



Article

Optimized Production of Fungal Polygalacturonase Using Cupuaçu (*Theobroma grandiflorum*) Peel as Substrate and Its Effect on Clarification of Cupuaçu Juice

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Abstract: Pectinolytic enzymes play a key role in many beverages manufacturing processes, improving their clarification and filtration steps. Fungal pectinases are considered promising green catalysts for industrial applications, and they can be produced using fruit-processing residues as substrate. In this study, we investigated the optimal conditions to produce polygalacturonase from *Aspergillus brasiliensis* in a solid-phase bioprocess, using cupuaçu (*Theobroma grandiflorum*) peel as substrate. Then, the pectinolytic extract was applied in the clarification of cupuaçu juice. A central composite design was used to determine the optimal fungal cultivation conditions. Thus, the optimal fungal cultivation (maximum production of 11.81 U/g of polygalacturonase) was obtained using cupuaçu peel with 80% moisture, at 34 °C, for 7 days in a medium containing 4.2% phosphorus and 2.6% nitrogen. The enzymatic extract showed greater activity at 60 °C and stability at a pH range between 5.0 and 7.0. The pectinolytic extract was able to clarify the cupuaçu juice, causing a 53.95% reduction in its turbidity and maintaining its antioxidant activity. Our results demonstrate that the cupuaçu peel can be used as a substrate to produce polygalacturonase, and the enzymatic extract produced can be applied in the cupuaçu juice processing, contributing to the circular economy.

Keywords: fungal pectinase; agro-industrial residues; cupuaçu juice; antioxidant activity; clarification



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1. Introduction

Pectinases are a group of enzymes responsible for the degradation of pectic substances from cell walls and have several industrial applications [1]. Pectic substances are heteropolysaccharides found in the cell wall of plant tissues, primarily composed of α -D-galacturonic acid, along with smaller amounts of rhamnose, arabinose, galactose, and other monosaccharides [2,3].

The amount of pectin and its composition vary according to the type of tissue and plant species [4], causing turbidity and high viscosity in fruit juices, which affect the market

value of these products [5]. High concentrations of pectin also lead to the formation of colloids, which constitute one of the main problems during the processing of clarification of fruit juices. Therefore, the removal of pectic substances is crucial for improving the appearance, texture, and flavor of the final product, especially beverages [5,6].

The removal of pectic substances can be carried out through filtration or centrifugation, resulting in clarified juice, although smaller suspended particles remain [5]. Different clarifying agents such as bentonite, potassium caseinate, gelatin, polyvinylpolypyrrolidone (PVPP), and silica gel can also be employed; however, these compounds may lead to the loss of bioactive properties of the product, such as antioxidant activity [7,8], but also could modify the color and aroma of fruit juices [9]. Enzymatic clarification of fruit juices using pectinases, on the other hand, has proven to be an efficient alternative for reducing turbidity [10–14]. Moreover, pectinases can be combined with other hydrolytic enzymes [14,15], which have been used in the clarification of juices commonly consumed worldwide, such as apple, grape, and orange juices [16].

Cupuaçu (*Theobroma grandiflorum*) is an economically significant Amazonian fruit, characterized by its acidic pulp, which is consumed both in its natural form and in a variety of processed products such as creams and desserts, including a chocolate-like product known as ‘cupulate’. Cupuaçu pulp contains organic acids, phenolic and volatile compounds, as well as amino acids, such as asparagine and glutamine, and a lipid composition that includes palmitic, oleic, and linoleic acids. In addition to its presence in the food industry, cupuaçu is also commercially utilized in the cosmetic sector for the production of body oils and shampoos [17]. According to data published by the IDAM (Institute for Sustainable Agricultural and Forest Development of Amazonas State), in the state of Amazonas, cupuaçu cultivation is traditionally carried out by family farming, involving approximately 5.2 thousand families across an estimated area of 6006 hectares, with a total volume of around 10.6 million fruits [18].

Cupuaçu juice is thick and viscous, with high concentrations of colloidal material and solid particles, which poses challenges for its industrial processing [19]. According to Cerón et al. [20], the processing of cupuaçu pulp generates approximately 65 kg of waste for every 100 kg of fruit in Brazil. Furthermore, the peel of the cupuaçu constitutes about 42% of the fruit’s total weight, highlighting the need for innovative approaches to the utilization of this by-product, both from an economic and environmental perspective [21]. The main aroma components of cupuaçu are ethyl butanoate, linalool, and ethyl hexanoate, whose sensory descriptors are related to fruit, citrus, and floral aromas. Due to its substantial amounts of polyphenols, cupuaçu is also considered a functional food that can help prevent inflammatory processes and provide antioxidant compounds, besides its high amount of polysaccharides, justifying the clarification treatment [17,19].

Besides being used in juice clarification, pectinolytic enzymes are also employed in wine production, in the degumming of cellulose fibers, in the recovery of essential oils, in the release of bioactive compounds, in the extraction of vegetable oils, in wastewater treatment, and in the textile industry, among many other applications [1,6,8]. Therefore, the global pectinase market is estimated to grow from US\$ 353.1 million in 2023 to US\$ 455.8 million in 2030 [22]. In addition, with a 25% share in the food and beverage enzyme market, pectinases represent a significant part of the biotechnology industry [23].

Industrial pectinases are produced by microorganisms, which are an attractive source of these biocatalysts due to their biodiversity, rapid growth, and susceptibility to genetic manipulation [15]. Enzymatic production can occur through submerged bioprocessing or solid-state fermentation. In solid-state fermentation, microbial growth occurs on a substrate with sufficient moisture to support microbial growth and metabolism [24]. The most used microorganisms in this type of bioprocess are filamentous fungi due to their

growth characteristics, which favor the use of agro-industrial waste as substrates, thus reducing production costs [25–27]. Fungal pectinases produced by solid-state bioprocessing have been extensively reported [4,11,28–30]. However, producing fungal enzymes on solid substrates requires optimizing parameters that directly affect the production of these molecules, such as pH, substrate moisture, inoculum, nutrients, temperature, and cultivation time [23].

In this sense, this study aimed to optimize the production of an enzymatic extract rich in polygalacturonase from the cultivation of the fungus *Aspergillus brasiliensis* using cupuaçu peel as a solid substrate. Additionally, the suitable conditions for clarifying cupuaçu juice using the enzymatic extract were determined, evaluating the reduction in turbidity as well as the maintenance of antioxidant activity. The accomplishment of these objectives could significantly value the cupuaçu peel—a by-product of juice production—by using them to produce polygalacturonase, an important adjuvant that enhances the juice's clarification, ultimately benefiting producers. In this way, to the best of our knowledge, this is the first study where the optimization of the production of an enzyme extracts rich in polygalacturonase from cupuaçu peel was studied, and at the same time, it was used to clarify the juice of this tropical fruit.

2. Materials and Methods

2.1. Material

The cupuaçu (*Theobroma grandiflorum*) fruit was sourced from the town of Manacapuru, Amazonas, Brazil, and procured at a local market in the city of Manaus. The fruits underwent a selection process, where those exhibiting abnormalities were excluded. After thoroughly washing the fruits with tap water, the shells were broken using a kitchen hammer.

2.2. Solid Residue

The peels were manually separated from the pulp, dried at 45 °C for 7 days in an air-circulating oven (TE-394/2-MP, Tecnal, Piracicaba, Brazil), and ground using a four-blade mill (MA 340, Marconi, Piracicaba, Brazil) equipped with a sieve with a 1.70 mm opening. The composition of cupuaçu peel was previously determined [21], showing 58.58% fiber, 0.49% lipid, 4.61% protein, and 2% ash content. After drying, the solid residue exhibited a moisture content of 6.5%.

2.3. Microorganism

The fungus *Aspergillus brasiliensis* ATCC 16404 was maintained on potato-dextrose-agar (PDA). For reactivation, the fungus was transferred to a plate containing PDA and incubated for 7 days at 28 °C. From the reactivated culture, a conidial suspension was produced, standardized to 1×10^7 spores/mL in a Neubauer chamber.

2.4. Pectinase Production

The enzyme production methodology was based on Falcão et al. [21]. The fungus was cultivated in 125 mL Erlenmeyer flasks containing 7 g of the dry, ground solid waste, supplemented with a solution containing NH_4NO_3 (nitrogen source) and KH_2PO_4 (phosphorus source), with enough distilled water to achieve 80% moisture. The medium was autoclaved (121 °C for 15 min) and inoculated with 50 μL of the suspension at 10^7 conidia/mL. The flasks were incubated in a B.O.D. chamber (Tecnal, TE-39I, Piracicaba, Brazil) at 34 °C.

The addition of nutrients (N and P) and cultivation time were evaluated using a central composite design (CCD) (Table 1). These three variables were identified in a previous study by our research group [31] as the most relevant to producing polygalacturonase by

A. brasiliensis on cupuaçu peel. The variables and their levels used in the CCD are presented in Table 1.

Table 1. Variables and factor levels used in the central composite design for studying polygalacturonase production using *Aspergillus brasiliensis* on cupuaçu peel.

Factor	Range and Levels				
	Axial (−1.68)	Lower (−1)	Central (0)	Higher (+1)	Axial (+1.68)
Phosphorous (P, %)	3.0	3.5	4.5	5.5	6.0
Nitrogen (N, %)	1.5	2.0	3.0	4.0	4.5
Cultivation time (t, days)	4	5	7	9	10

P = phosphorous supplementation (KH_2PO_4); N = nitrogen supplementation (NH_4NO_3).

Thus, with the results from the CCD and the obtained response surfaces, the optimal cultivation conditions for pectinase production by *A. brasiliensis* using cupuaçu waste were determined. After defining the optimal conditions, they were applied in a larger volume culture, where the cultivation parameters were proportionally scaled up to produce sufficient enzyme extract for clarification assays.

2.5. Enzymatic Extraction

The enzymatic extraction was performed according to the methodology described previously by Falcão et al. [21]. Thus, after the cultivation time established by the CCD, 20 mL of ultrapure water was added to the flasks, which were then subjected to an ultrasonic bath (USC-5000, Unique, Indaiatuba, Brazil) for 2 min, at 25 °C. Subsequently, the flasks were shaken at 150 rpm for 10 min, with no heating. The separation of the solid medium was carried out by vacuum filtration using a Büchner funnel. The enzymatic extracts were stored at −20 °C and used in further assays.

2.6. Enzyme Activity Assay

The activity of the polygalacturonase extract was determined based on the methodology described by Phutela et al. [32]. Initially, a solution containing polygalacturonic acid in 50 mM sodium acetate buffer, pH 5.0, was prepared. The assays consisted of mixing 125 µL of enzymatic extract and 125 µL of polygalacturonic acid. The solution was incubated in a thermo-shaker (K80-100, Kasvi, São Paulo, Brazil) for 40 min at 37 °C. After the reaction time, 250 µL of DNS was added, and the mixture was incubated again for 5 min at 100 °C. After cooling, 500 µL of distilled water was added, and absorbance readings were taken at 540 nm using a UV-Vis spectrophotometer (Shimadzu, UV-1800, Kyoto, Japan) [33]. The assays were performed in triplicate.

The activity of polygalacturonase was determined by quantifying the reducing sugars released during the reaction, using a standard curve of D-galacturonic acid ($y = 1.0704x - 0.1488$; $R^2 = 0.9948$). One unit of enzymatic activity (U) was defined as the amount of enzyme required to produce 1.0 µmol of reducing sugars per mL of enzymatic extract. The results are presented in U per gram of dry substrate.

2.7. Characterization of the Enzymatic Extract

The enzymatic extract was subjected to a pectinolytic activity assay, as described previously in Section 2.5, varying the reaction temperature between 30 °C and 70 °C. The pH was evaluated between 4.0 and 7.0, using acetate buffer for pH adjustment. The experiments were performed in triplicate. The characterization methodology was based on Ahmed et al. [13] with adaptations.

2.8. Enzymatic Clarification of Cupuaçu Juice

The enzymatic clarification of cupuaçu juice was conducted according to the methodology described by Barman et al. [10] used for the clarification of banana juice. The cupuaçu juice was prepared with the pulp, which was commercially obtained in Manaus, Amazonas State, Brazil. The pulp was weighed and diluted in distilled water at a ratio of 100 g of pulp to 200 mL of water, following the manufacturer's instructions (Frutifica Inc., Manacapuru, Brazil). The juice obtained was then filtered through cotton gauze, 9 threads/cm² (Ahmed S/A, Curvelo, Brazil), and incubated with the enzymatic extract at different concentrations (*v/v*). The enzymatic extract exhibited a polygalacturonase activity of 4.13 U/mL. The reaction was conducted in a total volume of 10 mL and maintained under magnetic stirring (752A, Fisatom, São Paulo, Brazil) at 120 rpm. The concentration of the enzymatic extract, clarification time, and temperature of the clarification process were evaluated through a factorial experimental design (2³) to determine the best process conditions. The studied variables and their levels are presented in Table 2.

Table 2. Variables and factor levels used in the 2³ factorial design for studying polygalacturonase production using *Aspergillus brasiliensis* on cupuaçu peel.

Factor	Range and Levels	
	Lower (−1)	Higher (+1)
Temperature (T, °C)	30	50
Time (t, min)	60	180
Enzyme loading (El, %, <i>v/v</i>)	5.0	10.0

After incubating the juice with the enzymatic extract, the absorbance was measured at 400 nm using a UV-Vis spectrophotometer (Shimadzu, UV-1800, Kyoto, Japan). The turbidity reduction percentage was then calculated based on the absorbance of the untreated (unclarified) juice. A control experiment, without the addition of the enzymatic extract, was also conducted, along with an experiment using the commercial enzyme Pectinex[®] LLX (Novozymes Latin America Ltd., São Paulo, Brazil). The enzyme activity of the commercial enzyme was measured using the method described in Section 2.6 and subsequently diluted to match the enzymatic activity of the fungal extract.

2.9. Antioxidant Activity of the Clarified Juices

The antioxidant activity of the enzymatically clarified juices was determined for the clarified juices after the experimental design trials. The DPPH free radical scavenging technique was employed, based on the methodology of Brand-Williams et al. [34]. The DPPH solution was prepared at 0.06 mmol/L in pure methanol. The assay was conducted using microplate spectrophotometry at 517 nm (LMR-96, Loccus, Cotia, Brazil). Ascorbic acid and quercetin were used as standards. The percentage of DPPH radical scavenging was obtained using the absorbance decay values of the sample compared to the control sample [35]. The experiments were performed in triplicate.

2.10. Statistical Analysis

Data from the CCD, used for optimizing polygalacturonase production, and from the factorial design, used for juice clarification and antioxidant trials, were analyzed using the Statistica software version 10.0 (TIBCO Software Inc., Palo Alto, CA, USA) with a significance level of $p < 0.05$. To examine differences in cupuaçu juice clarification and antioxidant activity results, a one-way analysis of variance (ANOVA) was also conducted using the same software and version. Tukey's test ($p < 0.05$) was used to identify significant

differences between treatments. ANOVA and Tukey's test ($p < 0.05$) were also applied to the results of the characterization of the enzymatic extract.

3. Results

3.1. Optimization of Pectinase Production

The results of enzymatic activity in each of the trials can be seen in Table 3. Thus, by analyzing the results in the table, it was clear that the highest values of polygalacturonase activity (EA = 10.32 U/g \pm 0.97) were obtained in the assays conducted at the central point of the DCCR (P = 4.5%; N = 3.0%; t = 7 days).

Table 3. Experimental design matrix and experimental results for the central composite design for polygalacturonase production using *Aspergillus brasiliensis* on cupuaçu peel.

Run	Design Matrix			Results	
	P (%)	N (%)	t (Days)	EA (U/g)	Predicted EA (U/g)
1	3.5 (−1)	2.0 (−1)	5 (−1)	1.32	3.16
2	5.5 (1)	2.0 (−1)	5 (−1)	0.00	1.62
3	3.5 (−1)	4.0 (1)	5 (−1)	6.49	4.14
4	5.5 (1)	4.0 (1)	5 (−1)	0.00	−0.06
5	3.5 (−1)	2.0 (−1)	9 (1)	8.56	8.36
6	5.5 (1)	2.0 (−1)	9 (1)	5.74	6.72
7	3.5 (−1)	4.0 (1)	9 (1)	7.24	6.44
8	5.5 (1)	4.0 (1)	9 (1)	4.42	2.24
9	3.0 (−1.68)	3.0 (0)	7 (0)	6.44	7.21
10	6.0 (1.68)	3.0 (0)	7 (0)	2.59	2.31
11	4.5 (0)	1.5 (−1.68)	7 (0)	10.44	7.77
12	4.5 (0)	4.5 (1.68)	7 (0)	1.60	4.74
13	4.5 (0)	3.0 (0)	4 (−1.68)	0.00	−0.75
14	4.5 (0)	3.0 (0)	10 (1.68)	4.23	5.47
15 (C)	4.5 (0)	3.0 (0)	7 (0)	9.93	10.32
16 (C)	4.5 (0)	3.0 (0)	7 (0)	11.10	10.32
17 (C)	4.5 (0)	3.0 (0)	7 (0)	10.35	10.32
18 (C)	4.5 (0)	3.0 (0)	7 (0)	9.60	10.32
19 (C)	4.5 (0)	3.0 (0)	7 (0)	11.81	10.32
20 (C)	4.5 (0)	3.0 (0)	7 (0)	9.22	10.32

(C) = central point; EA = enzymatic activity; P = phosphorous supplementation (KH_2PO_4); N = nitrogen supplementation (NH_4NO_3); t = cultivation time. Predicted EA = enzymatic activity calculated from the mathematical model.

The predicted values of enzymatic activity were obtained with Equation (1), with the coefficients defined by an analysis of variance (ANOVA) from the statistically significant variables (Tukey test, $p < 0.05$). The coefficient of determination obtained (R^2) was 0.86, and it was found that the predicted values were similar to those obtained experimentally.

$$\text{EA} = 10.32 - 1.46 \times \text{P} - 1.97 \times \text{P}^2 - 0.90 \times \text{N} - 1.44 \times \text{N}^2 + 1.85 \times \text{t} - 2.82 \times \text{t}^2 - 0.64 \times \text{P} \times \text{N} + 0.27 \times \text{P} \times \text{t} - 0.97 \times \text{N} \times \text{t} \quad (1)$$

Through the response surface graph, representing the interaction between the clarification time (t) and phosphorous source (P) (Figure 1a), between t and N (nitrogen source) (Figure 1b), and between P and N (Figure 1c), it is observed that the central ranges studied represent the optimal condition to produce fungal polygalacturonase. The 7-day cultivation time, as well as intermediate values of N and P, proved to be more favorable for polygalacturonase production by *A. brasiliensis* on cupuaçu peel.

By the statistical analysis, the results demonstrated that the critical values for polygalacturonase production were the following: P = −0.28; N = −0.37; t = 0.37, corresponding to 4.2% of the phosphorus source (KH_2PO_4), 2.6% of the nitrogen source (NH_3NO_4), and

7 days of cultivation time on the cupuaçu peel. These optimal values of the studied variables in the CCD were used for cultivation in larger volumes, aiming to obtain pectinolytic extract for application in fruit juice clarification.

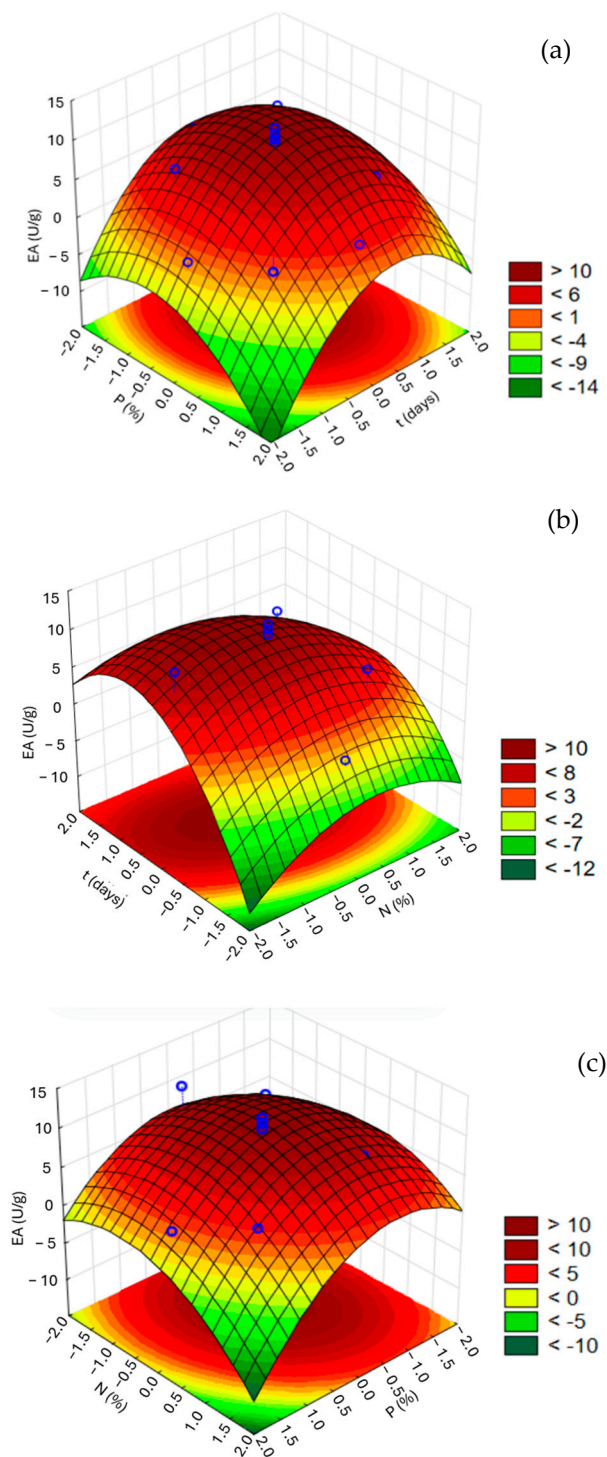


Figure 1. Response surface plots generated by the central composite design indicating the optimized variables: (a) cultivation time (t) and phosphorous source (P); (b) cultivation time (t) and nitrogen source (N); (c) phosphorous source (P) and nitrogen source (N) for polygalacturonase activity (EA) using *Aspergillus brasiliensis* on cupuaçu peel.

3.2. Characterization of the Pectinolytic Extract

The data obtained for the enzymatic activity of the pectinolytic extract produced by *A. brasiliensis* for the several trials and for each temperature and pH value are shown

in Figure 2. The optimal temperature for the enzymatic activity of the produced fungal polygalacturonase was 60 °C (Figure 2a). At this temperature, the fungal polygalacturonase extract showed the highest residual enzymatic activity. On the other hand, at temperatures from 30 to 50 °C, the extract showed values around 50% of its initial enzymatic activity, and at 70 °C, 60% of the pectinolytic activity was observed. These results are interesting from an industrial and biotechnological point of view, as they indicate a thermostable enzyme.

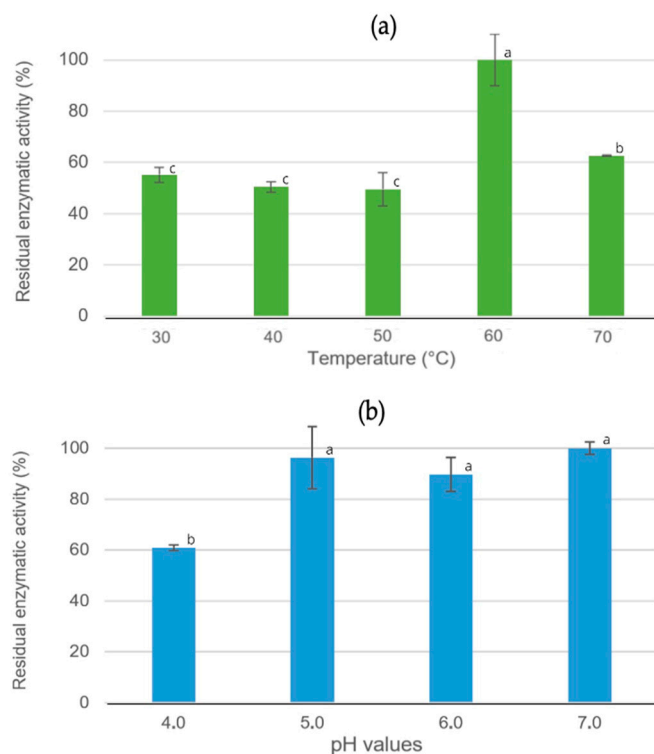


Figure 2. Enzymatic activity of the pectinolytic extract produced by *Aspergillus brasiliensis* on cupuaçu peel at different temperatures (a) and at different pH values (b). The values that share the same letter do not present a statistically significant difference between them, according to the Tukey test ($p < 0.05$).

In the assays determining the optimal pH value for the pectinolytic activity of the crude extract, it was observed that pH 7.0 provided the highest polygalacturonase activity; however, there was no statistical difference between pHs 5.0 and 6.0 ($p < 0.05$). Thus, this result demonstrated that the enzyme has a high enzymatic activity between a considerable pH range (pH 5.0 to 7.0) (Figure 2b). In addition, for acidic conditions (pH 4.0), a significant reduction in enzymatic activity was detected (only an average residual enzymatic activity of 60%).

After defining the optimal conditions to be used in the enzymatic reaction ($T = 60$ °C and pH 7.0), the enzymatic activity of polygalacturonase of 16.19 U/g was obtained (57% increase).

3.3. Enzymatic Clarification of Cupuaçu Juice

The results obtained for the enzymatic clarification of cupuaçu juice, and for the antioxidant activity of the clarified juice, are shown in Table 4. The absorbance at 400 nm of the juice was reduced (based on the absorbance of the untreated juice) in all enzymatic clarification trials that composed the factorial design, achieving approximately a 53.9% reduction in absorbance in trial 8, which showed no significant difference compared to trials 1, 4, and 5 ($p < 0.05$). On the other hand, using the commercial enzyme (Pectinex® LLX), a similar enzymatic clarification was obtained. In that case, a reduction of 59.2% in

absorbance of cupuaçu juice was achieved ($T = 50\text{ }^{\circ}\text{C}$; $t = 60\text{ min}$; $\text{El} = 10.0\%$, v/v). It can thus be observed that the fungal enzymatic extract showed a clarifying action similar to that obtained with the commercial pectinase used.

Table 4. Matrix of 2^3 factorial design for cupuaçu juice clarification, using the enzymatic extract rich in polygalacturonase produced by *Aspergillus brasiliensis* on cupuaçu peel and the antioxidant activity of the clarified juice.

Run	Design Matrix			Results	
	T ($^{\circ}\text{C}$)	t (min)	El (%)	AR (%)	AA (%)
1	30 (−1)	60 (−1)	5.0 (−1)	50.9 ± 2.7 a	32.6 ± 1.5 c
2	50 (1)	60 (−1)	5.0 (−1)	29.9 ± 9.6 b	38.7 ± 0.7 b
3	30 (−1)	180 (1)	5.0 (−1)	44.7 ± 2.5 a,b	52.6 ± 10.4 a
4	50 (1)	180 (1)	5.0 (−1)	52.6 ± 1.2 a	36.9 ± 3.4 b
5	30 (−1)	60 (−1)	10.0 (1)	49.7 ± 1.36 a	31.2 ± 5.3 c
6	50 (1)	60 (−1)	10.0 (1)	38.1 ± 6.7 a,b	39.6 ± 1.8 b
7	30 (−1)	180 (1)	10.0 (1)	42.8 ± 3.5 a,b	40.0 ± 0.6 b
8	50 (1)	180 (1)	10.0 (1)	53.9 ± 1.9 a	21.3 ± 5.6 d
CE	50 (1)	60 (−1)	10.0 (1)	59.2 ± 2.7 a	51.2 ± 3.5 a

T = temperature of the clarification process; t = time of the clarification process; El = enzyme loading (v/v). AR = absorbance reduction at 400 nm (based on the absorbance of the untreated juice); AA = antioxidant activity. CE = commercial enzyme (Pectinex[®] LLX). The values that share the same letter do not present a statistically significant difference between them, according to the Tukey test ($p < 0.05$).

In the Pareto diagram (Figure 3a), it can be observed that the interaction between the clarification time and temperature, as well as time alone, showed statistical significance ($p < 0.05$), both with a positive effect on reducing the absorbance at 400 nm of the cupuaçu juice. Figure 3b shows the response surface graph with the interaction between the incubation time and clarification temperature and where two regions of greater absorbance reduction are observed, i.e., low temperatures and low incubation times, and with higher values of both variables T (the temperature of the clarification) and t (the time of the clarification). Considering that the polygalacturonase activity of the enzymatic extract produced by *A. brasiliensis* is maximal at $60\text{ }^{\circ}\text{C}$, conducting the clarification process at this temperature would be ideal. However, the Pareto diagram shows that temperature has a negative effect on enzymatic clarification (measured by absorbance reduction at 400 nm), although it did not show a statistically significant effect ($p < 0.05$).

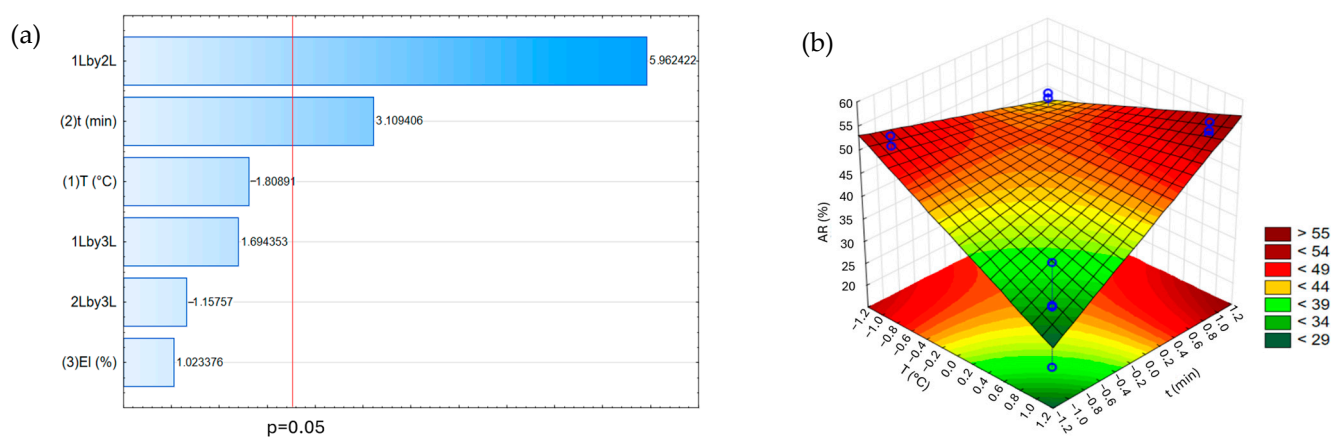


Figure 3. (a) Pareto chart for the effects of independent variables on the absorbance reduction of cupuaçu juice. (b) Response surface for the interaction of the independent variables on cupuaçu juice clarification, using the pectinolytic extract produced by *Aspergillus brasiliensis* on cupuaçu peel. T = temperature of the clarification process; t = time of the clarification process; El = enzyme loading; AR = absorbance reduction at 400 nm (based on the absorbance of the untreated juice).

The enzymatically clarified juice showed antioxidant activity ranging from 21.3 to 52.6% (Table 4). The non-clarified juice showed an antioxidant activity of $53.5\% \pm 3.5$, without a statistical difference ($p < 0.05$) when compared to trial 3 and to the commercial enzyme used. These results indicate that the use of the fungal pectinolytic extract under the conditions of trial 3 (temperature of $30\text{ }^{\circ}\text{C}$; time 180 min) was able to maintain the antioxidant capacity of the cupuaçu juice. In addition, the Pareto diagram in Figure 4 shows the variables that had a significant effect on the antioxidant activity of the enzymatically clarified cupuaçu juice.

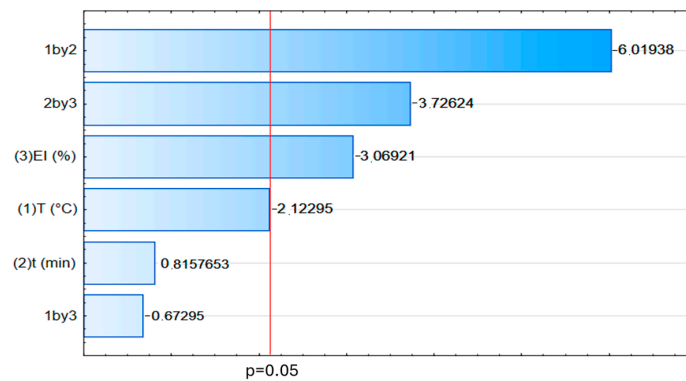


Figure 4. Pareto chart for the effects of independent variables on the antioxidant activity of the clarified cupuaçu juice; 1 = temperature of the clarification process (T); 2 = time of the clarification process (t); and 3 = enzyme loading (El).

The enzyme load and the temperature were significant ($p < 0.05$), with a negative effect on antioxidant activity, meaning lower enzyme concentrations and lower temperatures increase the antioxidant activity of cupuaçu juice. Additionally, two interactions showed statistical significance: the clarification time and temperature and the clarification time and enzyme load used in the process, both with a negative effect. In Figure 5, the response surface graphs of the interactions between the temperature and clarification time and between enzyme loading and clarification time (Figure 5a and Figure 5b, respectively) are shown, as well as their effects on antioxidant activity. For the interaction between temperature (T) and the time of the clarification process (t), it is observed that the longer incubation time and the lower temperature used in the process induced a greater antioxidant activity in the clarified juice. For the interaction between the time of clarification (t) and enzyme loading (El), it is observed that extended clarification times and lower enzyme loads improved the antioxidant activity.

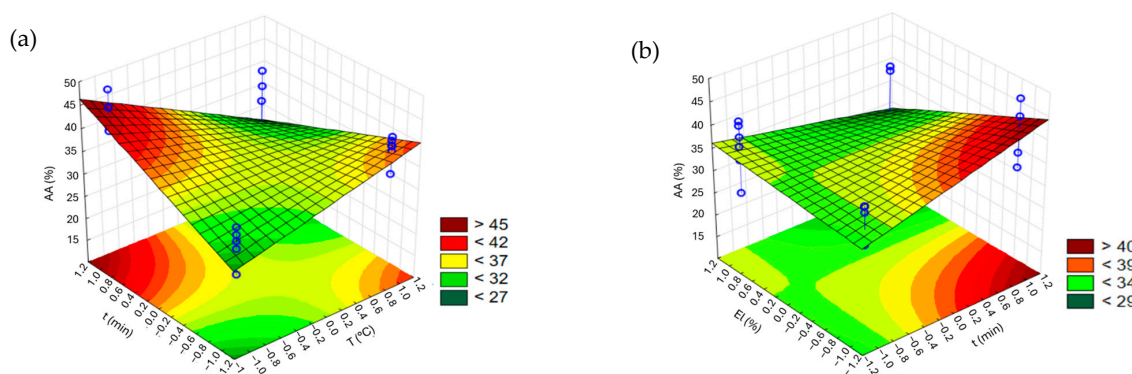


Figure 5. Response surface for the interaction of (a) time and temperature of clarification, and (b) time and enzyme loading, on the antioxidant activity of the clarified cupuaçu juice. AA = antioxidant activity determined by the DPPH method. T = temperature of the clarification process; t = time of the clarification process; El = enzyme loading.

Therefore, among the evaluated conditions, 180 min was the most favorable time for clarifying cupuaçu juice, combined with a temperature of around 30 °C and an enzyme load of 5.0%. These conditions resulted in a satisfactory clarification, while maintaining a significantly higher antioxidant activity.

4. Discussion

Fungi are diverse organisms with morphological plasticity and metabolic versatility, making them unique in enzyme production for industrial purposes [23]. Enzymes produced by fungi of the genus *Aspergillus* are generally considered safe (GRAS) and, being extracellular, can be easily recovered [36], reducing production costs. Furthermore, the use of waste as a solid substrate, simulating the natural habitat of the fungus, represents a promising strategy within the context of the circular economy by enabling the utilization of agro-industrial by-products [27,37]. In our study, we aimed to valorize the peels of a locally important Amazonian fruit for the cultivation of *A. brasiliensis*, targeting the production of polygalacturonase, an enzyme widely used in the beverage industry [3,15,38].

Solid-phase bioprocesses require the optimization of cultivation conditions for enzyme production, as the residue used may not contain all the nutrients needed for fungal growth [23,27]. The centesimal composition of cupuaçu peel was evaluated in a previous study, in which it was used for the production of fungal cellulase [21]. This residue has a high fiber concentration and low lipid and protein content. Regarding the inorganic composition of cupuaçu peel, essential elements for microbial growth were found early, such as potassium (2.96 ppm), magnesium (2.78 ppm), sulfur (2.20 ppm), phosphorus (0.40 ppm), calcium (209 ppb), iron (124 ppb), zinc (99 ppb), sodium (90 ppb), copper (72 ppb), and manganese (10 ppb). Thus, after preliminary tests [31], phosphorus and nitrogen sources, as well as the fungal cultivation time, were identified as the most important variables for pectinase production by *A. brasiliensis* using cupuaçu peel as a substrate, and therefore, in our study, we investigated the best conditions through a CCD.

Optimizing solid-phase cultivation conditions promotes increased enzyme production and/or shorter cultivation times. Ketipally et al. [29] performed a selection among various residues for use in solid-phase bioprocesses, achieving a polygalacturonase production of 4.83 U/mg in 7 days of cultivation on orange peel using *A. nornius* MR103. After optimizing the cultivation time, pH, temperature, carbon source, and nitrogen source, activity reached 5.94 U/mg. In another study, Barman et al. [10] optimized the substrate concentration, time, and temperature of *A. niger* cultivation for pectinase production on banana peel, reaching 6.6 U/mL for crude pectinolytic extract in less than 3 days of cultivation.

Thus, in our research, by the implementation of the CCD, it was possible to optimize polygalacturonase production by *A. brasiliensis* using cupuaçu peel as a substrate. The optimal cultivation time was 7 days, a result similar to that obtained previously by Akhter et al. [28] for *A. niger* cultivated on wheat bran. However, Nsude et al. [39] and Shanmugavel et al. [40] reported that only 4 days was the optimal condition for *A. tamaritii* to produce pectinase. According to Okonji et al. [41], pectinolytic fungi usually reach maximum production around 3 to 7 days of cultivation; they optimized pectinolytic activity of *A. fumigatus* using 3.0% ammonium sulfate as a nitrogen supplement for orange peel in 6 days, with optimal conditions similar to those found in our study.

Nitrogen sources play a crucial role in fungal cultivation, as they influence growth and enzyme activity [41]. Organic nitrogen sources, such as yeast extract and peptone, provide more complex compounds that require enzymatic degradation before utilization by fungi, in addition to supplying additional compounds such as vitamins and essential amino acids. In contrast, inorganic nitrogen sources, such as ammonium nitrate, urea, and ammonium sulfate, are lower in cost and directly assimilable, providing nitrogen in a more

accessible form and fostering faster and more homogeneous growth [42]. In the study by Ketipally et al. [29], peptone was essential for maximum polygalacturonase production by *A. nornius* MR103 on orange peel. Conversely, ammonium chloride was considered the best nitrogen source for pectinase production by *Cladosporium chlamydosporigenum* AUMC 11340 (26.6 U/mL) after 9 days of submerged fermentation [43]. Fawole and Odunfa [44] reported that ammonium nitrate and ammonium sulfate were good nitrogen sources for pectinase production in *A. niger*, while glycine and tryptophan were ineffective. These results follow the same trend as our study, because a concentration of 2.6% ammonium nitrate was considered ideal for enriching the cupuaçu peel used as a substrate for pectinase production by *A. brasiliensis*.

Defining the optimal conditions for enzyme activity is fundamental for the desired application, i.e., maximizing the biocatalyst's efficiency. The optimal temperature for the pectinolytic extract produced by *A. brasiliensis* on cupuaçu peel was 60 °C, which is consistent with previous studies characterizing pectinases produced by fungi of the genus *Aspergillus*. According to Moharram et al. [43], it is uncommon for filamentous fungi to produce pectinase with activity below 40 °C, even in psychrophilic or psychrotolerant fungi. And, according to Okonji et al. [41], the pectinase from *A. fumigatus* achieved a maximum activity of 6.00 U/mL at 60 °C and pH 5.0, with significant reductions in polygalacturonase activity at all other pH values tested. However, in our research, the highest polygalacturonase activity was obtained at pH 7.0, with the extract's activity remaining above 80% at pH values of 5.0 and 6.0. In the study conducted by Ahmed et al. [13] with *A. niger* cultivated on orange peel in submerged fermentation, the authors found pH 5.0 and 50 °C to be the optimal conditions for the purified enzyme. Therefore, the optimal temperature identified for the pectinolytic extract in our study aligns with expectations for pectinases from fungi of the genus *Aspergillus*.

On the other hand, the results for the optimal pH were inconsistent with those typically observed for pectinases from *Aspergillus* sp. These pectinases are usually classified as acidic pectinases [41], with higher enzymatic activities at pH values lower than those observed in the present study. Therefore, further research is needed to determine the cause of this deviation, which may be related to the cultivation medium of the enzymatic extract, as this has not been previously explored in the literature.

Most industrial fruit-based beverages go through a clarification process to prevent turbidity and sedimentation in the bottle, which at the same time, contribute to the improvement of the sensory characteristics of beverages. According to results obtained in our research, the pectinolytic extract produced by *A. brasiliensis* applied in the clarification of cupuaçu juice, reduced the absorbance by 53.4% after 180 min of contact with the enzyme at 10% and 50 °C. This result can be considered very promising for the potential use of this pectolytic extract in the clarification of fruit juices, in particular for cupuaçu juice. These results are similar to those of Ahmed and Sohail [13], who achieved 61% clarification of orange juice and also highlighted the importance of increasing the incubation time of the juice with the enzyme during the process. Patel et al. [5] corroborate these findings, since they also observed that longer times improved the enzymatic clarification process for apple and blueberry juices. However, more studies must be carried out in order to analyze the impact, namely, of the clarification temperature on the physicochemical stability and sensory characteristics of cupuaçu juice.

Although promising results were observed for the clarification of cupuaçu juice using the pectinase extract from *A. brasiliensis*, similar performance was not achieved for mango juice clarification, where only a 12.33% reduction in juice absorbance was observed after treatment with the extract [45]. Therefore, each food matrix must be thoroughly evaluated to understand the potential applications of the enzymatic extract.

Furthermore, as was observed in our study for cupuaçu juice clarification, temperature also proved to be a relevant factor in clarifying orange juice, as reported by Ahmed and Sohail, [13]. According to these authors, lower temperatures aided the clarification process. Considering that the optimal temperature obtained in our study for the enzyme produced by *A. brasiliensis* was 60 °C, using lower temperatures could lead to a decrease in the enzymatic activity. However, between 30 and 50 °C, our results demonstrated that catalytic activity remained above 50% (Figure 2). In addition, according to the results from the experimental design (Table 4), this activity was sufficient to reduce the initial turbidity of cupuaçu juice with efficiency comparable to that of commercial enzymes.

The fact that the enzymatic extract was produced from cupuaçu residue as a substrate in fungal cultivation may have contributed to the efficiency of juice clarification produced with cupuaçu pulp. This is corroborated by the positive results obtained by Barman et al. [10], where banana peel was used as a substrate to produce pectinases, applied in the clarification of banana juice. The authors used a partially purified enzyme with an activity of 20.6 U/mL and achieved an 85% reduction in absorbance. In this case, enzyme purification proved essential for increasing clarification efficiency, as it tripled enzyme activity. Thus, purifying the polygalacturonase produced by *A. brasiliensis* should be considered in future studies.

The fruit juice clarification process can result in the loss of nutrients relevant to the beverage. Haile et al. [12] clarified lemon, apple, and mango juices using pectinase produced by the bacterium *Serratia marcescens*. For all juices, a decrease in total phenolic content and antioxidant activity was observed after the process. Additionally, in the study by Gani et al. [46], a significant decrease in antioxidant activity was demonstrated in pear juice extracted with pectinase. This negative result was also observed with the use of clarifying agents containing polysaccharides and proteins [7]. In the present investigation, however, the antioxidant activity of the cupuaçu juice was kept after clarification with the pectinase produced by *A. brasiliensis*. Cupuaçu pulp has low antioxidant activity, as reported by Kuskoski et al. [47] and Santos et al. [48]. However, enzymatic clarification, therefore, proves to be an essential step for processing cupuaçu pulp into juice, not only to reduce turbidity but also to keep a health-promoting property.

The antioxidant activity of clarified cupuaçu juice was significantly affected by the enzyme load used in the process. Lower concentrations of the pectinolytic extract resulted in higher antioxidant activities. It is thus observed that the presence of the enzymatic extract is important for maintaining antioxidant activity, but enzyme concentrations should be kept at lower levels, which is also economically beneficial. Patricio et al. [11] report that enzymatic treatments with pectinases can help maintain phenolic compounds in juices, which likely explains the preservation of antioxidant activity in the enzymatically clarified cupuaçu juice.

5. Conclusions

This study demonstrated that, through solid-state fermentation, it was possible to use cupuaçu peel as a substrate to produce polygalacturonase by the fungus *A. brasiliensis*. Furthermore, the polygalacturonase activity of the enzyme extract could be optimized by determining the best cultivation conditions. The enzyme produced is thermostable and functions across a wide pH range, which is beneficial for industrial applications. Finally, it was shown that this enzyme can be used in the processing of cupuaçu juice, reducing its turbidity and improving its potential nutritional quality in terms of antioxidant activity, thus presenting significant economic potential for producers.

Future studies should aim to optimize the clarification process, especially through the purification of the produced pectinases, and also carry out a more in-depth study on

the impact of these enzymatic extracts on the chemical composition of cupuaçu juices and other fruit juices, particularly in terms of their phenolic composition and sensory profile. Additionally, evaluating the long-term stability of the produced extract under varying temperature and pH conditions will contribute to assessing its potential for industrial applications. In this way, this study can be the first approach to this topic, which is expected to be very encouraging in terms of the production and use of these enzyme extracts in the food industry.

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