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Production, Pre-Purification and Encapsulation of Proteolytic Enzyme Secreted by Filamentous Fungus

Produção, Pré-Purificação e Encapsulamento de Enzima Proteolítica Secretada por Fungo Filamentoso

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Abstract
Encapsulation of enzymes allows the preservation of their biological activities in various environmental conditions, and is important for industrial application. Thus, the objective of this work was to produce, partially purify and encapsulate the microbial protease enzyme obtained by semisolid fermentation. The enzyme extract was produced from the fermentation of substrate with different physicochemical compositions by the fermentation agent the filamentous fungus *Aspergillus oryzae*. An experimental mixture design was used to map out the experimental region and determine the optimal ratios between substrates. The enzyme extract obtained from the optimized formulation was subjected to partial purification in ammonium sulfate, dialysis, and then lyophilized. With the freeze-dried enzyme the enzymatic determination, milk coagulation, thermogravimetric analysis, and differential thermal analysis were performed. The encapsulation process was carried out using the ionic gelation technique in the presence of the polymer sodium alginate and calcium chloride dihydrate. X-ray diffractometry (XRD) identified that the studied babassu mesocarp, wheat bran and babassu coconut fiber samples showed amorphous structural features, presence of cellulose and lignin. The protease obtained in the formulation of the optimized medium, precipitated and freeze-dried, presented milk coagulation capacity, proteolytic activity of 261.5 U/g and through thermal analysis it was verified that the material has thermal stability around 40 °C. It was possible to encapsulate the enzyme extract and preserve the enzymatic activity and the

coagulation capacity of the milk. The results showed the optimization of the medium formulation, as well as the potential for industrial application of the purified and encapsulated enzyme, preserving the activity and enabling the application and reuse of the enzyme.
Keywords: Semisolid fermentation. *Aspergillus oryzae*. Mixture planning. Encapsulation.

Resumo
O encapsulamento de enzimas permite a preservação de suas atividades biológicas em diversas condições ambientais, tendo importância na aplicação industrial. Dessa forma, o objetivo deste trabalho foi produzir, purificar parcialmente e encapsular a enzima microbiana protease obtida por fermentação semissólida. O extrato enzimático foi produzido a partir da fermentação de substrato com diferentes composições físico-químicas através do agente de fermentação o fungo filamentoso *Aspergillus oryzae*. Um planejamento experimental de mistura foi utilizado para determinar a região experimental e as proporções ideais entre os substratos. O extrato enzimático obtido pela formulação otimizada foi submetido ao processo de purificação parcial em sulfato de amônio, diálise e na sequência liofilizado. Com a enzima liofilizada realizou-se a determinação enzimática, coagulação do leite, análise termogravimétrica e análise térmica diferencial. O processo de encapsulamento foi realizado através da técnica de gelificação iônica na presença do polímero alginato de sódio e cloreto de cálcio dihidratado. A difratometria por raios-X (DRX) identificou que as amostras mesocarpo do babaçu, farelo de trigo e fibra do coco babaçu estudadas apresentaram características estruturais amorfas, presença de celulose e lignina. A protease obtida na formulação do meio otimizado, precipitada e liofilizada apresentou capacidade de coagulação do leite, atividade proteolítica de 261,5 U/g e através das análises térmicas foi verificado que o material possui estabilidade térmica em torno de 40 °C. Foi possível encapsular o extrato enzimático e preservar a atividade enzimática e com capacidade de coagulação do leite. Os resultados evidenciaram a otimização da formulação do meio, como também o potencial de aplicação industrial da enzima purificada e encapsulada preservando a atividade e possibilitando a aplicação e reutilização da enzima.
Palavras-chave: Fermentação semissólida. *Aspergillus oryzae*. Planejamento de mistura. Encapsulamento.

1. Introduction

Enzymes are biological proteins and macromolecules that catalyze specific substrates into products, exhibiting different properties (YUEN, *et al.*, 2019; DEMIRKAN, *et al.*, 2017).

There are enzymes of microbial, animal and vegetal origin, in which, enzymes of microbial origin are the most chosen in the industry, due to their stability, economic viability, ease of modification, product optimization, high yield, consistency, fast production with accessible culture media and high catalytic performance. Microbial enzymes are gaining enormous importance due to their efficiency, compatibility and stability in industrial processes (AQUARONE, *et al.*, 2001; ALMEIDA, *et al.*, 2011). Among the enzymes of microbial origin, there is the protease enzyme, which has application in several industrial sectors such as: detergents, food (cheese making, baking, beer clarification), pharmaceutical (hair removal), animal feed production and waste management (LIMA, 2016; DEMIRKAN, *et al.*, 2017; KOBLITZ, 2019).

In the food industry, the vegetable proteases, specifically papain, used in meat tenderizing, hydrolyze meat proteins, presenting high affinity for actin and good activity on collagen, contributing to the softness of the food and also used in the process of beer clarification, hydrolyzing the peptides and preventing them from solubilizing and avoiding turbidity, contributing to the process of clarification and formation of foams. The animal proteases, renin and chymotrypsin, used in the coagulation of milk, act in the ability to form excellent clotting and texture. The microbial proteases, excreted by the fungus *Aspergillus oryzae,* used in the ripening of cheese, act to preserve the nutritional value, contributing to changes in aroma, flavor and texture. Fungal enzymes are used in baking, where they act to modify the protein network by breaking peptide bonds, contributing to the hydrolysis of soft products (AQUARONE, *et al.*, 2001; KOBLITZ, 2019).

The use of enzyme production in industry requires efficiency and low cost, and agroindustrial residues are excellent alternatives because they have a low market price, substrates of great potential in the production of commercial enzymes (MUKHTAR, *et al.*, 2013; HARANGOZÓ, *et al.*, 2015). In addition, fungal enzymes are highly desirable for solid-base fermentation because they penetrate hard substrates and accelerate the hydrolysis process (YUEN, *et al.*, 2019).

The semi-solid fermentation is defined and characterized by the growth of microorganisms on a solid matrix, presenting a water activity value that allows the growth and metabolism of the microorganism, but that, at the same time, does not exceed the maximum water retention capacity of the solid matrix (PALMA, 2003). Semisolid fermentation is traditionally used to obtain enzymes of fungal origin because of its lower cost in energy demand, equipment and reduced wastewater treatment, as the interest of the industry for enzyme-producing microorganisms, which have a wide applicability, is increasing (VINIEGRA-GONZÁLEZ, 1997; PEREIRA, 2014). The use of cheap agro-industrial wastes as substrates in semi-solid fermentation aids in the fermentation of enzyme production, since the mixture of these substrates makes viable microbial growth and provides a better surface area and accessibility of nutrition to the microorganism (RIGO, *et al.*, 2021).

The babassu is a native plant of Brazil, where, only in the Northeast region there is an area of about 14 million hectares with babassu, most of it (54.2%) concentrated in the state of Maranhão (LORENZI, 2004; BATISTA *et al.*, 2006). Its fruit is composed of the epicarp, which is the extreme layer of the babassu fruit. It has a fibrous structure, corresponding to 12% of the fruit and is reddish-yellow in color. The mesocarp is the layer below the epicarp, corresponds to 23% of the fruit, has a floury appearance and is rich in starch. Endocarp protects the kernels, it corresponds to 58 % of the fruit. The central part of the fruit is composed of seeds (almonds) from which the vegetable oil is extracted, 7% of the fruit (LORENZI, 2004; VIEIRA, *et al.*, 2011; SILVA, *et al.*, 2019). Babassu becomes an excellent substrate because of its range of properties.

Enzyme activity can also be diminished or inhibited by small molecules (impurities) present in the process. In industrial processes, the use of enzymes under adverse and extreme conditions can alter their stability and decrease their useful life, due to factors such as changes in temperature, pH or ionic forces, and can lose their full catalytic capacity (YUEN, *et al.*, 2019; BARROSO, *et al.*, 2021).

In order to minimize expenses and offer benefits both in research and industrial processes, enzyme encapsulation plays an important role and advantages, such as: increase the number of enzyme molecules per unit area, increase enzyme reuse, avoid enzyme contamination in the final product, improve enzyme activity, and avoid loss of activity (DEMIRKAN, *et al.*, 2017).

There are several enzyme encapsulation and immobilization techniques, which are divided according to their particle formation mechanism into physical, chemical and the physico-chemical (BARROSO *et al.*, 2021). In this work the physicochemical method was used, which includes the ionic gelation technique that is accessible and low cost.

The technique is based on the ability to crosslink natural polymers including alginate, chitosan, pectin and cellulose, which have charges when exposed to monovalent, divalent or trivalent ions. These biopolymers are easy to manipulate, have desirable properties and are stable at pHs above 3.5 and are of great interest due to their biocompatibility, biodegradability, non-toxicity and have chemical stability (RIBEIRO, 2014; DEMIRKAN, *et al.*, 2017).

Thus, the objective of this work was to produce, purify and encapsulate the microbial protease enzyme obtained by semisolid fermentation using regional substrate.

2. Methodology

This research work was developed at the advanced campus of the Federal University of Maranhão, in the city of Imperatriz, Maranhão, in the Microbiology and Cereal Technology laboratories of the Food Engineering course.

The substrates used were: babassu coconut mesocarp (BM) obtained from the Association of Coconut Breakers of the city of Cidelândia, MA/BR, the babassu husk fiber (BF) was provided by

Tobasa Bioindustrial de Babaçu located in the city of Tocantinópolis, TO/BR and the wheat bran (FT) was purchased at the local commerce in the city of Imperatriz, MA/BR.
The main equipment used in this research were: Laminar flow with air recirculation - Pachane, Vertical Autoclave CS - Prismatec, BOD (Biochemical Oxygen Demand) Incubator - SOLAB SL - 117, Refrigerated Centrifuge - Daiki, Analytical Balance - Marte, UV/Visible Spectrophotometer with scanning - GEHAKA UV 340G, Freeze Dryer LS3000 TERRONI.

2.1 Fermentation agent

The microorganism used in the fermentations was the filamentous fungus *Aspergillus oryzae CCBP 001*, belonging to the collection of Embrapa Agroindústria Tropical and kindly provided to the Federal University of Maranhão. The spores of the fungus were activated in test tubes with tilted potato dextrose agar medium and incubated at 30°C for 7 days in a BOD incubator (SOLAB, SL - 117), this process was repeated by transferring spores from the tilted agar to a new tube with tilted potato dextrose agar and incubated at 30°C for 7 days in a BOD incubator. In the third activation step the spores from the slant agar were inoculated into 10 g wheat bran medium moistened with 5 mL of 1.7 % NaH_2PO_4 and 2 % sterile $(NH_4)_2SO_4$ solution at 1 atm at 121 °C in 125 mL Erlenmeyer flask and incubated for 5 days at 30 °C in a BOD incubator (SOLAB, SL - 117). To loosen the spores from the wheat bran particles, 50 mL of 0.3 % (v/v) Tween solution was used and stirred with a glass rod, then the suspension was filtered through sterile gauze and the inoculum size was determined using the spore count in a Neubauer chamber.

2.2 X-Ray Diffraction (XRD)

The X-ray diffraction measurements were performed in the LDRX laboratory at UFMA - Imperatriz, Advanced Campus.

XRD is a technique used to identify the crystallinity of compounds, whether organic or inorganic, thus the technique is one of the important factors to determine the structural properties of lignocellulosic materials, present in this research.

The BM, FT and BF samples were subjected to XRD analysis with the aid of a PANanalytical Empyrean diffractometer, using angular scanning (2θ) from 5° to 50°, with an angular step of 0.02° and a time per step of 2 s.

2.3 Semisolid Fermentation Medium and Mixing Plan

A study of the formulation of the fermentation medium was carried out through the experimental design of Simplex-Centroid mixture with Interior Points, aiming to understand the proportions of substrate in the formulation to obtain higher proteolytic activity.

In the Simplex-Centroid Mixture Experimental Design the substrates were used in proportions to compose the mixtures containing 10 g in each trial, in a total of 10 experimental trials, as shown in Table 1, correlating the planning with the growth of *Aspergillus oryzae* in 96 h fermentation. The inoculum was 10^7 spores of *Aspergillus oryzae* per gram of medium, incubated for 96 h at 30 °C in a BOD (SOLAB, SL - 117). For all assays, enzyme extractions were obtained by adding distilled water and incubating for 1 h at 30 °C and filtering to separate the supernatant. The supernatant was stored at -6 °C for proteolytic enzyme determination and other tests performed in this study.

Table 1 - 3-factor experimental design: simplex-centroid + interior points, babaçu mesocarp (BM), wheat bran (FT) and babaçu coconut fiber (BF).

Experimental Points	(BM)	(FT)	(BF)
1	100 %	0 %	0 %
2	0 %	100 %	0 %
3	0 %	0 %	100 %
4	50 %	50 %	0 %
5	50 %	0 %	50 %
6	0 %	50 %	50 %
7	33 %	33 %	33 %
8	66 %	17 %	17 %
9	17 %	66 %	17 %
10	17 %	17 %	66 %

The results obtained in the experimental planning were statistically treated by analysis of variance (ANOVA) with 90 % confidence using STATISTIC software version 7.0.

2.4 Proteolytic Activity Determination

To determine proteolytic activity the method of FREITAS et. al., (2015) was used, the enzyme extract was diluted in 1 mL of 0.5% azocasein solution in 50 mM acetate buffer, pH 5 to the tubes, followed by incubation in a water bath at 37 °C, for 40 min. The reaction was stopped with trichloroacetic acid, centrifuged for 15 min at 3000 rpm, and the supernatant reacted with KOH (5%), followed by reading in the UV/Visible spectrophotometer with scanning. The enzyme activity determined was considered a unit of proteolytic activity (U) defined as the amount of enzyme that produces a 0.01 difference in absorbance between the blank and the sample per minute of reaction.

2.5 Partial purification of proteolytic extract

A fermentation to obtain larger volumes of enzyme extract was carried out using the medium formulation with the best substrate ratio for obtaining the enzyme in the mixture plan. After obtaining the enzyme extract, it was submitted to precipitation in ammonium sulfate solution at 60% saturation concentration (Bracht and Ishii-Iwamoto, 2003), in the ammonia dissolution, an ice bath was used to keep the solution at a low temperature.

Then the precipitated protein was dialyzed on cellulose membrane for desalting, subsequently the protein fraction was freeze-dried in a TERRONI LS3000 Freeze-dryer under vacuum conditions at -40 °C. After concentration of the extract, protease activity, milk coagulation and thermal analyses were performed.

2.6 Coagulation of milk by proteolytic extract

The freeze-dried enzyme extract was dissolved at the proportion of 1mg/20 mL in 10 mM calcium chloride solution (pH = 8.22), the coagulation time was measured using the mixture of 1 mL of the enzyme dissolved in 10 mM calcium chloride with 1 mL of the Piracanjuba skim milk solution dissolved in 10 mM calcium chloride (12 %), and incubated at 37 °C in the water bath until the milk coagulates.

2.7 Characterization by Thermal Analysis

The thermogravimetric analysis (TGA) and differential thermal analysis (DTA) measurements were performed at the Thermal Analysis Laboratory (LAT) of the UFMA - Imperatriz, Advanced Campus. TGA and DTA measurements of the freeze-dried enzyme extract were performed simultaneously on a Shimadzu DTG-60 thermogravimetric analyzer, in an open α-alumina crucible, under inert nitrogen atmosphere (50 mL/min), over a range from 25 to 700 °C, with a heating rate of 10 °C/min.

2.8 Encapsulation

To encapsulate the freeze-dried enzyme extract, the following reagents were used: Sodium alginate - (Sigma-Aldrich), calcium chloride PA, Dihydrate (Prochemicals) and deionized water.

A solution of 1 % sodium alginate and 1 % calcium chloride was prepared, and these solutions were left under magnetic stirring for 5 h. The freeze-dried extract was solubilized in deionized water under 3 h of magnetic stirring. 20 mL of the 1% sodium alginate solution was added to the enzymatic extract solution (Figure 1a), after 1 h under magnetic stirring, the mixture (enzymatic extract and 1% sodium alginate) was dripped into the 1% calcium chloride solution (Figure 1b). The resulting solution containing the spherical enzyme particles was allowed to stand at room temperature for 1 h. After the microspheres were filtered, the diameter of 10 spheres was measured (sample mean).

Milk coagulation test and determination of proteolytic activity were performed with the encapsulated enzymes.

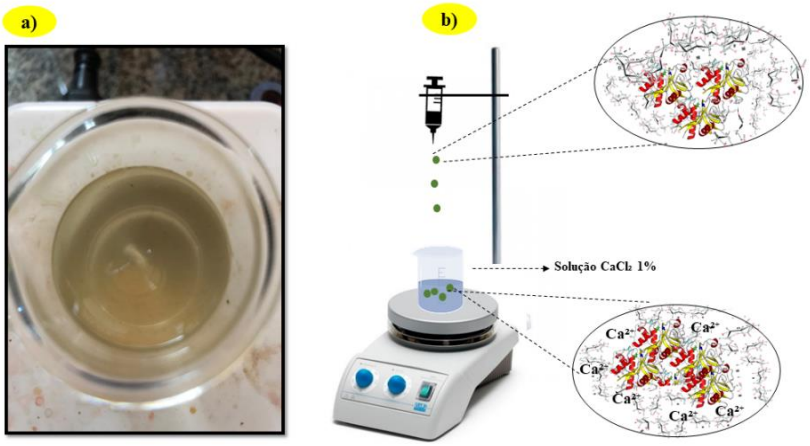

Figure 1 - Procedure for encapsulation of enzyme extract, a) mixture of enzyme extract with 20 mL sodium alginate 1%; b) representation of encapsulation method.

In the determination of the proteolytic activity and coagulation of the milk of the encapsulates, the same procedure of items 2.3 and 2.5 was used, modifying only the exchange of the diluted extract for the number of capsules.

3. Results and Discussion

3.1 X-Ray Diffraction

Figure 2 presents the diffractograms obtained for the substrates (babassu mesocarp (BM), wheat bran (FT) and babassu coconut fiber (BF)) in the angular range from 5 to 50°, 2θ. As observed, in the diffractogram of the BM sample, it reveals three partially amorphous halos between 2θ = 13° and 25°, which is more intense when compared to the diffractogram of FT and BF. The halos centered at 2θ = 15° and 17° of the BM sample, are characteristic of the presence of lignin and the

amorphous halo at 2θ = 23°, can be attributed to cellulose. In the diffractogram of the FT sample, we observe an amorphous halo between 2θ = 15° to 25°, in its less intense form that is characteristic of the presence of cellulose. While in the diffractogram of the BF sample, we analyzed two amorphous halos, the first centered at 2θ = 16° characteristic of the presence of lignin and at 2θ = 22° attributed to the presence of cellulose. Furthermore, the absence of crystalline peaks in the XRD pattern indicates that the material has an amorphous structure, and with this, we can indicate the breakdown of these lignocellulosic materials during semisolid fermentation process.

According to the literature, the broadened peaks centered around 2θ = 15°-18° are characteristic of the presence of lignin and those located around 2θ = 20 - 25° can be attributed to the cellulose contained in the residue, these compounds being characteristic of lignocellulosic materials (ANDRADE-MAHECHA, 2012; SILVA, 2021).

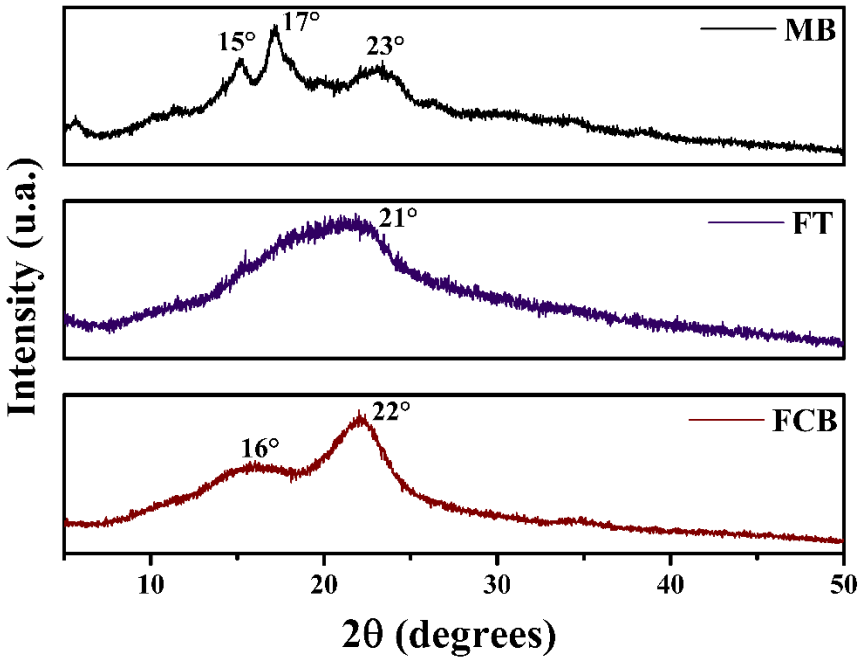

Figure 2 - X-ray diffraction pattern of the BM, FT and BF samples.

3.2 Substrate Composition and Proteolytic Activity

In Figure 3, we can observe the growth of the fungus *Aspergillus oryzae* CCBP 001 at 30 °C after 96 h of fermentation in Erlenmeyer flasks with 35 % humidity for different substrate formulations with the data of protease activity productions, as observed in Table 2.

The highest protease activity occurred in the medium with 100 % wheat bran (51.625 U/g), the second highest production was 41.75 U/g when mixing 50 % wheat bran and 50 % babassu coconut fiber (experimental point 6), as observed in Table 2. The third best formulation for obtaining protease activity was the experimental point 9 with 66 % wheat bran 17 % babassu coconut fiber and 17 % babassu mesocarp where 39.25 U/g was obtained, and the fourth best production with 33.75 U/g presenting equal proportions of substrates.

The four best results that showed considerable proteolytic activity were due to the conditions of temperature, humidity, aeration, and substrate in the medium. According to the literature, the substrates used in the formulation of the medium present in their structure's cellulose, hemicellulose, lignin, starch, proteins and fibers that make these solid substrates serve as carbon and energy sources for the development of *Aspergillus oryzae*, thus confirming that fungal enzymes are highly desirable for solid-base fermentation because they penetrate hard substrates and accelerate the hydrolysis process (SILVA et. al., 2018). Aiming at cost and benefit, it was indicated to optimize the substrate containing the babaçu coconut fiber.

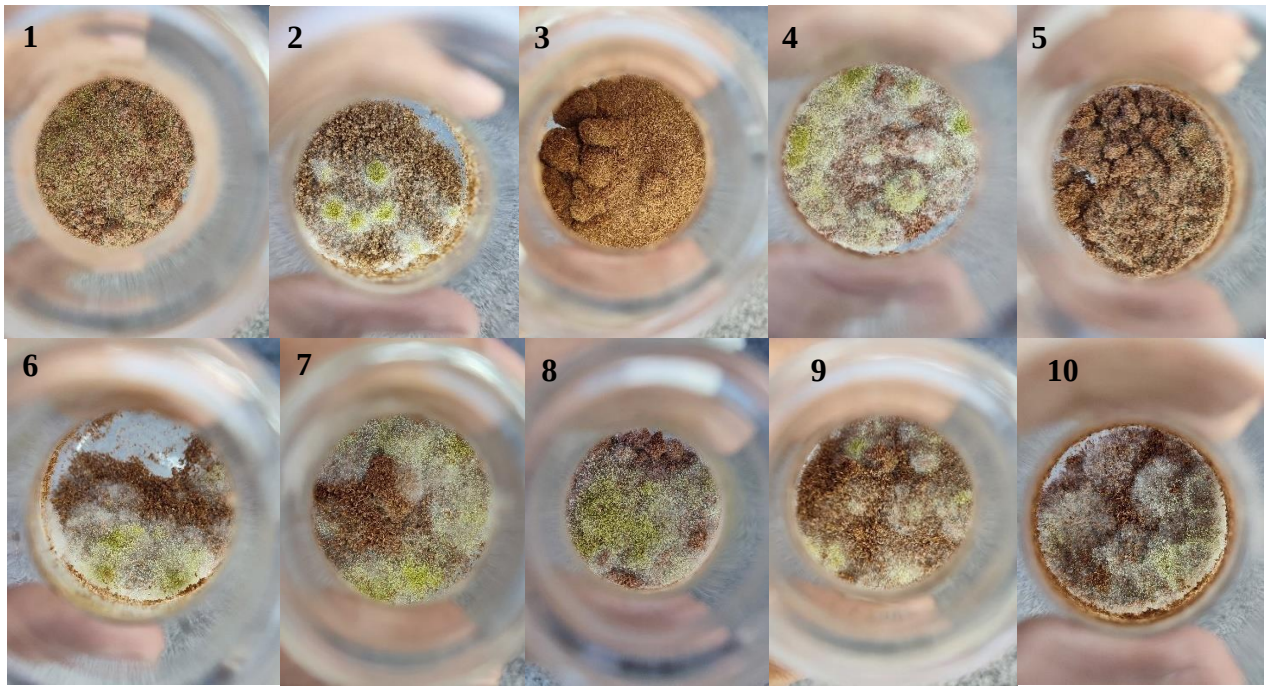

Figure 3 - Growth of *Aspergillus oryzae* in 96 h fermentation with 10 mixture trials.

Table 2 - Experimental design with amounts in grams of the actual independent variables related to the production of proteolytic activity.

Experimental Points	*Mass of Mesocarp of Mesocarp (g)*	*Mass of Wheat Bran (g)*	*Mass of Coconut Fiber babaçu (g)*	*protease activity (U/g)*
1	10	-	-	0,75
2	-	10	-	51,63
3	-	-	10	6,13
4	5	5	-	30,00
5	5	-	5	2,88
6	-	5	5	41,75
7	3,33	3,33	3,33	33,75
8	6,67	1,67	1,67	16,13
9	1,67	6,67	1,67	39,25
10	1,67	1,67	6,67	8,00

3.3 Statistical Analysis of Experimental Design

For the statistical analysis of the experimental planning, initially the results were adjusted to the linear model, with 10 terms, in which it was observed that only the regression coefficient that considers linearity in the formulation with wheat bran showed significant evidence, for 90% confidence. Table 3 presents the results of the model coefficients and their respective confidence intervals. The analysis of variance (ANOVA) indicated a percentage of variance of 0.8966 and a calculated $F_{Calculated}$ of 30.4.

Table 3 - Analysis of variance for protease activity in the mixture design.

Source of variation	*Sum squared*	*Degrees of freedom (v)*	*Quadratic Mean*	$F_{Calculated}$	F_{Tabled}	*p-value*
Regression	2740,755	2	1370,378	30,4	3,26	0,000355
Waste	315,926	7	45,132			
Total	3056,681	9	339,631			
% explained variance (R^2)			0,8966	R^2_{fitted}	0,8671	

After obtaining the proteolytic activity of the mixture assay, we observe in Figure 4a, the ternary linear model contour curve of the mixture experimental planning for the protease activity as a function of substrates proportions, being visualized in Figure 4b, in which, as the proportions of wheat bran increases it is possible to obtain higher proteolytic activity on the red surface, where, the mixture of by-products were babassu mesocarp (BM), wheat bran (FT) and babassu coconut fiber (BF). It is possible to analyze that the planning of substrate mixture for the fermentative medium is satisfactory for the choice of the medium in which protease production results in higher values of proteolytic activity, where, the first highest protease activity was 51.625 U/g and second with production of 41.75 U/g.

And with that, the use of substrate mixtures presents a better energy balance and a lower environmental impact than pure substrates.

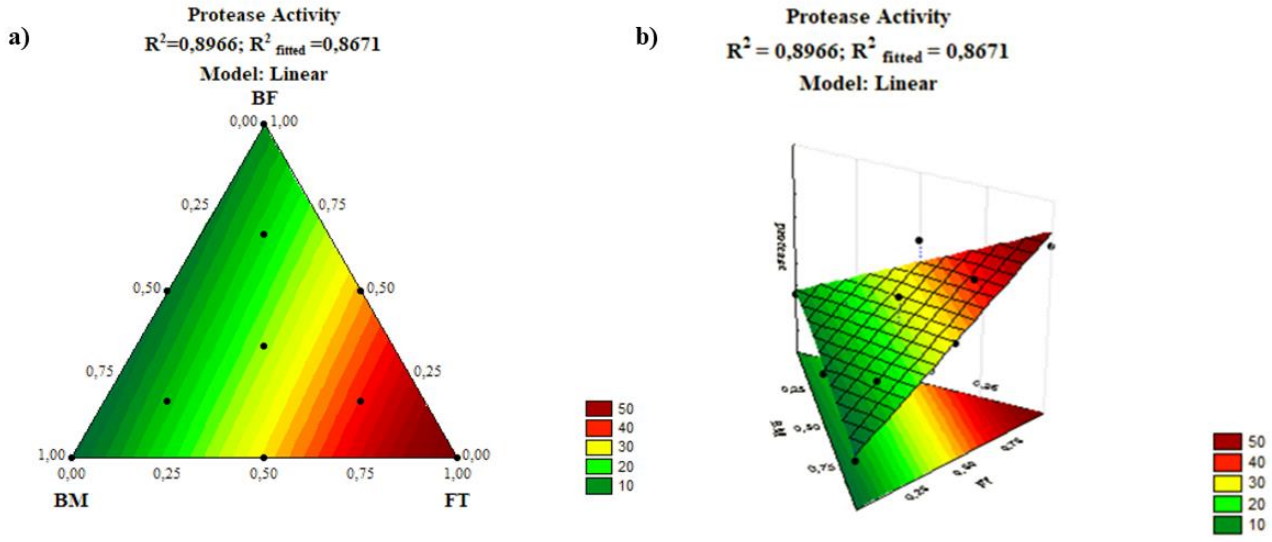

Figure 4 - Mixture planning plot (a) Contour surface; (b) Ternary response surface aiming to obtain higher proteolytic activity.

Semisolid fermentation presents advantageous characteristics when using substrates with high energy sources and low water activity.

The babassu mesocarp is a rich source of starch (68.30%), containing vitamins and minerals (FRANÇA et. al., 2014). The ternary formulation of babassu mesocarp, wheat bran and babassu coconut fiber presents a substrate with great potential for proteolytic enzyme production, where the composition of starch, cellulose and fiber are decisive factors in the semi-solid fermentative process. Wheat contains about 70% carbohydrates and the other 30% are proteins, vitamins and minerals. Wheat bran, on the other hand, has an average of 31 % cellulose, 26 % hemicellulose, 24 % lignin, and 7 % ash, and is a substrate considered as a source of nutrients for enzyme production (BAKKER,

2017; SANTOS *et al.*, 2008). Thus, the present study, using only wheat bran, showed a higher amount of proteolytic activity.

Besides being rich in proteins, fatty acids and carbohydrates, wheat bran also has physical characteristics such as texture and porosity that can facilitate the dispersion of fungi, contributing to higher enzyme production when compared to the other fermentative in question (PANDEY, 2013). Babassu coconut fiber has water absorbing, fibrous, sandy and lignocellulosic properties (SILVA et. al., 2018), together with the compositions of binary mixture of wheat bran with babassu coconut fiber, this substrate paper an important role in enzyme production.

In Figure 5, analyzing the raw residuals of the results obtained with the mixture planning we can observe that the substrates have a straight-line segment in the linear fit, justifying by the proximities of the points being significant the amounts of substrates used in this work.

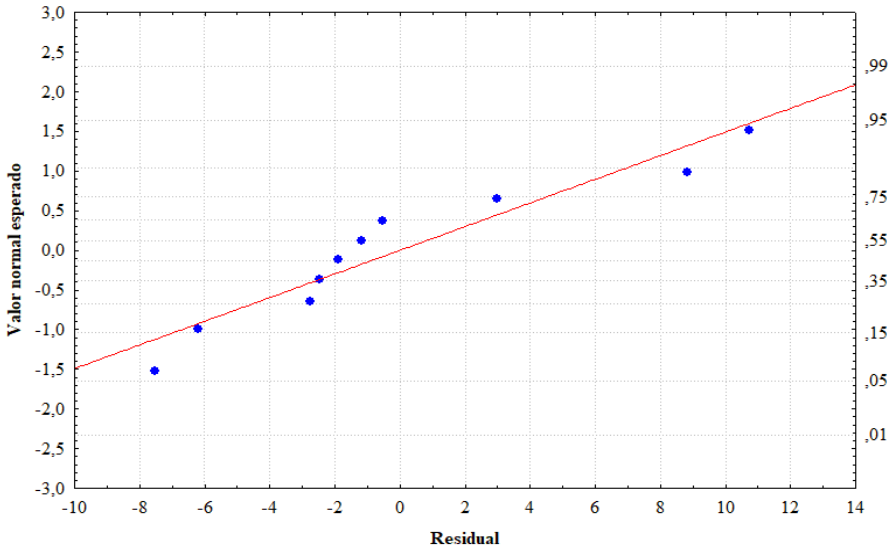

Figure 5 - Analyses of raw residuals from mixture planning in the linear model.

3.4 Enzyme Optimization

From the statistical analyses it was observed that the linear model presents a more adequate behavior in the analysis of variance (ANOVA), thus, the second best production was optimized to include the babassu coconut fiber which is a regional product of great value in the communities of coconut breakers in the Maranhão region, and because it is a very sandy residue that is easy to obtain, sustainable reuse and of great influence in solid state fermentative processes due to the rapid rate of oxygen diffusion in the water film around the fibrous particles.

Using the experimental point 6 (Table 2) of the second-best condition of the mixture experimental planning, obtained from the favorable conditions of substrate, moisture and enzyme activity, according to Figure 6, we observed the growth of the fungus *Aspergillus oryzae CCBP 001* at optimizations of temperature of 30 °C with 96 h of fermentation, moisture of 40 % and substrate composition of 50 % wheat bran and 50 % babassu coconut fiber. The extraction proceeded in the same way as the mixture plan, obtaining 500 mL of enzyme extract. With this composition and a favorable humidity condition for the growth of *Aspergillus oryzae*, the enzymatic extract was obtained with a pH of 7.92 and production of 85 U/g proteolytic activity.

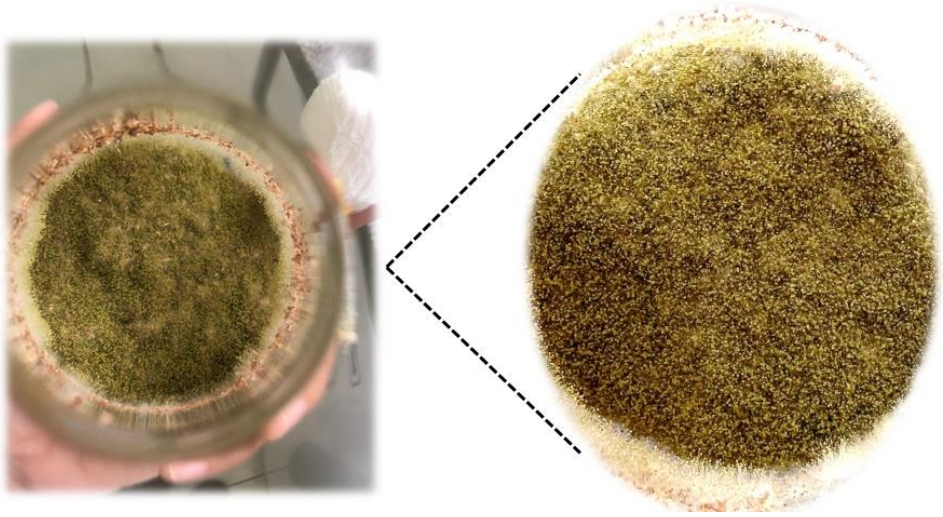

Figure 6 - Growth of *Aspergillus oryzae* in 96 h fermentation with FT and BF.

3.5 Enzyme Purification and Freeze-Drying

On solubilization of 0.017 g proteolytic extract powder in 10 mL distilled H2O, a protease production of 261.5 U/g was obtained. In comparison with the activity obtained from the extract diluted with distilled water alone, the concentration of proteolytic enzymes is much higher when the freeze-dried extract is used. The performance of protease in coagulating milk for cheese production can be evaluated by the relationship between the ability to form clots and the total hydrolysis of the milk caseins (KOBLITZ, 2019). Then, for the coagulation of skim milk, using 1 mL of the extract diluted in $CaCl_2$ at a temperature of 37 °C in a water bath, it was found that the milk coagulated in less than 5 min, confirming the concentration of the enzyme after purification and freeze-drying.

Therefore, adding proteolytic enzymes directly to milk or curd is a good alternative to speed up this process (JUSTINA, *et al.*, 2018).

3.6 Thermal Analysis

In Figure 7, we analyze the behavior of the freeze-dried enzyme extract by means of the TGA and DTA curves with increasing temperature. In which, for the TGA-DTA measurements, 2.019 mg of enzyme extract mass was used for a temperature range from 25 °C to 701 °C.

According to the behavior of the DTA curve, an endothermic event is observed between 30 °C and 117 °C, where it shows a mass loss of approximately 11.4 %, about 0.230 mg, by the TGA curve, attributed to the exit of the residual water molecules and associated with the endothermic peak of the DTA curve at 52 °C, confirming the dehydration of the enzyme extract. In the second event presented in the TGA curve, corresponding to the loss of 54 % mass, about 1.085 mg was observed in the interval from 231 °C to 486 °C, being related to the exothermic peak of the DTA curve at 364 °C, confirming the decomposition of the protein substances, vitamins and fibers of the extract and remaining only ashes, the minerals.

Thus, through thermal analysis we observed that the enzymatic extract has thermal stability until 40 °C, because from this temperature on the extract begins to dehydrate and decompose organic compounds, which are substances that can bring unpleasant tastes and odors, and thus, from the temperature increase the extract begins to lose its enzymatic activity.

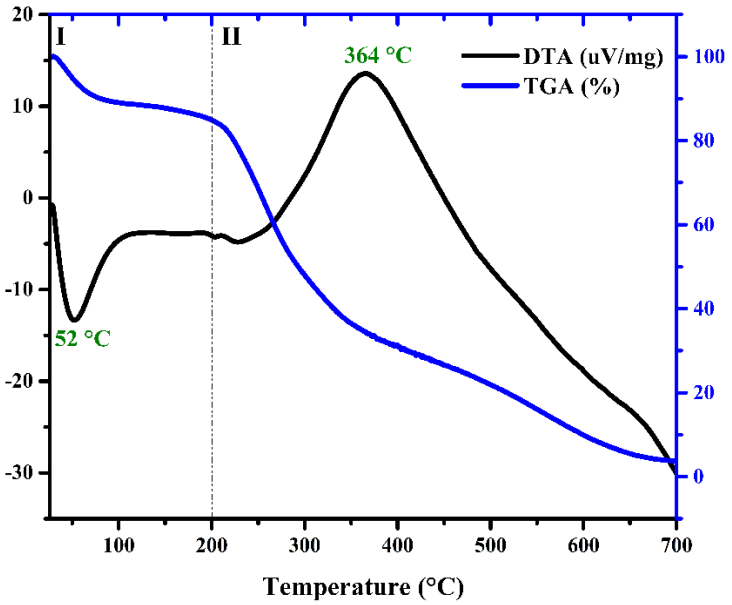

Figure 7 - TG and DTA curves of freeze-dried enzyme extract.

In studies on enzyme activity and thermal stability of β-Gatactosidase from *kluveromyces lactis* and *Aspergillus oryzae*, Bosso (2012), found a difference between the activity of enzymes extracted from yeast *K. lactis* and filamentous fungus *A.oryzae* in lactase hydrolysis, where the enzyme from *K. lactis* showed good stability at 40 °C while the enzyme from *A. oryzae* acted better at 55 °C.

In the work of Magalhaes *et al.*, (2019) on the Production and characterization of proteolytic enzymes from *Lentinus crinitus* (L.) Fr. 1825 DPUA 1693 from the Amazonian biome (Polyporaceae), it was observed that the protease enzyme from *Lentinus crinitus* shows stability at temperature of 60 °C.

3.7 Enzyme Encapsulation

Using the ionic gelation technique, it was possible to observe that the method was fast and simple. For the analyses of the enzyme encapsulates 10 samples were selected, where these 10 represent the sample size of all the encapsulates obtained during the process. They had a mean diameter of 0.1965 cm, standard deviation of 0.0298 and a coefficient of variation of 0.1521. Figure 8 shows a better visualization of the capsules obtained under the microscope.

The sodium alginate and calcium chloride used for encapsulating the enzymes contributed to proteolytic activity, since metal ions have been described in the literature to increase and stabilize enzyme activity, where the ions may be involved in catalytic processes, participating in redox or electron transfer reactions (MAGALHÃES, *et al.*, 2019).

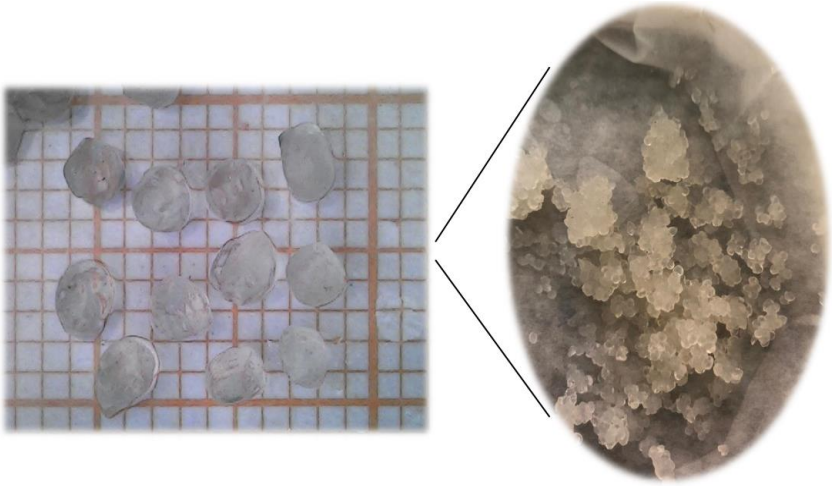

Figure 8 - Enzyme encapsulation.

Once the enzyme encapsulates were obtained, protease activity tests and milk coagulation tests were performed. Using 20 samples for the proteolytic activity test and 25 samples for the milk coagulation. Confirmed the achievement of proteolytic activity by the formation of a pink-colored compound. The test served to analyze the efficiency of the enzyme obtained, in which the encapsulated enzyme released enzymes and protease degradation occurred, therefore, the encapsulated enzyme can be easily separated from the reaction solution by centrifugation, determining proteolysis. And the 25 samples added in 1 mL of milk solubilized with 10 mM $CaCl_2$ coagulated the milk in 3 h and 25 min, where we observe in Figure 9. Despite the extended coagulation time, the encapsulated enzyme has a good application in the ripening of artisan cheeses.

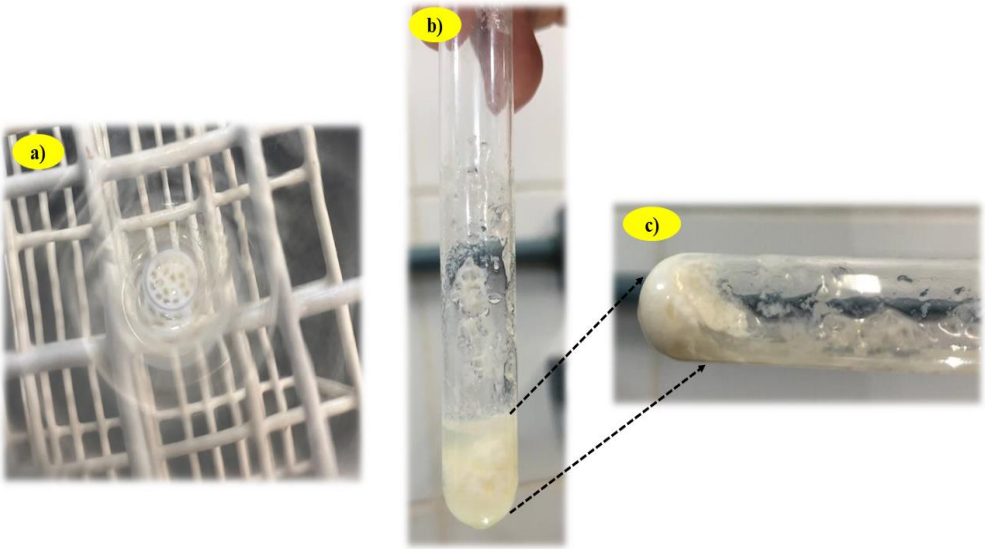

Figure 9 - Coagulation of milk with enzyme encapsulates from 3 h and 25 min at 37 °C in water bath.

However, according to the literature and experimental studies, the direct addition of the enzyme extract may have disadvantages, such as a large loss of enzyme in the whey, poor enzyme distribution in the curd, formation of undesirable taste and texture due to the direct attack on the substrate by the enzymes. And one way to get around this effect is the use of encapsulated enzymes, where they are physically separated from the substrate, which prevents direct attack in the early stages of maturation, and are released to the environment in a controlled manner during the maturation time (JUSTINA, *et al.*, 2018; GULER-AKIN *et al.*, 2012).

Thus, the application of encapsulated proteolytic enzymes is a highly efficient and cost-effective technique and is progressively replacing conventional approaches at laboratory scale and

industrial levels for matured products and in the production of artisanal cheeses, which use enzymes with temperature stability up to 40 °C.

4. Conclusion

In this work it was possible to obtain proteolytic activity of the filamentous fungus *Aspergillus oryzae CCBP 001* by semisolid fermentation, using substrates babassu mesocarp, wheat bran and babassu coconut fiber through mixture planning. It was found that through the mixture planning the substrate with wheat bran was the best substrate for enzyme production and the composition of 50% wheat bran and 50% babassu coconut fiber obtained the second-best enzyme production. Formulations containing wheat bran in higher proportion also obtained expressive results regarding protease activity, being: wheat bran combined with babassu coconut fiber and wheat bran combined with babassu coconut fiber and babassu coconut mesocarp. Therefore, the statistical analysis of mixture planning was of utmost importance in the optimization of *Aspergillus oryzae* enzymatic obtaining.

X-ray diffractometry (XRD) identified that the substrate samples studied presented partially amorphous characteristics with the presence of amorphous cellulose and lignin halos.

In the optimization to obtain the second-best result of proteolytic activity of *Aspergillus oryzae CCBP 001*, it was possible to obtain an enzyme extract with 85 U/g of enzymatic activity in conditions of 40% humidity at 30 °C. The drying of the proteolytic enzyme extract made it possible to determine the activity in larger quantities, enzyme concentration, determination of thermal stability, encapsulation of the extract and higher yield in milk coagulation.

The purified and lyophilized enzyme had a protease yield of 261.5 U/g, where we confirmed that the enzyme has higher concentration in the solid state without water molecules in the medium. Purification of the enzyme by precipitation with ammonium sulfate showed sufficient activity, coagulating the milk in less than 5 min.

Through TGA and DTA analyses, it was observed that the freeze-dried enzyme extract presents thermal stability up to 40 °C, suffering then dehydration, decomposition of organic compounds at higher temperatures.

The encapsulated enzymatic extract showed stability and proteolytic activity. The encapsulated enzymatic extract coagulated the milk in a longer time, but in an efficient and gradual way.

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