fermentation

MDPI

Article

Pectinase Production from Cocoa Pod Husk in Submerged Fermentation and Its Application in the Clarification of Apple Juice

Anderson Steyner Rozendo [1], Luciana Porto de Souza Vandenberghe [1,*], Patricia Beatriz Gruening de Mattos [1], Hervé Louis Ghislain Rogez [2] and Carlos Ricardo Soccol [1]

[1] Department of Bioprocess Engineering and Biotechnology, Federal University of Paraná, Centro Politécnico, Curitiba 81531-980, PR, Brazil; andersonsteyner@gmail.com (A.S.R.); patricia.mattos@ufpr.br (P.B.G.d.M.); soccol@ufpr.br (C.R.S.)

[2] Centre for Valorization of Amazonian Bioactive Compounds (CVACBA), Institute of Biological Sciences, Federal University of Pará (UFPA), Belém 66075-750, PA, Brazil

* Correspondence: lvandenberghe@ufpr.br

Abstract: The present work aimed to use cocoa pod husk (CPH) and its extracted pectin as a potential substrate for the production of pectinase and to test the enzyme produced in the clarification process of apple juice. CPH with a particle size of <0.84 mm was employed for pectinase production by a selected strain of *Aspergillus niger* NRRL 2270. The optimization of the physicochemical conditions of the production medium led to an enzymatic activity of 602.03 U/g dry CPH, which was obtained under the following conditions: 110.25 g/L of CPH, 5% w/v pectin extract, 0.05 g/L of yeast extract, incubation at 28 °C, and pH 4, representing a 176% increase in enzymatic activity under the evaluated conditions. The production kinetics of pectinase showed maximum enzymatic activity at 96 h. Subsequently, the enzymatic extract was precipitated, microfiltered, and ultrafiltrated, resulting in 4852.50 U/mg of specific activity. The enzymatic activity after recovery and purification processes corresponded to 819 U/g dry CPH. Finally, a clarification stage of apple juice was carried out, in which the produced pectinase (CauPec) showed turbidity of 448.89 NTU compared to 417.89 NTU for the commercial enzyme and a viscosity of 1.86 cP, CauPec, and 1.19 cP, commercial pectinase, as well as soluble solids of 8.0 for commercial pectinase and 8.73 for CauPec. Therefore, it can be concluded that CPH and its pectin extract were excellent substrates for the production of pectinases, whose formulation is highly stable and can be applied in the clarification of apple juice.

Keywords: cocoa pod husk; *Aspergillus niger*; pectinase; recovery; purification; juice clarification

check for updates

Citation: Rozendo, A.S.; Vandenberghe, L.P.d.S.; de Mattos, P.B.G.; Rogez, H.L.G.; Soccol, C.R. Pectinase Production from Cocoa Pod Husk in Submerged Fermentation and Its Application in the Clarification of Apple Juice. *Fermentation* **2024**, *10*, 337. https://doi.org/10.3390/fermentation10070337

Academic Editor: Patrizia Romano

Received: 30 May 2024
Revised: 19 June 2024
Accepted: 21 June 2024
Published: 28 June 2024

Copyright: © 2024 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (https://creativecommons.org/licenses/by/4.0/).

1. Introduction

Pectinases (EC. 3.2.1.15), endo-polygalacturonases, are a group of enzymes composed of hydrolases, lyases, and esterases, which are enzymes that degrade pectic substances that are the structural polysaccharides present in plant cells [1]. The wide variety in pectinase enzyme groups is related to the different forms of pectin present in the plant cell wall. There are several examples of the use of the *Aspergillus niger* species in studies of pectinase production [2].

Pectinases are classified as polygalacturonase (PG), polymethylgalacturonase (PMG), pectin lyase (PL), pectate lyase (PAL), and pectin methylesterase (PME) [1]. Pectinolytic enzymes can be applied in the extraction and clarification of juices and wines, the extraction of essential oils, the pretreatment of residues, in wastewater with significant pectin content, and in the paper, cellulose, and textile industries [2]. However, production costs are of considerable importance. So, one alternative to this issue would be the use of low-cost substrates, such as agro-industrial residues.

Cocoa (*Theobroma cacao* L.) belongs to the *Sterculiaceae* family and is commonly known as cocoa tree, cocoa, or the tree of life. It is a plant native to the South American continent, especially in the Amazon region [3]. The main producers of cocoa fruits are the Ivory Coast, Ghana, Nigeria, Indonesia, Cameroon, and Brazil. Together, they contribute approximately 90% of the world's production [4]. Regarding world production, the gross value of cocoa production reached, in 2022/2023, 4.45 million tons [5]. Cocoa beans are the main commercial product derived from the cocoa tree. A significant portion of cocoa pod husks (CPHs) is generated during processing, which is usually discarded on-site immediately after cocoa bean extraction [6]. CPH accounts for approximately 52–76% of the mass of the cocoa fruit. Taking into consideration worldwide production, the generation of CPHs can be in the order of 3.70 million tons annually. Their disposal in the environment can cause serious environmental problems, such as degradation by microorganisms [4], mainly fungi, which are capable of degrading these biomasses. In favorable climatic conditions, CPH can become a breeding ground for harmful fungal pathogens, like *Marasmius perniciosus*, *Phytophthora palmivora*, and *P. megakarya*. The *Phytophthora* species cause pod rot, or black pod disease, which affects susceptible cocoa plant genotypes by forming small dark lesions on the pods that quickly spread across the surface and into the internal tissues, including the beans and pulp. In the 1980s, Brazil experienced a severe outbreak of the witches' broom fungus (*Moniliophthora perniciosa*), which resulted in the death of thousands of trees and a significant reduction in almond production for nearly twenty years. This phytosanitary issue is thought to have been exacerbated by improper disposal and management of cocoa waste. Mismanagement of CPH can compromise the quality of the next harvest and the health of the plants, leading to substantial economic losses [6].

According to Valladares-Diestra et al. [7], CPH is composed of 14.4% cellulose, 10.50% hemicellulose, and 27.63% lignin. Despite being considered an environmental issue, CPHs' characteristics make them potential raw materials for use in the biotechnological sector. One of these characteristics is the considerable presence of pectin. CPHs contain between 5.3 and 7.1% pectin in their composition [3]. This makes it possible to use them for pectin extraction and even for the production of pectinolytic enzymes [1].

Other uses of CPH were reported, such as the ones described by Rebello et al. [2], who showed that it can be used as a low-cost adsorbent for the retention of pollutants, such as industrial dyes or heavy metals. Studies in the food industry have used CPH to produce flour and biscuits, and it is also a nutritional fortification agent [8]. Additionally, the high presence of potassium in CPH ash (77.53%) can be properly managed for biofertilizer production. Other applications of CPH were reported [9,10]. CPHs were used to obtain microfibrillated cellulose [11], nanocellulose [12], nanocrystalline cellulose [13], and carboxymethyl cellulose [14].

Therefore, this study aims at producing pectinase enzymes through submerged fermentation using CPH as a substrate by *Aspergillus niger*. In addition to pectinase enzyme production, this study also seeks to test the enzyme produced, purified through membranes, and formulated in the apple juice clarification process.

2. Materials and methods

2.1. Material

The CPHs used in this study were sourced from the cocoa-producing region in the state of Pará, with the cocoa being the Forastero variety and produced at Konagano Farm, located in Ramal do Anuerá, s/n, Tomé-Açu-PA, Brazil at 02°25′08″ S latitude and 48°09′08″ W longitude. The material was graciously provided by the University Federal of Pará, a partner institution of the CAPES PROCAD 2013-BIOCAU Project. After the separation and particle size classification of the CPH, physical–chemical characterization and lignocellulosic characterization of the material was conducted according to the National Renewable Energy Laboratory (NREL) procedures [15–18]. Only fractions 1.18–2.00 mm and smaller than 0.84 mm were used for a better homogenization and mass transfer of nutrients during fermentation.

2.2. Microorganism and Inoculum Preparation

The inoculum was prepared as follows: 60 g of white rice were weighed and cooked for 10 min. The cooked rice grains were drained, placed in Erlenmeyer flasks, and sterilized in an autoclave for 15 min at 121 °C and 1 atm. Subsequently, the rice was inoculated with a spore suspension of *Aspergillus niger* NRRL 2270 obtained from a slant tube, and then the flasks were incubated in an oven for up to six days at 30 °C. The spore suspension was obtained by suspending the spores grown on rice grains in the Erlenmeyer flasks in a deionized water solution containing 1 drop of previously sterilized Tween 80 after agitation and filtration. A suspension with a concentration of 1×10^7 spores/mL was obtained and used as inoculum.

2.3. Pectinase Production by Submerged Fermentation

The culture medium initially used during optimization tests was composed of (g/L) CPH 50.0 (particle size < 0.84 mm); glucose 5.0; yeast extract 0.05; $(NH_4)_2SO_4$ 5.0; $MgSO_4$ 0.5; KH_2PO_4 2.5; $FeSO_4$ 6.3×10^{-4}; $ZnSO_4$ 6.2×10^{-4}; and $MnSO_4$ 1.0×10^{-5}, as per Reginatto et al. [19], with the commercial pectin replaced by CPH. Fermentation was conducted in 250 mL Erlenmeyer flasks with 100 mL of medium and incubated in an orbital shaker at 28 °C for 120 h under agitation of 100 rpm. The experiments were conducted in triplicate.

2.4. Optimization of Pectinase Production

The steps for optimizing pectinase production were carried out based on the culture medium described in Section 2.3. Yeast extract and $(NH_2)_2CO$ were previously tested in a preliminary step of experiments before optimization steps. Another important test was carried out with the addition of a supplementary source of pectin (extracted from CPH) to analyze its influence on pectinase production. The medium was also prepared using a pectin solution (50 g/L) extracted from CPH in a water bath at 100 °C for 20 min and was subsequently filtered, where it was referred to as pectin extract. Finally, an experiment was conducted using only CPH as a carbon source. All experiments were performed in triplicate.

2.4.1. Step 1—Optimization—Incomplete Factorial Design $3^{(3-1)}$

After preliminary tests, an incomplete factorial design was applied $3^{(3-1)}$ to study the influence of different levels of CPH concentration, pectin extract, and yeast extract on pectinase synthesis. Table 1 presents the uncoded and coded levels of each studied variable.

Table 1. Coded and uncoded levels of the studied factors—$3^{(3-1)}$ factorial design.

Variables (g/L)	Levels		
	−1	**0**	**+1**
CPH	25	50	75
Yeast extract	0.00	0.05	0.10
Pectin extract	0	50	75

2.4.2. Step 2—Optimization—Central Composite Rotatable Design

A Central Composite Rotatable Design (CCRD) experimental design was employed, with a triplicate at the central point. This step aimed to investigate the influence of factors' levels: CPH concentration and pectin extract concentration on pectinase production. These factors have shown to be significant in the last step of optimization. The concentration was kept constant at 0.05 g/L. Table 2 presents the coded and uncoded levels of the studied factors.

Table 2. Coded and uncoded levels of the studied factors—CCRD.

Variables (g/L)	Levels				
	−1.41	**−1**	**0**	**+1**	**+1.41**
CPH	39.75	50	75	100	110.25
Pectin extract	36	40	50	60	64

2.5. Kinetics of Pectinase Production

Kinetics of pectinase production was carried out using optimized conditions of medium composition (CPH (110.25 g/L), pectin extract (50 g/L), yeast extract (0.05 g/L), $(NH_4)_2SO_4$ (5 g/L), $MgSO_4$ (0.5 g/L), KH_2PO_4 (2.5 g/L), $FeSO_4$ (6.3×10^{-4} g/L), $ZnSO_4$ (6.2×10^{-4}), and $MnSO_4$ (1.0×10^{-5})), which were defined according to Section 2.4. Erlenmeyer flasks with a volume of 250 mL were incubated in an orbital shaker for 7 days at 28 °C and 120 rpm.

2.6. Pectinase Precipitation, Recovery, and Semi-Purification

Crude pectinase extract passed through precipitation with ammonium sulfate at 80% (*w*/*v*). The samples were maintained in ice baths during precipitation. The precipitate was separated through centrifugation at 4000 rpm and 15 min [20]. The precipitate was then resuspended in a 0.1 M citrate buffer at pH 4.0 and homogenized. The samples were then filtrated under microfiltration (MF, 0.2 µm) and ultrafiltration (UF, *membrane weight cut-off*—MWC of 30 kDa) in a tangential filtration system, VivaFlow 200 (Sartorius Stedim, Gottingen, Germany), with a transmembrane pressure of 1 bar. Enzyme activity, specific activity, % of enzyme recovery, and purification factor were determined according to Poletto et al. [21]. The produced enzyme was called CauPec.

2.7. Determination of Pectinase Activity

Pectinase activity was determined according to the method for determining polygalacturonase (PG) activity. The method was performed according to Handa et al. [22] with modifications. The determination of reducing sugars was performed using the 3,5-Dinitrosalicylic Acid (DNS) method [23], with galacturonic acid (Sigma-Aldrich, St. Louis, MO, USA) as the standard. The absorbances of the samples were read in a PowerWave X5 microplate reader (Biotek®, Winooski, VT, USA) at 540 nm. One unit of PG activity was defined as the amount of enzyme that releases 1 µmol of D-galacturonic acid per minute at 50 °C and pH 4.0.

2.8. Enzyme Application in Apple Juice Clarification

For the enzymatic clarification assays, Fuji apples purchased from a local supermarket were used. Initially, the apples were washed under running water, cut, and blended in a blender along with deionized water at a ratio of 250 mL of water for every 4 apples. Sodium metabisulfite (0.1%) was added to the suspension to prevent enzymatic browning. The apple juice was then frozen at −18 °C until the clarification reaction.

The clarification process was carried out as follows. In Erlenmeyer flasks, 50 mL of the previously extracted juice was added along with 10 mL of the pectinolytic enzyme at a concentration of 450 U/mL. Three samples were prepared: one with the enzymatic product produced in this work (CauPec), one with the addition of the commercial pectinase enzyme (pectinase from *Aspergillus niger*—Sigma), and one with the volume corresponding to the enzyme replaced by deionized water, serving as the blank for the assay. The flasks were incubated in a water bath with orbital shaking at 50 °C for 60 min. After this time, the flasks were incubated at 85 °C for 15 min to inactivate the enzymes. Finally, the flasks were placed in an ice bath until they reached room temperature. Subsequently, the samples were vacuum filtered using Whatman Grade 1 qualitative filter paper (a pore size of 11 µm).

2.9. Analytical Methods of Samples after Apple Juice Enzymatic Clarification

2.9.1. Flow Rate

The flow rates of all samples were determined after vacuum filtration, where the filtration times (t) and the respective volumes (v) were recorded. The flow rate was calculated using Equation (1).

$$\text{Flow rate} = \frac{v}{t} \quad (1)$$

2.9.2. Turbidity

Turbidity is understood as a physical property of fluids, where the presence of suspended particles interferes with the transparency of the liquid. To determine this variable, a PoliControl AP2000 (PoliControl, Diadema, SP, Brazil) digital turbidimeter was used.

2.9.3. pH

The pH determination of the samples was performed using a LUCA-210 pH meter (Tecnopon, Brazil). Prior to each measurement, the device was properly calibrated with pH 4 and 7 buffer solutions.

2.9.4. Viscosity

The viscosity of a fluid corresponds to its resistance to deformation or flow. The absolute viscosity of the sample, also called the dynamic viscosity coefficient, was determined using Equation (2). For this purpose, an Ostwald viscometer (SPLabor, Brazil) was used to determine the flow times of the samples. Viscosity calculations were made using deionized water as a reference.

$$\frac{\mu_{H2_2O.(t.\rho)}}{t_{H_2HO.}\ \rho_{H_2O}} \quad (2)$$

2.9.5. Brix

The readings of total suspended solids were taken using a Handheld MyBrix refractometer (Metler Toledo, Barueri, SP, Brazil) with a reading scale from 0 to 32 Brix, with the values expressed in °Brix.

2.10. Statistics

All of the results are expressed as average ± standard deviation (in triplicate). Analysis of variance (ANOVA) was used to assess the data significance, where $p \leq 0.05$ was considered statistically significant. Statistica Ultimate Academic, version 14 (StatSoft, South America, São Caetano do Sul, SP, Brazil) was used in the statistical analysis of results in the optimization steps.

3. Results and Discussion

CPH composed of 38.8% lignin, 9.20% hemicellulose, and 18.8% cellulose was initially employed in pectinase production by submerged fermentation using *A. niger* NRRL 2270, which was previously selected, reaching an enzyme activity of 304.17 U/g dry CPH (15.21 U/mL) after 96 h.

Preliminary tests were carried out to evaluate two different nitrogen sources (yeast extract and $(NH_2)_2CO$) and the necessity of adding a supplementary source of pectin; the pectin extract that was obtained from CPH to induce the synthesis of pectinases by the strain (Table 3). As expected, the addition of an extra source of pectin led to an activity of 468.69 ± 2.0 U/g dry CPH, corresponding to an increase of 64.89%. So, pectin extract was then included as a medium component and was also studied in the next steps of optimization.

Table 3. Preliminary tests of pectinase production using CPH by submerged fermentation.

Essay	Medium Components (g/L)										Pectinase Activity (U/g Dry CPH)
	CPH	Glucose	Yeast Extract	$(NH_4)_2SO_4$	$MgSO_4$	KH_2PO_4	$FeSO_4$	$ZnSO_4$	$MnSO_4$	$(NH_2)_2CO$	
1 *	50	5	0.05	5	0.5	2.5	6.3×10^{-4}	6.2×10^{-4}	1.0×10^{-5}	0	304.17 ± 1.57
2	50	0	0.05	5	0.5	2.5	6.3×10^{-4}	6.2×10^{-4}	1.0×10^{-5}	0	272.50 ± 2.10
3	50	0	10.55	0	0.5	2.5	6.3×10^{-4}	6.2×10^{-4}	1.0×10^{-5}	0	186.61 ± 0.47
4	50	0	0	0	0.5	2.5	6.3×10^{-4}	6.2×10^{-4}	1.0×10^{-5}	2.26	255.57 ± 2.56
5 **	50	0	0.05	5	0.5	2.5	6.3×10^{-4}	6.2×10^{-4}	1.0×10^{-5}	0	468.69 ± 2.0
6	50	0	0	0	0	0	0	0	0	0	333.65 ± 0.78

* Control medium; ** medium prepared with CPH + pectin extract.

3.1. Step 1—Incomplete Factorial Experimental Design $3^{(3-1)}$

Seeking to continue the selection of components to produce pectinases, an incomplete factorial experimental design $3^{(3-1)}$ was conducted with three factors: the concentration of CPH, yeast extract, and pectin extract. Below, Table 4 shows pectinase activity values according to different essays.

Table 4. Incomplete factorial design $3^{(3-1)}$—influence of the studied factors on pectinase activity.

Essay	Factors (g/L)			Pectinase Activity (U/g Dry CPH)
	CPH	Yeast Extract	Pectin Extract	
1	25	0	0	233.68 ± 0.02
2	25	0.05	75	138.99 ± 0.02
3	25	0.10	50	252.49 ± 0.30
4	50	0	75	236.65 ± 0.10
5	50	0.05	50	232.03 ± 0.02
6	50	0.10	0	267.33 ± 0.02
7	75	0	50	341.63 ± 0.02
8	75	0.05	0	232.69 ± 0.10
9	75	0.10	75	237.31 ± 0.03
10 (C)	50	0.05	50	199.70 ± 0.10
11 (C)	50	0.05	50	238.63 ± 0.10

It is possible to see that the highest enzymatic activity (341.63 ± 0.02 U/g) occurred in run 7, with the highest concentration of CPH and pectin extract at a concentration of 50 g/L, but there was no addition of a nitrogen source. The lowest enzymatic activity (138.99 ± 0.02 U/g) was observed in run 2, which consisted of the highest concentrations of CPH and pectin extract and the presence of yeast extract at 0.05 g/L.

ANOVA showed that the effects of the independent variables evaluated were significant with a p value < 0.05. The coefficient of determination for this analysis was R^2 equal to 0.9625 (Supplementary Materials). The variable with the greatest significant effect was yeast extract, which, according to the Pareto diagram (Supplementary Materials), when used in lower concentrations, tends to increase the response in terms of pectinase activity, suggesting that higher C/N ratios are favorable to enzymatic synthesis. The interaction effects between the studied variables were not significant. Given this situation, it was decided to carry out a new optimization step to determine the best concentration for CPH and pectin extract.

3.2. Step 2—CCRD to Study the Effect of the Concentration of CPH and Pectin Extract

A CCRD was employed to investigate the influence of the following factors: CPH concentration and pectin extract concentration on the production of pectinases. The concentration of yeast extract was set at 0.05 g/L, the same that was employed in the control production medium, since in previous tests, its effect was not significant. The Pareto diagram (Supplementary Materials with the factors' effects indicates that the independent

variable, CPH concentration, had a significant effect ($p < 0.05$) on pectinase activity(Figure 1). It can also be seen that the interactions between the variables studied were not significant in the tested levels but had a positive effect.

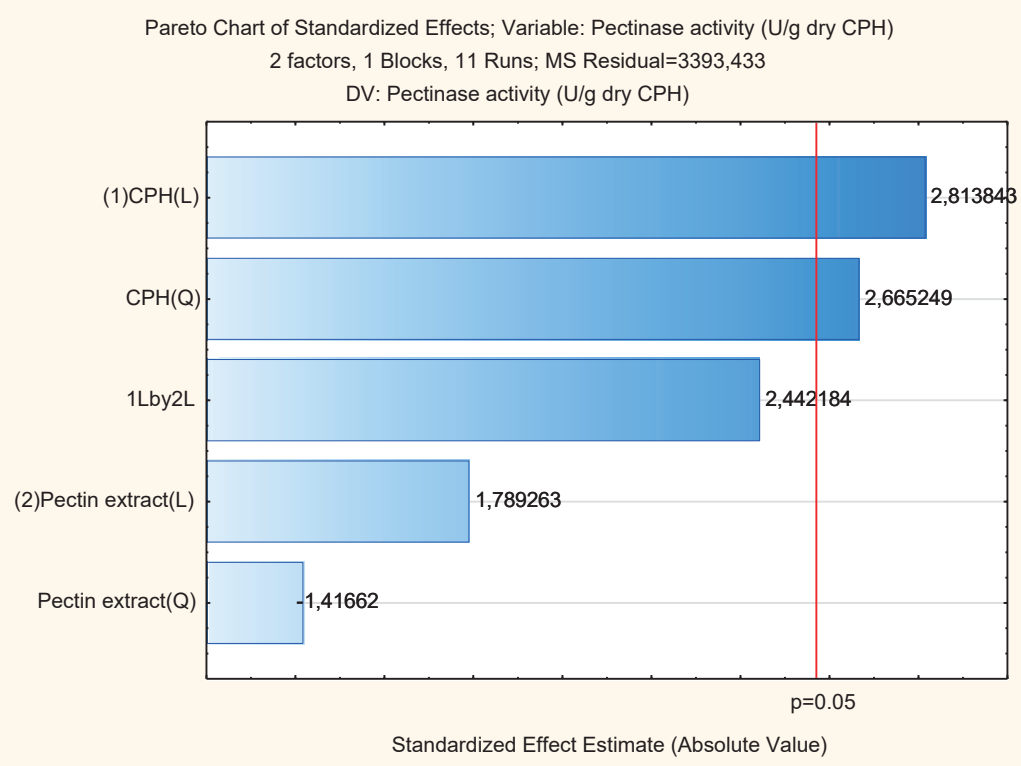


Figure 1. Pareto chart—Step 2 optimization—the effect of CPH and pectin extract concentrations on pectinase activity (U/g dry CPH).

Table 5 shows the enzymatic activities for CCRD design. Pectinase activity of 602.03 U/g dry CPH (30.10 U/mL) was achieved with 110.25 g/L of CPH, 5% pectin extract (w/v), and 0.05 g/L of yeast extract, and was, therefore, established with optimized conditions. Considering the enzymatic activity obtained at the beginning of the experimental tests, of 220 U/g dry CPH (11 U/mL), it can be considered that there was a 2.73-fold increase in pectinase activity within the conditions evaluated.

Table 5. CCRD—a study of the effect of the concentration of variables on the production of pectinase.

Run	Factors (g/L)		Pectinase Activity (U/g Dry CPH)
	CPH	Pectin Extract	
1	50	40	307.54 ± 0.02
2	50	60	308.09 ± 0.02
3	100	40	293.79 ± 0.03
4	100	60	578.87 ± 0.01
5	39.75	50	456.00 ± 0.01
6	110.25	50	602.03 ± 0.01
7	75	36	327.33 ± 0.01
8	75	64	332.83 ± 0.02
9 (C)	75	50	354.28 ± 0.03
10 (C)	75	50	387.82 ± 0.03
11 (C)	75	50	366.92 ± 0.02

In Figure 2, the response surface for CCRD is presented, where it is possible to visualize the profile of pectinase activity as a function of the independent variables, CPH

concentration and pectin extract concentration. Higher variables' concentrations promote higher pectinase activity.

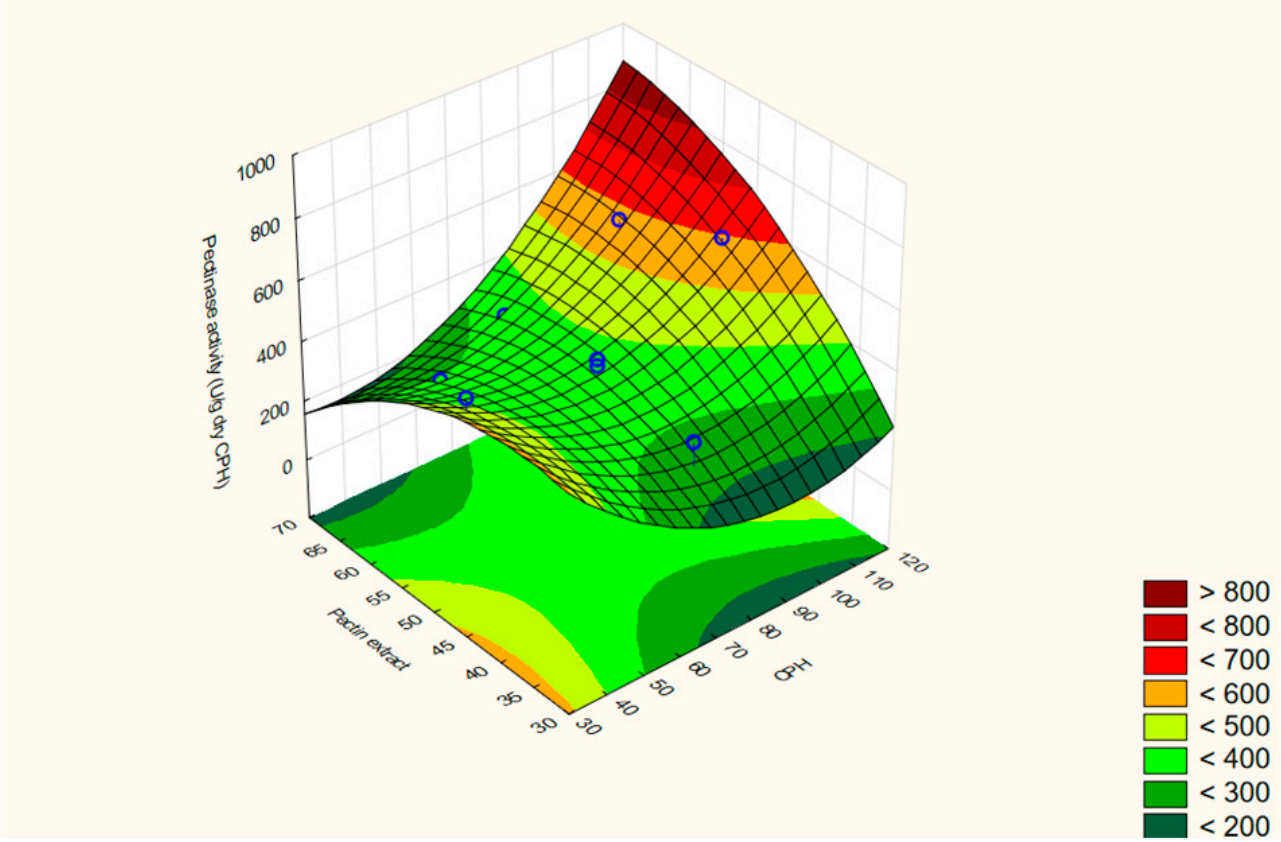


Figure 2. Response surface—CCRD—Step 2 optimization—Influence of CPH and pectin extract on pectinase activity (U/g dry CPH).

The response surface plots illustrate a trend of increased pectinase activity related to increases in CPH concentration. However, very high concentrations of CPH would result in increased viscosity in the medium, thus operationally limiting production processes in bioreactors. Analysis of variance, ANOVA (Supplementary Materials) showed that the effect of the variable CPH concentration was significant for a p value of < 0.05. The coefficient of determination for this analysis was R^2 equal to 0.8530, with an adjusted R of 0.70922. Predicted versus observed values of responses can be also analyzed (Supplementary Materials). The model that describes the behavior of the enzymatic activity response as a function of the variables' CPH concentration and pectin extract concentration was determined by Equation (3), where Z is pectinase activity (U/g dry CPH), X is CPH concentration (g/L), and Y is the concentration of pectin extract (g/L).

$$Z = 786.69 - 27.63 \times x + 0.10 \times x\hat{}2 + 17.60 \times y - 0.35 \times y\hat{}2 + 0.28 \times x \times y \quad (3)$$

After optimization, pectinase activity obtained in this work was comparable and/or higher than other previously reported. In a study on the production of pectinases by *A. niger*, carried out by Sandri and Silveira [24], they obtained a maximum production of 68 U/g. In solid-state fermentation (SSF) for the production and optimization of polygalacturonase from mango peels, using the filamentous fungus *Fusarium moniliforme*, Afzia et al. [25] produced pectinase with *Aspergillus flavus* using dried Assam lemon peels. After optimization, pectinase activity reached 3.68 U/mL. *Yarrowia phangngaensis* was investigated for its ability to produce pectinase using agro-wastes as substrates. In this case, the authors reported a maximum pectinase activity of 1.61 U/mL [26].

3.3. Kinetics of Pectinase Production

Seeking to observe the production profile of pectinases, a kinetic study was carried out under optimized medium composition conditions (Figure 3). It can be observed that the maximum pectinase activity was reached at 96 h of cultivation, achieving 551.30 U/g, which corresponds to 27.57 U/mL, indicating that the process can be stopped by the fourth day. The activity remained practically constant, staying at 440.00 U/g at the end of 168 h. The maximum productivity was 5.74 U.g/h (or 0.28 U/mL·h). It is also observed that the pH values tend to decrease throughout the fermentation process, reaching 4.85 after 24 h of cultivation and 2.62 at the final fermentation time of 168 h. This decrease is possibly associated with the production of metabolites, such as organic acids, by the microorganism under the cultivation conditions. In fact, the strain *A. niger* NRRL 2270 (ATTC 11414) is known as a citric acid producer [27].

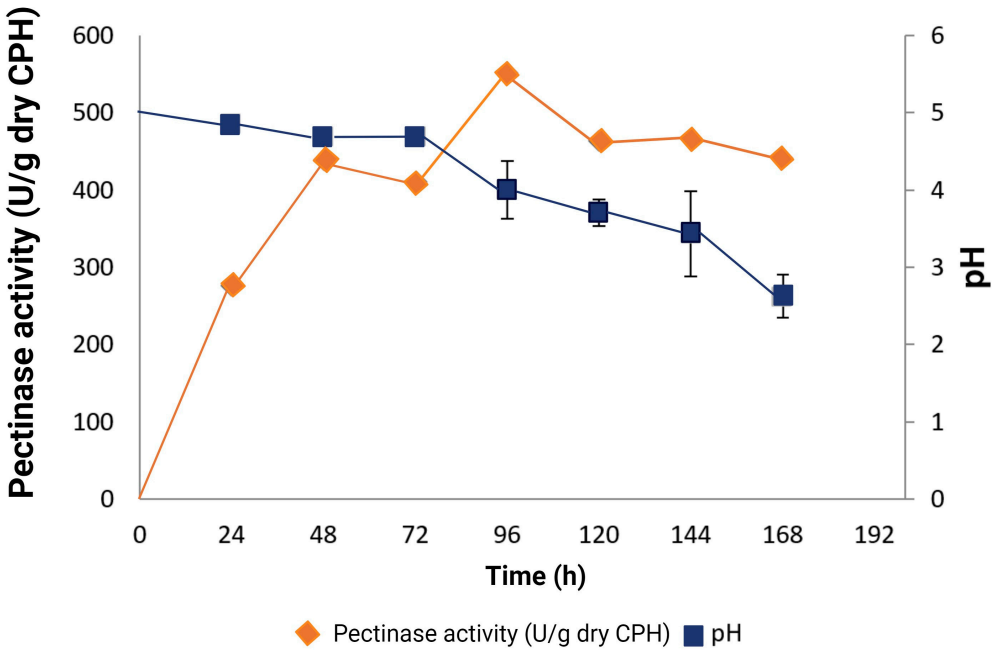


Figure 3. Kinetics of pectinase production using CPH and pectin extract.

In a study conducted by Biz et al. [28], the highest pectinase activity by a strain of *A. niger* using orange bagasse as a substrate in SSF was 77 U/g in 19 h of production, corresponding to a productivity of 4.0 U/g.h. In contrast, pectinase production using orange peel residues in submerged fermentation by an *A. niger* strain reached 77.2 U/mL after 5 days or 0.64 U/mL·h [29]. Despite the different pectinase production values over the cultivation period, it is important to consider that production depends on the microorganism, type of fermentation, and nutrient concentration in the cultivation medium [1].

3.4. Application of Semi-Purified and Formulated Pectinases in Apple Juice Clarification

The crude extract from the pectinase production was initially microfiltered using a membrane with a pore size of 0.2 µm, aiming to remove suspended particles and macromolecules. After MF, the permeate fraction was then subjected to UF using a 30 kDa membrane. After passing and recirculating the enzyme through this membrane until a pre-established retentate volume was achieved, the enzyme of interest was concentrated, as evidenced by the increased pectinase activity at the end of the UF process, indicating the concentration of the enzyme of interest. After separation and recovery by MF and UF, the obtained enzymatic activity was 40.95 U/mL, corresponding to 819 U/g dry CPH and a specific activity of 4852.50 U/mg, representing a purification factor of 6.73. Regarding the enzymatic activity achieved at the end of the production medium optimization, the

increase obtained after separation and purification was 36%. Finally, the enzyme extract was formulated with sodium benzoate and xylitol.

One of the main applications of pectinases is in the beverage industry due to their ability to improve fruit pressing and clarification, which significantly increases yields and enhances color and clarity [1]. In Figure 4, the stages of the apple juice clarification process developed in this work are shown.

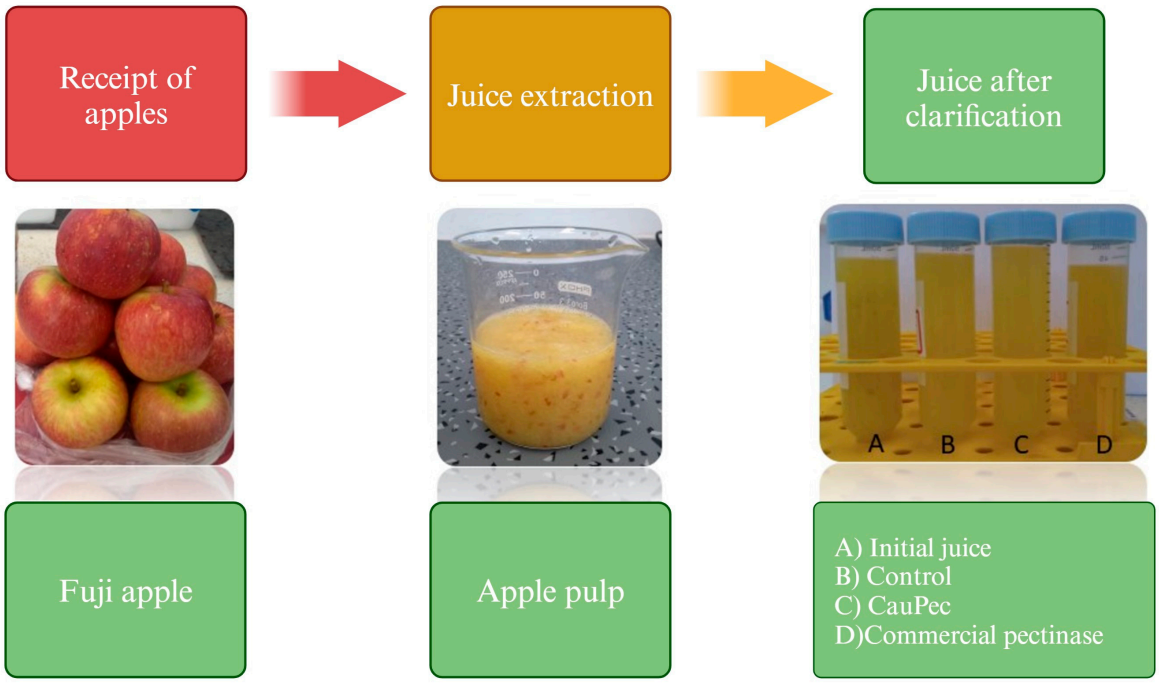


Figure 4. Steps of the apple juice enzymatic clarification process.

An apple juice with 8.13 °Brix (81.13 g/L total sugars), organic acids (9.5 µmol/mL of galacturonic acid), and pH 4.21 was employed in enzymatic clarification using produced pectinase CauPec. Essays were carried out with an incubation time of 60 min, the pH value for the treated juice was close to the values found for the untreated juice (control), and the juice treated with commercial pectinase (Sigma) was around 4.0. The turbidity values observed indicate a value of 589.00 NTU for the control experiment, while for the juices treated with CauPec and pectinase (Sigma), the values were significantly lower and were 415.83 NTU and 408.89 NTU, respectively. Compared to the control, the turbidity of the apple juice decreased by 30.5%. It is important to highlight that the CauPec enzyme was obtained from an ultrafiltered fraction, which may contain other enzymes, and, therefore, the possible action of other enzymes in the extract should also be considered, according to Cerreti et al. [30]. A significant and synergistic effect of the combined use of pectinase and protease enzymes, for example, can occur in terms of turbidity in juice clarification.

After enzymatic treatment, the viscosity of apple juice was 1.86 cP for CauPec and 1.29 cP for commercial pectinase compared to the control without treatment (1.18 cP). Juice pulps are composed of pectin-rich material. When this material is hydrolyzed, the previously insoluble pectin becomes available in an aqueous medium, increasing its viscosity [31]. To assess the action of the tested enzymes regarding viscosity, it was possible to observe that this parameter showed similar results for both evaluated enzymes, with an increase in viscosity compared to the control. These results alone are insufficient to indicate which enzyme exhibited greater catalytic activity.

Another parameter analyzed was the volumetric flow rate for the samples after enzymatic hydrolysis. The volumetric flow rate for the three samples showed similar values,

indicating that the enzymatic effect did not present significant differences. The release of fibers and smaller compounds by the enzyme action may be influencing the flow rate.

Regarding the °Brix parameter, it can be said that there was a gradual increase with enzymatic treatment. The control sample, without enzymatic treatment, showed 8.13 °Brix, while the sample treated with the CauPec enzyme showed 9.57 °Brix, indicating a higher amount of total soluble solids in the sample, representing an 18% increase compared to the control. In this case, it can be due to the enzymatic degradation of the cell wall of apple pulp, depending on the type of enzyme [32], which results in the release of soluble solids by the degradation of some constituent polymers. They observed that enzymatic extraction also increased the concentration of total soluble solids in juice from various fruits. Additionally, it is important to consider the use of various enzymes in different combinations to increase the total soluble solids content of the juice, for example, pectinase and cellulases. A study on banana juice clarification conducted by Barman et al. [33] revealed that the greatest clarification effect was achieved when the raw banana juice was incubated for 60 min with a 2% concentration of partially purified pectinase.

Considering the results of the apple juice clarification process, it is possible to infer that the rate of enzymatic hydrolysis depends on various factors, such as incubation time, temperature, and enzyme concentration [30]. An enzymatic extract of pectinases from *A. niger*, used for strawberry juice clarification, led to a 60% reduction in turbidity and a 40% reduction in juice viscosity [24]. Afzia et al. [25] applied crude pectinase in grape juice clarification, showing decreased optical density (1.45 ± 0.06 to 1.17 ± 0.07) and viscosity (1.76 ± 0.10 to 1.46 ± 0.07 mPa·s), increased reducing sugar content (13.90 ± 0.10 to 16.96 ± 0.06 g/100 mL juice), and total soluble solids (17.77 ± 0.16 to 18.50 ± 0.30 ∘Brix). Optimum juice clarification was achieved by treating 10 mL of apple juice with pectinase turbidity and clarity reduction percentages of 39.33% and 59.84%, respectively [26].

Given the relevance of clarification processes in the juice industry, the impact of using pectinase enzymes for the facilitation and optimization of such processes is evident. The clarification process can be considered a necessary step in fruit juice processing, enhancing the visual aspect of the final product and thus increasing consumer market appreciation [30].

4. Conclusions

CPH supplemented with its pectin extract-based culture medium showed great potential for pectinase production. Optimal conditions were defined for the process reaching a pectinase activity of 551.30 U/g dry CPH (or 27.57 U/mL) with a productivity of 0.28 U/mL·h. In addition, pectinase separation and recovery processes, involving precipitation, MF, and UF, were highly efficient. The produced enzyme, called CauPec, was formulated with sodium benzoate and xylitol additives and applied in apple juice clarification processes, showing significant and comparable results to commercial enzymes. Future essays will involve process scale-up, enabling applications for industrial scales and the investigation of drying processes, such as spray drying, to assess enzyme stability after the process step, as well as evaluate long-term enzyme stability. With these strategies, a medium- to high-value bioproduct can be obtained using cocoa chain residues at lower costs, which could be interesting for the industry, adopting biorefinery concepts and non-waste technology approaches.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/fermentation10070337/s1, Table S1. ANOVA—$3^{(3-1)}$ factorial experimental design—Step 1—Optimization (Pectinase activity (U/g dry CPH); R-sqr = 0.96252; Adj: 0.81259 ($3^{(3-1)}$ fractional factorial design, 1 block, 9 runs ([No active dataset]) in Workbook_Step_1_$3^{(3-1)}$) 3 3-level factors, 1 Blocks, 11 Runs; MS Residual = 434.0556). Figure S1. Response surface—First step optimization—$3^{(3-1)}$ experimental design Table S2. ANOVA–CCDR–Step 2–Optimization pectinase activity. ANOVA; Var.: Pectinase activity (U/g dry CPH); R-sqr = 0.85461; Adj: 0.70922 (2**(2) central composite, nc = 4 ns = 4 n0 = 2 Runs = 10 ([No active dataset]) in Workbook_Step_2_CCRD) 2 factors, 1 Blocks, 11 Runs; MS Residual = 3393.433 DV: Pectinase activity (U/g dry CPH). Figure S2.

Pareto chart—First step optimization—$3^{(3-1)}$ experimental design. Figure S3. Predicted values versus observed values—Step 2 Optimization—CCDR.

Author Contributions: Conceptualization, A.S.R. and L.P.d.S.V.; methodology, A.S.R. and L.P.d.S.V.; formal analysis, A.S.R. and L.P.d.S.V.; investigation, A.S.R.; writing—original draft preparation, A.S.R., H.L.G.R., P.B.G.d.M. and L.P.d.S.V.; writing—review and editing; Review, L.P.d.S.V. and L.P.d.S.V.; supervision, L.P.d.S.V.; project administration, L.P.d.S.V.; funding acquisition, L.P.d.S.V. and C.R.S. All authors have read and agreed to the published version of the manuscript.

Funding: Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES-PROEX, PPGEBB-UFPR) and the Conselho Nacional de Desenvolvimento Científico e Tecnológico do Brasil (CNPq) Process number 5440080/2022-3.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: The original contributions presented in the study are included in the article/Supplementary Materials, further inquiries can be directed to the corresponding author.

Acknowledgments: The authors thank the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES-PROEX) and the Conselho Nacional de Desenvolvimento Científico e Tecnológico do Brasil (CNPq) for the research funding.

Conflicts of Interest: The authors declare no conflicts of interest.

References

1. John, J.; Kaimal, K.S.; Smith, M.L.; Rahman, P.K.; Chellam, P.V. Advances in upstream and downstream strategies of pectinase bioprocessing: A review. *Int. J. Biol. Macromol.* **2020**, *162*, 1086–1099. [CrossRef] [PubMed]
2. Rebello, S.; Anju, M.; Aneesh, E.M.; Sindhu, R.; Binod, P.; Pandey, A. Recent advancements in the production and application of microbial pectinases: An overview. *Rev. Environ. Sci. Biotechnol.* **2017**, *16*, 381–394. [CrossRef]
3. Vandenberghe, L.P.d.S.; Valladares-Diestra, K.K.; Bittencourt, G.A.; de Mello, A.F.M.; Vásquez, Z.S.; de Oliveira, P.Z.; Pereira, G.V.d.M.; Soccol, C.R. Added-value biomolecules' production from cocoa pod husks: A review. *Bioresour. Technol.* **2021**, *344*, 126252. [CrossRef]
4. Okiyama, D.C.G.; Navarro, S.L.B.; Rodrigues, C.E.C. Cocoa shell and its compounds: Applications in the food industry. *Trends Food Sci. Technol.* **2017**, *63*, 103–112. [CrossRef]
5. ICCO. Quarterly Bulletin of Cocoa Statistics. In ICCO. 2024. Available online: https://www.icco.org/february-2024-quarterly-bulletin-of-cocoa-statistics/ (accessed on 24 May 2024).
6. Vásquez, Z.S.; de Carvalho Neto, D.P.; Pereira, G.V.M.; Vandenberghe, L.P.S.; de Oliveira, P.Z.; Tiburcio, P.B.; Rogez, H.L.G.; Góes Neto, A.; Soccol, C.R. Biotechnological approaches for cocoa waste management: A review. *Waste Manag.* **2019**, *90*, 72–83. [CrossRef] [PubMed]
7. Valladares-Diestra, K.K.; Porto de Souza Vandenberghe, L.; Ricardo Soccol, C. A biorefinery approach for pectin extraction and second-generation bioethanol production from cocoa pod husk. *Bioresour. Technol.* **2021**, *346*, 126635. [CrossRef] [PubMed]
8. Panak Balentić, J.; Ačkar, Đ.; Jokić, S.; Jozinović, A.; Babić, J.; Miličević, B.; Šubarić, D.; Pavlović, N. Cocoa Shell: A By-Product with Great Potential for Wide Application. *Molecules* **2018**, *23*, 1404. [CrossRef] [PubMed]
9. Antwi, E.; Engler, N.; Nelles, M.; Schüch, A. Anaerobic digestion and the effect of hydrothermal pretreatment on the biogas yield of cocoa pods residues. *Waste Manag.* **2019**, *88*, 131–140. [CrossRef] [PubMed]
10. Suhartini, S.; Hidayat, N.; Hadi, M.W. Co-digestion of cocoa pods and cocoa leaves: Effect of C/N ratio to biogas and the energy potential. *IOP Conf. Ser. Earth Environ. Sci.* **2021**, *733*, 012139. [CrossRef]
11. Avicenna, A.; Jimat, D.N.; Salleh, H.M.; Anuar, H. Effect of Fungal Pretreatment by Aspergillus Niger Assisted by Ultrasonication on Microfibrillated Cellulose Obtained from Cocoa Pod Husk. *J. Adv. Res. Mater. Sci.* **2020**, *21*, 1–13.
12. Jimat, D.N.; Putra, S.S.S.; Jamal, P.; Nawawi, W.M.F. Isolation of Nanocellulose Fibers (NCF) from Cocoa Pod (*Theobroma cacao* L.) via Chemical Treatment Combined with Ultrasonication. In *Advances in Nanotechnology and Its Applications*; Jameel, A.T., Yaser, A.Z., Eds.; Springer: Singapore, 2020; pp. 97–105.
13. Akinjokun, A.I.; Petrik, L.F.; Ogunfowokan, A.O.; Ajao, J.; Ojumu, T.V. Isolation and characterization of nanocrystalline cellulose from cocoa pod husk (CPH) biomass wastes. *Heliyon* **2021**, *7*, e06680. [CrossRef]
14. Ogunneye, A.L.; Ibikunle, A.A.; Sanyaolu, N.O.; Yussuf, S.T.; Gbadamosi, M.R.; Badejo, O.A.; Lawal, O.S. Optmized carboxymethil cellulose preparation from cocoa pod husks by surface response methodology. *J. Chem. Soc. Niger.* **2020**, *45*, 1–13.
15. Sluiter, A.; Hames, B.; Ruiz, R.; Scarlata, C.; Sluiter, J.; Templeton, D.; Crocker, D. *Determination of Structural Carbohydrates and Lignin in Biomass*; National Renewable Energy Laboratory: Golden, CO, USA, 2012.
16. Sluiter, A.; Hames, B.; Hyman, D.; Payne, C.; Ruiz, R.; Scarlata, C.; Sluiter, J.; Templeton, D.; Wolfe, J. *Determination of Total Solids in Biomass and Total Dissolved Solids in Liquid Process Samples*; National Renewable Energy Laboratory: Golden, CO, USA, 2008.

17. Sluiter, A.; Hames, B.; Ruiz, R.; Scarlata, C.; Sluiter, J.; Templeton, D. *Determination of Ash in Biomass*; National Renewable Energy Laboratory: Golden, CO, USA, 2008.
18. Sluiter, A.; Ruiz, R.; Scarlata, C.; Sluiter, J.; Templeton, D. *Determination of Extractives in Biomass*; National Renewable Energy Laboratory: Golden, CO, USA, 2008.
19. Reginatto, C.; Rossi, C.; Miglioranza, B.G.; dos Santos, M.; Meneghel, L.; da Silveira, M.M.; Malvessi, E. Pectinase production by *Aspergillus niger* LB-02-SF is influenced by the culture medium composition and the addition of the enzyme inducer after biomass growth. *Process Biochem.* **2017**, *58*, 1–8. [CrossRef]
20. Pedrolli, D.P.C.; Monteiro, A.; Gomes, E.; Carmona, E.C. Pectin and pectinases: Production, characterization and industrial application of microbial pectinolytic enzymes. *Open Biotechnol. J.* **2009**, *3*, 9–18. [CrossRef]
21. Poletto, P.; Borsóic, C.; Zeni, M.; da Silveira, M.M. Downstream processing of pectinase produced by *Aspergillus niger* in solid state cultivation and its application to fruit juices clarification. *Food Sci. Technol.* **2015**, *35*, 391–397. [CrossRef]
22. Handa, S.; Sharma, N.; Pathania, S. Multiple Parameter Optimization for Maximization of Pectinase Production by Rhizopus sp. C4 under Solid State Fermentation. *Fermentation* **2016**, *2*, 10. [CrossRef]
23. Miller, G.L. Use of Dinitrosalicylic Acid Reagent for Determination of Reducing Sugar. *J. Anal. Chem.* **1959**, *31*, 426–428. [CrossRef]
24. Sandri, I.G.; da Silveira, M.M. Production and Application of Pectinases from *Aspergillus niger* Obtained in Solid State Cultivation. *Beverages* **2018**, *4*, 48. [CrossRef]
25. Afzia, N.; Shill, N.; Kalita, B.J.; Sit, N. Optimization of conditions for production of pectinase in solid-state fermentation with *Aspergillus flavus* using dried Assam lemon peel powder as substrate. *Meas. Food* **2024**, *14*, 100166. [CrossRef]
26. Ezeh, N.O.; Nwagu, T.N.; Amadi, O.C.; Okolo, B.; Moneke, A.; Agu, R. Extracellular pectinase production from a novel Yarrowia phangngaensis XB3 grown on banana waste and its application in fruit juice clarification. *Biocatal. Agric. Biotechnol.* **2023**, *47*, 102614. [CrossRef]
27. ATCC. Aspergillus Niger van Tieghem 11414TM. In ATCC. 2024. Available online: https://www.atcc.org/products/11414 (accessed on 18 June 2024).
28. Biz, A.; Finkler, A.T.J.; Pitol, L.O.; Medina, B.S.; Krieger, N.; Mitchell, D.A. Production of pectinases by solid-state fermentation of a mixture of citrus waste and sugarcane bagasse in a pilot-scale packed-bed bioreactor. *Biochem. Eng. J.* **2016**, *111*, 54–62. [CrossRef]
29. Ahmed, I.; Zia, M.A.; Hussain, M.A.; Akram, Z.; Naveed, M.T.; Nowrouzi, A. Bioprocessing of citrus waste peel for induced pectinase production by Aspergillus niger; its purification and characterization. *J. Radiat. Res. Appl. Sci.* **2016**, *9*, 148–154. [CrossRef]
30. Cerreti, M.; Liburdi, K.; Benucci, I.; Spinelli, S.E.; Lombardelli, C.; Esti, M. Optimization of pectinase and protease clarification treatment of pomegranate juice. *Food Sci. Technol.* **2017**, *82*, 58–65. [CrossRef]
31. Nadaroglu, H.; Taskin, E.; Adigüzel, A.; Güllüce, M.; Demir, N. Production of a Novel Pectin Lyase From Bacillus pumilus (P9), Purification and Characterisation and Fruit Juice Application. *Rom. Biotechnol. Lett.* **2010**, *15*, 5167–5176.
32. Sharma, H.P.; Patel, H.; Sugandha. Enzymatic added extraction and clarification of fruit juices—A review. *Crit. Rev. Food Sci. Nutr.* **2017**, *57*, 1215–1227. [CrossRef]
33. Barman, S.; Sit, N.; Badwaik, L.S.; Deka, S.C. Pectinase production by Aspergillus niger using banana (Musa balbisiana) peel as substrate and its effect on clarification of banana juice. *J. Food Sci. Technol.* **2015**, *52*, 3579–3589. [CrossRef]

Disclaimer/Publisher's Note: The statements, opinions and data contained in all publications are solely those of the individual author(s) and contributor(s) and not of MDPI and/or the editor(s). MDPI and/or the editor(s) disclaim responsibility for any injury to people or property resulting from any ideas, methods, instructions or products referred to in the content.