrifuged material, the supernatant was carefully removed and transferred to sterile and closed test tubes. Of the supernatant material (enzymatic solution), half was stored in a refrigerated environment of 4°C, and the other half was filtered in a 0.22 μ m millipore membrane, being subsequently kept under the same refrigeration conditions.

2.7 Enzyme Activity Analysis (AE)

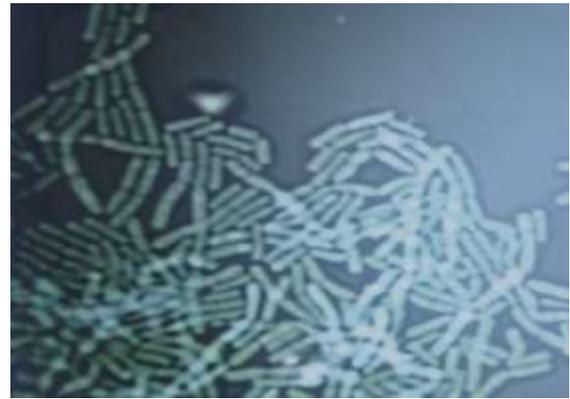
2.7.1 Avaliação do Índice de Amilolise (IA)

The amyolysis index (AI) is a potential estimate of the amyolytic activity of microorganisms on the starch substrate, obtained by visual assessment and calculated by the ratio between the “diameter of the amyolysis halo and the diameter of the colony”. AI values above 2.0 denote excellent production capacity and enzyme action. Amyolysis halos are well visualized after the development of the growth plate on iodine vapor (Astolfi Filho et al., 1986).

Figure 2 - Bacteria, bacillus type (a,b) observed under a microscope (1,000x), image captured with projection on a notebook screen.



a) Gram (+)



b) Streptobacillus arrangement

Source: Authors (2021).

B) Molecular: Taxonomy Classification.

Sequência:>591-1

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CAAGTCGAGCGAATGGATTAAGAGCTTGCTCTTATGAAGTTAGCGGCGGACGGGTGAGTAACACGTGG
GTAACCTGCCATAAGACTGGGATAACTCCGGGAAACCGGGGCTAATACCGGATAACATTTTGAACNGCATGGT
TCGAAATTGAAAGGCGGCTTCGGCTGTCACTTATGGATGGACCCGCGTCGCATTAGCTAGTTGGTGAGGTAACG
GCTCACCAAGGCAACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAGACACGGCCCAGA
CTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCAACGCCGCGTGAGTGA
TGAAGGCTTTCGGGTCGTA AAACTCTGTTGTTAGGGAAGAACAAGTGCTAGTTGAATAAGCTGGCACCTTGACG
GTACCTAACAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGTAATACGTAGGTGGCAAGCGTTATCCGG
AATTATTGGGCGTAAAGCGCGCGCAGGTGGTTTTCTTAAGTCTGATGTGAAAGCCCACGGCTCAACCGTGGAGGG
TCATTGGAAACTGGGAGACTTGAGTGCAGAAGAGGAAAGTGGAATTCCATGTGTAGCGGTGAAATGCGTAGAG
ATATGGAGGAACACCAGTGGCGAAGGCGACTTTCTGGTCTGTA ACTGACACTGAGGCGCGAAAGCGTGGGGAG
CAAACAGGATTAGATACCCTGGTAGTCCACGCCGTA AACGATGAGTGCTAAGTGTTAGAGGGTTTCCGCCCTTT
AGTGCTGAAGTTAACGCATTAAGCACTCCGCCTGGGGAGTACGGCCGCAAGGCTGAAACTCAAAGGAATTGAC
GGGGGCCCGCACAAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGTCTTGACAT
CCTCTGAAAACCCTAGAGATAGGGCTTCTCCTTCGGGAGCAGAGTGACAGGTGGTGCATGGTTGTCGTCAGCTC
GTGTCGTGAGATGTTGGGTTAAGTCCC GCAACGAGCGCAACCCCTTGATCTTAGTTGCCATCATTAAAGTTGGGCAC
TCTAAGGTGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCTTATGACCTGG
GCTACACACGTGCTACAATGGACGGTACAAAGAGCTGCAAGACCGCGAGGTGGAGCTAATCTCATAAAACCGTT
CTCAGTTCGGATTGTAGGCTGCAACTCGCCTACATGAAGCTGGAATCGCTAGTAATCGCGGATCAGCATGCCGC
GGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCACGAGAGTTTGTAAACCCGAAGTCGGTGGG
GTAACCTTTTTTG GAGCCAGCCGCTAAAG
```

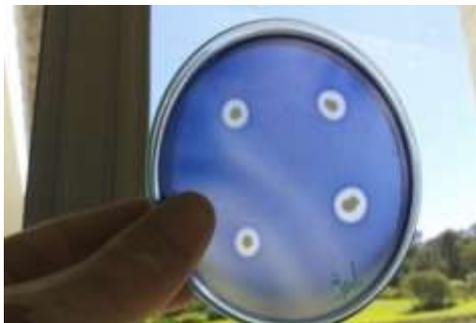
Taxonomy classification: *Bacillus cereus* Frankland and Frankland 1887. Cellular organisms; Bacteria; Terrabacteria group; Firmicutes; Bacilli; Bacillales; Bacillaceae; Bacillus; Bacillus cereus group.

3.2 Amylolysis Test

The amylolysis test of the bacterium *Bacillus cereus* resulted in the formation of amylolysis halos around the

bacterium colonies, after developing the plaque with iodine vapor, presenting IA = 3.3 (10/3). Starch hydrolysis was given as a function of the release and action of amylolytic enzymes in the medium by the bacteria *B. cereus*, shown in Figure 3.

Figure 3 - Amylolysis halos around *Bacillus cereus* colonies.

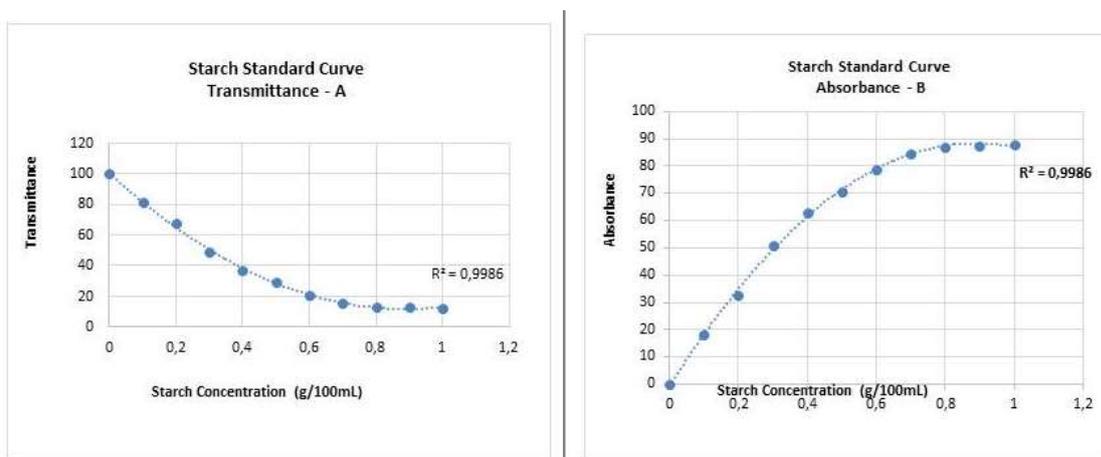


Source: Authors (2021).

3.3 Starch Standard Curve

The standard curve for starch (graphs 1 A and B) served as a basic parameter to analyze the degree of starch degradation in the analysis of enzymatic reactions (AE).

Graph 1 - Starch Standard Curve – Transmittance (A) and Absorbance (B) in λ 620 nm.



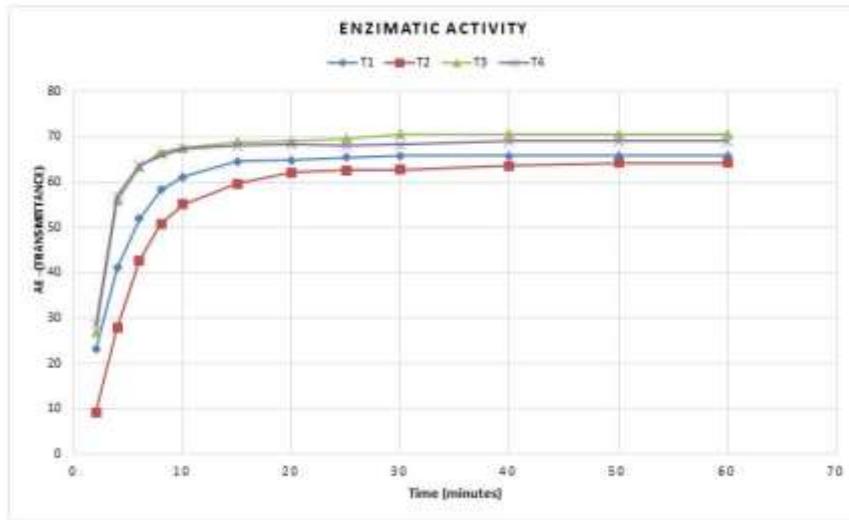
Source: Authors (2021).

The standard curve graph (Transmittance and Absorbance) serves as a linearity model with the concentration of starch present in the medium, allowing the identification of starch concentrations consumed in the enzymatic process throughout the process through mathematical calculations.

3.4 Evaluation of the Enzymatic Activity of Amylases on the Starch Substrate

The result of the Enzymatic Activity (AE) of the amylases synthesized by the microorganism *Bacillus cereus*, in the different treatments of the supernatant T1, T2, T3, T4 proved the effectiveness of the action of this microbial enzyme on the starch substrate, as shown in graphs 2, 3, 4 and 5 (Starch degradation over time), by transmittance and absorbance readings.

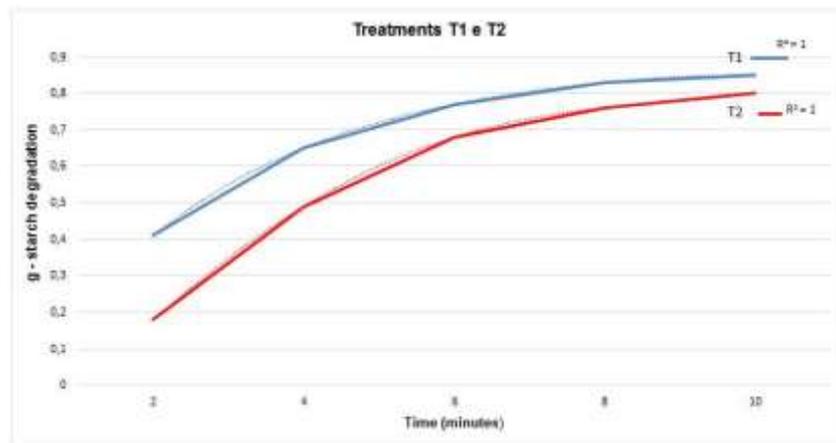
Graph 2 - Enzymatic activity (AE: amylases in starch Substrate x Time).



Source: Authors (2021).

In this Graph 2, it is possible to verify the degradation of the starch substrate as the transmittance increases over time. The curves presented in the treatments (T1 to T4) represent the characteristic behavior of a first-order enzymatic reaction.

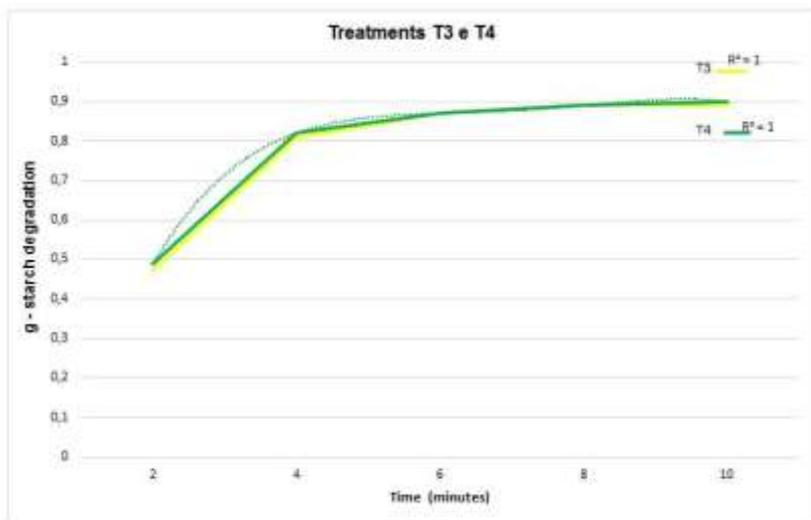
Graph 3 - Enzyme activity in starch degradation in grams x time (10 minutes): comparisons of T1 treatments (100 μ L Enzyme centrifuged); and T2 (10 μ L Enzyme Centrifuged).



Source: Authors (2021).

From the curves in Graph 3, it can be seen that the T1 treatment has a higher enzyme concentration (100 μ L of supernatant), which is why it presented a higher rate of starch degradation in the medium compared to T2.

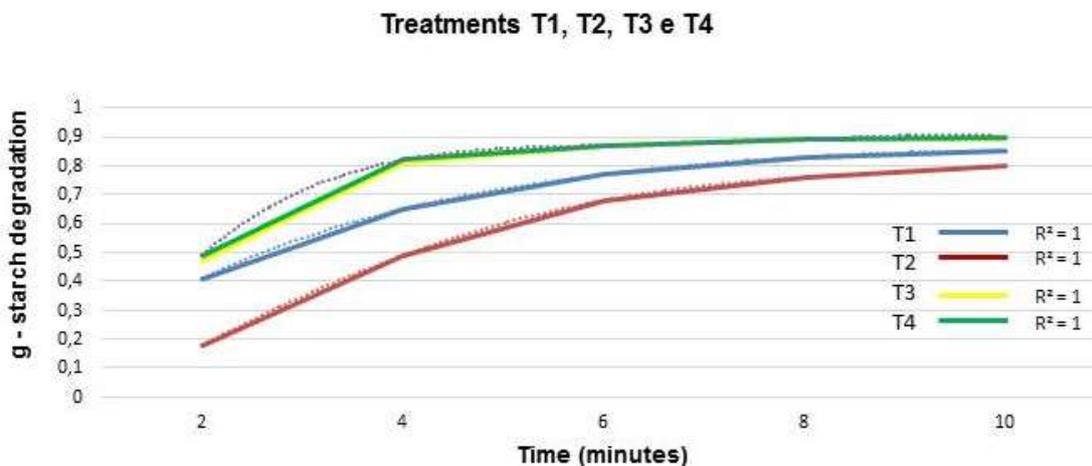
Graph 4 - Enzyme activity on starch degradation in grams x time (10 minutes), comparative of Treatments: T3 (10 μ L membrane filtered supernatant of 0.22 μ m) and T4 (100 μ L membrane filtered supernatant of 0.22 μ m).



Source: Authors (2021).

The curves of the enzymatic reaction in the starch substrate in treatments 3 and 4 allow us to infer that the filtration of the supernatant in millipore membrane ensures the activity of the amylase enzyme in a more uniform way, since the difference between the concentrations of the treatments did not interfere in the kinetics of the reaction.

Graph 5 - Enzymatic activity on starch degradation in grams x time (10 minutes), comparison of Treatments T1, T2, T3 and T4.



Source: Authors (2021).

In the graph above, it can be clearly observed that the treatments (T3, T4), which were subjected to the process of centrifugation and filtration in millipore membrane, had higher enzymatic activity than the treatments (T1, T2) which were subjected only to the centrifugation process of the supernatant.

4. Discussion

Microscopy analyzes of the microorganism indicated that it was of the *Bacillus cereus* species. The ratio of the diameters of amylolysis halos by the diameters of the colonies of this microorganism indicated a high production capacity of

amylolytic enzymes, resulting in an Amylolytic Index (AI) of 3.3. By analyzing Graph 5, which shows the Enzyme Activity curves for the four treatments, it can be seen that the maximum Enzyme reaction speed, in general, occurred around the first 10 minutes for all treatments. However, Treatments 3 and 4 already showed a deflection of the curve in the first 4 minutes and the maximum speed after 6 minutes. Regarding the initial enzymatic activities, between the treatments "enzymatic solutions of the centrifuged supernatants and of the 0.22 µm millipore membrane filtrates" of *Bacillus cereus* growth media, they proved that the filtration in 0.22 µm millipore membrane (curves of the T3 and T4 treatments, present in the graph 4) it was more efficient to separate the enzymes from other residues present in the supernatant, which appeared to interfere slightly in the initial process of the reaction. Thus, between Treatments T1 and T2 (supernatants only submitted to centrifugation), there was a delay in the initial reaction time of treatment T2 in relation to T1, since treatment T2 contained a 10 times smaller aliquot of centrifuged supernatant (enzyme solution); which did not occur between treatments T3 and T4 (supernatants submitted to filtration in 0.22 µm millipore membrane), even though the T4 treatment received an aliquot 10 times greater than the T3 treatment. Thus, the importance of enzyme purification treatments is highlighted, since the filtration process of *Bacillus cereus* culture supernatants on 0.22 µm millipore membranes (T3 and T4) was more efficient in terms of amylase activity when compared to the centrifugation process. Among all the treatments evaluated, the treatment "T3" stood out, which showed greater efficiency in the starch hydrolysis process compared to the other treatments, since its enzymatic activity had higher initial reaction speed, with an aliquot of 10µL of solution enzymatic, and promoted the greatest degradation of the starch substrate around 92% within 30 minutes of the hydrolysis process.

5. Conclusions

The tests developed were very fruitful, it can be proven that the microorganism "*Bacillus cereus*" produced amylase enzymes with high potential for amylolytic action, which may be suitable for industrial processes in the various areas of interest to humanity, requiring the optimization of growth parameters microbial and amylase production, such as: culture media, pH, temperature, rpm, time, growth factors, amylase inducers, as well as the use of down-stream technologies to purify biosynthesized enzymes to scale-up processes. In this sense, it is concluded that biotechnology is a science of theoretical and practical excellence, viable for the prospecting of microorganisms with faculties in generating bioproducts, both for scientific uses and for industrial purposes; it is also inferred that the bacterium *Bacillus cereus* is a microorganism with biotechnological potential for the production of other substances of high importance in the field of animal and human health, such as antimicrobials and probiotics, in addition to the amylase enzyme, proven in this work.

In future works, we will aim at standardizing and optimizing the bioprocess, such as: temperature; pH; Rpm; use of growth media with the addition of ions and inducers to improve the microorganism's adaptation and accelerate the growth phase, as well as to direct gene expression to the synthesis of amylases; evaluation of enzymatic activity by varying the substrate concentration; purification of the supernatant with different filter elements.

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