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Saccharification of Agricultural Wastes and Clarification of Orange Juice by *Penicillium rolfsii* CCMB 714 Pectinase

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Abstract: Pectinases are enzymes used in several industrial processes. Seven agroindustrial wastes—jackfruit seed meal (*Artocarpus heterophyllus*), cocoa seed peel (*Theobroma cacao*), cocoa husks (*Theobroma cacao*), passion fruit husks (*Passiflora edulis*), mangosteen husks (*Garcinia mangostana*), malt residue (*Hordeum vulgare*) and the peach palm waste (*Bactris gasipaes* Kunth.)—were evaluated to produce a crude extract containing pectinase activity by *Penicillium rolfsii* CCMB 714. The jackfruit seed meal was chosen as the best substrate for solid-state fermentation, which was optimized with 4 mL of water as a wetting agent for 2 days at 35 °C and with a 0.5% nitrogen source, whereby the pectinase production increased by 44% (362.09 U/g). The obtained crude extract was characterized and applied to wastes saccharification and orange juice clarification. The pectinase showed better activity at a pH of 3.0 to 5.0 and 55 °C, it stably maintained over 80% of activity at 30–50 °C for up to 60 min and 1 mM $CuSO_4$ increased the pectinase activity by 17%. The saccharification of agroindustrial wastes (cocoa husks, mangosteen husks and passion fruit husks) resulted in 126.55 µmol/mL of reducing sugars from passion fruit husks, which represents an increase of 126% after optimization (45 °C for 22 h). For the clarification of orange juice, it was possible to reduce the absorbance of the juice by 55%. These results elucidate the potential of the low-cost pectinase solution from *P. rolfsii* CCMB 714 cultivated in jackfruit seed meal for both the enzymatic pretreatment of plant biomass and the application in beverage industries.

Keywords: enzymes; filamentous fungi; agroindustrial waste; saccharification; pectinase

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1. Introduction

A wide range of enzymes have important industrial and biotechnological uses, and among them, pectinase is one of the most commercially produced enzymes for biotechnological applications [1]. Pectinase acts on pectic substances, which are polysaccharides found in the cell wall of plants, and thus forms pectic acid [2]. Based on its action site, the pectinases can be classified as endopectinases, which randomly cleave at the internal linkages of pectin, and exopectinases, which cleave at the external units of the long chain polysaccharides [1].

This enzyme has different industrial applications [3], such as the clarification of grape and wine making [4] and the saccharification of plant biomass [5,6].

Ecological processes produce low-cost microbial enzymes from the use of plant biomass as the fermentation medium of the process. From this perspective, agroindustrial waste is considered an efficient solid support for the growth of micro-organisms and consequent enzymatic production [7,8].

Among enzyme-producing fungi, the genus *Penicillium* is the most widely exploited for this purpose [7,9–11]. However, due to its diversity, some species such as *P. rolfsii* require further studies. The species produces some enzymes, such as xylanases [12], cellulases, hemicellulases [13] and tannases [14]; however, there are no known reports in the literature on the production of pectinases.

For commercial exploitation, efficiency in enzyme production is a decisive factor. To this end, statistical tools are widely used to optimize production and application and reduce time and resources [15–17]. Among the available tools, the experimental design analyzes the independent variables and locates the optimal values for the best responses [18]. However, an artificial neural network (ANN) can be used to predict responses based on experimental results and thus produce knowledge through learning [19]. ANN models become accurate computational models of optimization techniques that have the ability to overcome research limitations such as the need to perform a large number of experiments and the planning restricted to the experimental domain [20]. In this way, the application of this methodology based on artificial intelligence results in more efficient enzyme production in solid-state fermentation compared to the classical optimization methodology [21,22].

The aim of the present work was to produce a crude enzymatic extract containing pectinase activity from *Penicillium rolfsii* CCMB 714 via solid-state fermentation using agroindustrial wastes as the substrate. To prove the versatility of this low-cost pectinase solution, the extract was applied in the saccharification of agroindustrial residues to obtain reducing sugar (which can be directed to different bioprocess) and in the clarification of orange juice (by hydrolyzing the pectic content). To achieve the results, statistical tools were applied, and a basic characterization of the pectinase activity was performed.

2. Materials and Methods

2.1. Culture Maintenance

The selected filamentous fungus was *Penicillium rolfsii* CCMB 714. This fungus was isolated from cocoa (*Theobroma cacao* L.) leaves from the Tropical Atlantic Forest region of Northeastern Brazil [14]. *Penicillium rolfsii* CCMB 714 was deposited in the Collection of Cultures of Micro-organisms of Bahia—CCMB (Feira de Santana, Brazil). A strain was stored in sterile distilled water at 4 °C and in 50% (*v*/*v*) glycerol at –18 °C. For reactivation, the fungus was grown in a PDA (Potato Dextrose Agar) medium at 30 °C for 7 days.

2.2. Agroindustrial Waste Obtention

The following seven agroindustrial wastes were obtained from local businesses (Ilhéus, Brazil): jackfruit seed meal (*Artocarpus heterophyllus*), cocoa seed peel (*Theobroma cacao*), cocoa husks (*Theobroma cacao*), passion fruit husks (*Passiflora edulis*) and mangosteen husks (*Garcinia mangostana*). The malt residue (*Hordeum vulgare*) was obtained from the microbrewery of the State University of Santa Cruz—UESC, Brazil, and the peach palm waste (*Bactris gasipaes* Kunth.) was obtained from the company Inaceres (Uruçuca, Brazil). All residues were dried in an oven at 50 °C for 24 h, ground in a knife mill until the appropriate grain size (5 mm) and stored in glass air-tight containers.

2.3. Solid-State Fermentation and the Crude Enzyme Extract Obtention

Fermentations were carried out in triplicate using 125 mL Erlenmeyer flasks containing 5 g of different wastes (evaluated individually) autoclaved at 121 °C/15 min (Logen, Brazil) and moistened with sterile distilled water. After homogenization, the different agroindustrial substrates were inoculated with 6 plugs of 1 cm in diameter of the mycelium of the fungus previously grown in PDA (Potato Dextrose Agar—Kasvi), and the culture was grown at 30 °C for 96 h [14]. After the incubation period, the crude multienzyme extract was obtained according to Bezerra et al. [22] with modifications, whereby 20 mL of sterile distilled water was added to the flasks, homogenized and kept under agitation (200 rpm) for 30 min at 25 °C. Then, the material was filtered in gauze and then centrifuged

at 15,000× *g* at 4 °C for 15 min. The clear supernatant was considered as the crude enzyme extract and was used for the described analyses.

2.4. Enzyme Assay

The enzyme activity was determined via the quantification of reducing sugar by using the 3,5-dinitrosalicylic acid method (DNS) [23] with a spectrophotometer (Bel Photonics 2000 UV, Piracicaba, Brazil) at 540 nm. The pectinase activity was measured by using 1% pectin citric (Biotec, Recife, Brazil) (*w*/*v*) in a sodium acetate buffer (50 mM/pH 4.5) at 40 °C for 30 min. The standard curve was constructed with D-galacturonic acid solutions, and one unit of enzymatic activity (U) was defined as the amount of enzyme capable of releasing 1 µmol of α-D-galacturonic acid per minute under the reaction conditions. The results were expressed in U/g (gram of dry substrate).

2.5. Study of Fermentation Conditions

The preselected jackfruit seed meal (5 g) was used as a solid substrate in a new fermentation step to initially investigate its supplementation with 1% (*w*/*w*) of three different nitrogen sources: cocoa skin, yeast extract and ammonium phosphate. Then, a Mixing Matrix was applied, with the levels of each component ranging from 0 to 0.33% (*w*/*w*); the response was measured as a function of the proportion between them, and the fermentation conditions were the same as in the previous step, and the best pectinase activity obtained was evaluated.

Based on the results obtained in the mixture matrix, an extended Box-Behnken experimental design was applied. The matrix was expanded to achieve a minimum number of experiments capable of training the artificial neural network (ANN), which is a statistical tool used in the subsequent step. The analyzed experimental variables were water volume (moisture) (*w*/*v*) (from 2.0 to 4.5 mL), time (from 2 to 12 days), cultivation temperature (from 15 to 40 °C) and concentration of nitrogen sources (from 0.5 to 3.0% p/p, using the previous results of the nitrogen sources obtained in the mixture matrix). The data obtained from the Box-Behnken matrix were analyzed by the ANN to obtain a computational model for the enzyme activity experiment. The ANN was applied using Matlab R2020b and a Deep Learning Toolbox. The ANN structure was a feedforward multilayer perceptron with four inputs: moisture volume, time, temperature and nitrogen source; one hidden layer using a hyperbolic tangent as the activation function; and one output (enzyme activity) using the linear activation function. The artificial neuron weight and biases training was performed by using the backpropagation method, which thus normalized the dataset, and by applying the Levenberg–Marquardt method with the mean of the squared error (MSE) cost function and early stopping with a maximum of 20 validation checks as the stopping criterion.

The number of artificial neurons in the hidden layer varied from 2 to 20. For each number of neurons, 20,000 tries were performed while changing the initial weights and biases and randomly dividing the dataset into training, validation and test sets. This exploratory training was required due to this case of applying an ANN to a small dataset composed of 19 experiment essays divided in 14, 3 and 3 pieces of data used to train, validate and test the model, respectively. The number of hidden-layer artificial neurons is defined by a uniform and large determination coefficient (R^2) among the training, validation and test sets, which give priority to simpler models. R^2 is a model generalization measure, and its uniformity is required to avoid overtrained models, while large R^2 values and a simpler model reduce overfitting. This methodology is based on an exhaustive search for an adequate model and is liable to suffer from data leakage [24], and it was successfully applied by Bezerra et al. [22] and De Menezes et al. [25].

In this way, it was possible to define the best fermentation conditions to obtain pectinase for the next step.

2.6. Physicochemical Characterization of Pectinases

To determine the optimum temperature of the enzyme, enzymatic assays were carried out at different temperatures (from 30 to 90 °C), and thermal stability was ensured by incubating the enzyme at different temperatures for up to 60 min, followed by the standard enzymatic assay. The optimum pH for enzyme activity was determined by incubating the substrate (1% *w*/*v* citrus pectin) prepared in different buffers at 0.05 M: sodium acetate (pH 3.0; 4.0 and 5.0), sodium phosphate (pH 6.0 and 7.0) and Tris-HCL (pH 8.0) following the assay of enzyme activity under standard conditions (40 °C/30 min) [16].

To evaluate the effect of ions and other reagents on the activity of the studied enzyme, the extract was incubated for 15 min at room temperature (25 ± 2 °C) with solutions containing 1 mM and 5 mM of the ion salts: Na_2CO_3, $NiSO_4$, $ZnSO_4$, $MgCl_2$, $CaCl_2$, $LiSO_4$, $MnSO_4$, $CuSO_4$ and the reagents Sodium Dodecyl Sulfate (SDS), Ethylenediamine Tetra-acetic Acid (EDTA) and β-mercaptoethanol. The enzyme activities were measured under standard enzyme assay conditions and were compared to the control (without the addition of ions and reagent), which was considered as 100%.

2.7. Application of Enzyme Extract with Pectinase Activity

2.7.1. Saccharification of Agroindustrial Waste

Three agroindustrial wastes, namely cocoa husks, mangosteen husks and passion fruit husks, were used for the saccharification and production of reducing sugars. The choice of agroindustrial wastes was due to its availability in the region and its chemical constitution rich in pectin. A total of 5 mL of enzyme extract (155.6 U/g of pectinase activity) was added to 0.25 g of wastes (50 g/L) and was incubated at 50 °C for 24 and 48 h [22]. After incubation, the material was filtered through a gauze and was centrifuged at 5000× *g* at 4 °C for 5 min. The obtained supernatant was used for the analysis of the reducing sugars by 3,5-dinitrossalissilic acid (DNS) [23]. All the assays were performed in triplicate, and the produced reducing sugar was expressed in μmol/mL.

The passion fruit husk was selected for the next stage of process optimization by using a response surface methodology (RSM). In this step, a central composite rotational design (CCRD) matrix was applied to evaluate the influence of the variables temperature (30.9 °C (−1.41), 35 °C (−1), 45 °C (0), 55 °C (+ 1) and 59.1 °C (+ 1.41)) and time (2 (−1.41), 5 (−1), 12 (0), 19 (+ 1) and 22 h (+ 1.41)) on saccharification. Statistical analyses and response surfaces were obtained from the 11 experimental runs analyzed using Statistica v. 8 (Statsoft, Tulsa, OK, USA). The parameter values used to enhance the production of reducing sugars were experimentally validated in triplicate.

The structure of the residue under the conditions before and after treatment with the enzyme extract with pectinase activity was analyzed with Fourier transform infrared spectroscopy (FTIR) in attenuated total reflection (ATR) mode to characterize the sample. Measurements were performed in the spectral range between 650 and 4000 cm^{-1} in a Perkin Elmer Spectrum 400 FT-IR/FT-NIR Spectrometer (PerkinElmer, UK). The obtained spectra were the averages of 10 scans with a resolution of 4 cm^{-1}, which was processed using Perkin Elmer Spectrum software (Spectrum_Multimedia_V2.1).

2.7.2. Orange Juice Clarification

The orange juice used in the experiments was manually obtained from fresh oranges from a local supermarket in Ilhéus, Brazil, using a screw-type juice extractor. To evaluate the potential of the enzyme extract to clarify orange juice, a CCRD matrix of 17 experiments was used with the following variables: time in minutes (66.4 (−1.68), 80 (−1), 100 (0), 120 (+1) and 133.6 (+1.68)), enzyme concentration (*v*/*v*%) (2.64 (−1.68), 4 (−1), 6 (0), 8 (+1) and 9.36 (+1.68)) and temperature (23.2 °C (−1.68), 30 °C (−1), 40 °C (0), 50 °C (+1) and 56.8 °C (+1.68)). In each experiment, 25 mL of orange juice was added to the pectinase enzymatic extract. After incubation, the juice was previously heated at 90 °C for 5 min to inactivate the enzyme, followed by centrifugation at 3000× *g* at 4 °C for 10 min. The juice was analyzed in a spectrophotometer with an absorbance reading at 660 nm, and the lower

absorbance showed the increase in the juice clarification [26]. The obtained results were evaluated using the software Statistica v.8 (Statsoft, Tulsa, OK, USA). The optimal point was experimentally tested in triplicate.

3. Results and Discussion

3.1. Screening of Pectinase Production Using Agro-Industrial Wastes as Substrates

After solid-state fermentation of the fungus *P. rolfsii* CCMB 714 using different agroindustrial wastes as substrates, it was observed that jackfruit seed meal was the best to produce pectinases (247.28 ± 28.5 U/g) (Figure 1). Based on the highest pectinase activity detected, the jackfruit seed meal was selected as the substrate to be used for the next steps of the solid-state fermentations.

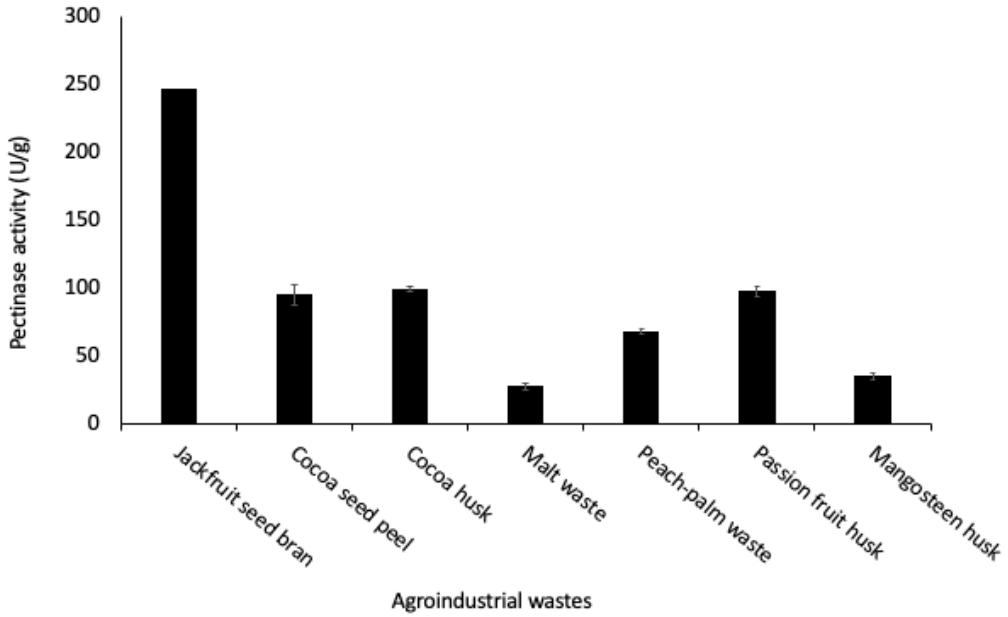


Figure 1. Production of pectinases by *P. rolfsii* CCMB-714 during solid-state fermentation at 30 °C for 96 h, using different agroindustrial wastes as substrates.

This positive result with the jackfruit waste was probably due to its constitution. The elemental composition of jackfruit waste varies, but on average it is approximately 44% carbon, 6% hydrogen, 38% oxygen, 10% nitrogen and small amounts of other elements such as sulfur, phosphorus and potassium [27,28]. Jackfruit has a low amount of calcium pectate in its structure; however, the present content varies greatly (1–2.28%) depending on the part of the plant, plant varieties, plant growth stage and plant tissues, as it participates in its hardening as rind, core [29,30] and seed (0.49–0.70%) [31]. The composition of the medium interferes with the production of pectinases, as also demonstrated with *Bacillus licheniformis* KIBE-IB3 cultivated with different agroindustrial wastes, where wheat bran was better at inducing the production of polygalacturonase (974 U/mg) after 24 h of cultivation, probably due to the presence of essential nutrients in this residue [32,33]. Likewise, to produce pectinase with *Aspergillus niger*, the use of orange pomace and sugarcane bagasse maintained the best conditions for pectinases in a tray bioreactor [34].

3.2. Study of Fermentation Conditions

Nitrogen is essential for micro-organisms, and different nitrogen sources can result in different responses for each micro-organism and each substrate [22]. The results showed that the addition of nitrogen sources increased the production of pectinase (327.86 U/g) by 33% in relation to the initially obtained value (Supplementary Materials Table S1). The responses obtained were adjusted to a quadratic model, and the quality of fit of the mathematical model was evaluated with an ANOVA with 95% reliability. The resulting

contour curve (Figure 2) showed the interaction between the factors and the response. The mean of the three points at 33.33% (327.86 ± 18.72 U/g) was very similar to the model predicted for the same situation (323.38 U/g); therefore, this point was selected for the next step. The adjusted coded model was:

$$\text{Pec (U/g)} = 242.31\cdot(\text{CSP}) + 251.39\cdot(\text{YE}) + 265.18\cdot(\text{AP}) + 262.71\cdot(\text{CSP})\cdot(\text{YE}) + 182.95\cdot(\text{CSP})\cdot(\text{AP}) + 188.13\cdot(\text{YE})\cdot(\text{AP})//R^2 = 0.9058;\ R^2_{adj} = 0.7487//F_{calc} = 5.767;$$

p-value = 0.900.

where Pec: pectinase, CSP: cocoa seed peel, YE: yeast extract and AP: ammonium phosphate.

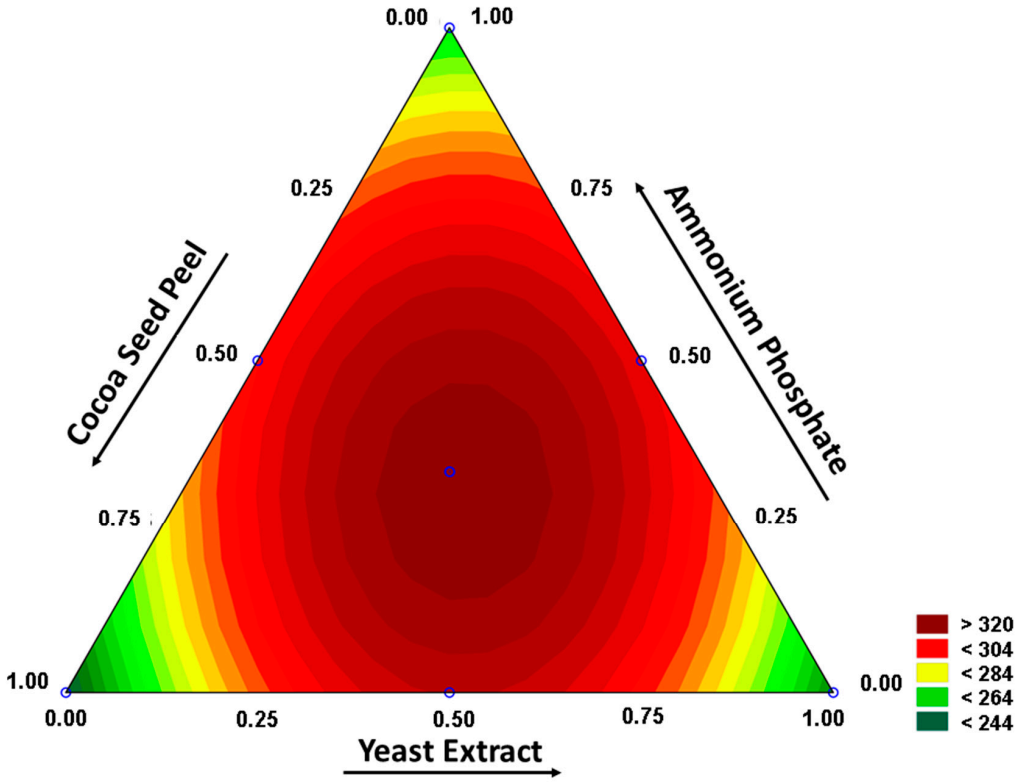


Figure 2. Contour curve for the effect of coded compositions of nitrogen sources: cocoa seed peel (CSP), yeast extract (YE) and ammonium phosphate (AP) on pectinase production (PEC, U/g).

Supplementary Materials Table S2 shows the results obtained by the analysis of variance and the evaluated mathematical models (linear, quadratic and special cubic), as well as the best-adjusted quadratic model used in the analysis and generation of the surface. The residues showed random variability.

The ANN resulting from the methodology described in Section 2.5 had four artificial neurons in the hidden layer obtained for the dataset, and the sets division is presented in Table 1. Experiments 17, 18 and 19 were the Box-Behnken central point triplet, which were transformed by only one dataset by the average of the enzymatic activity, which subsequently reduced the 22 experiments to 19 pieces of data. The other present information presented is the ANN estimated enzyme activity, estimation residue and MSE and R^2 for the total dataset and the training, validation and test sets. Supplementary Materials Figure S1 shows the data used to evaluate the model quality by comparing its residues' normalized histogram of residuals to a normal probability distribution function.

Table 1. Experiment planning and estimate values by ANN model, residuals and performance measures for production of pectinases by *P. rolfsii* CCMB–714. The analysis was the experimental relationship between moisture (M, volume), time (t, days), temperature (T, °C), nitrogen source (NS, %), enzyme activity (EA, U/g) and estimated enzyme activity (EEA).

Experiment	M (mL)	t (Days)	T (°C)	NS (%)	EA (U/g)	ANN Estimated (EEA) *	Residue (EA-EEA)	MSE	R^2
				Training set					
1	2.5	4	20	1	199.91	199.33	0.58		
2	2.5	4	20	2	273.20	272.65	0.55		
3	2.5	4	30	1	273.20	267.71	5.49		
7	2.5	8	30	1	197.95	212.22	−14.27		
8	2.5	8	30	2	346.56	287.90	58.66		
9	3.5	4	20	1	227.12	227.38	−0.26		
10	3.5	4	20	2	306.58	307.24	−0.66		
11	3.5	4	30	1	257.23	258.95	−1.72	351.12	0.87
14	3.5	8	20	2	233.80	232.21	1.59		
15	3.5	8	30	1	253.17	240.20	12.25		
17	3	6	25	1.5					
18	3	6	25	1.5	196.16 **	198.13 **	−1.97 **		
19	3	6	25	1.5					
20	2	10	15	2.5	312.89	284.08	28.81		
21	4	2	35	0.5	362.09	377.71	−15.62		
22	4.5	12	40	3	290.83	288.36	2.47		
				Validation set					
12	3.5	4	30	2	281.54	290.38	−8.84		
13	3.5	8	20	1	187.86	190.32	−2.46	28.04	0.98
16	3.5	8	30	2	255.35	255.46	−0.11		
				Testing set					
4	2.5	4	30	2	330.10	355.33	−25.23		
5	2.5	8	20	1	181.12	180.68	0.44	326.90	0.93
6	2.5	8	20	2	186.05	204.60	−18.55		
							Total Dataset	299.02	0.90

* Data rounded to 2 decimals. ** Central point average.

The optimization of the parameters and the medium that improves pectinase production is very important to obtain a high enzyme quantity and suitable cost effectiveness. The proposed methodology applied to a small dataset aims to reduce the overtraining and overfitting effects to the model prediction while resulting in a sufficiently complex model to interpolate the dataset. Equivalently, it is possible "to learn" the experimental relationship between moisture (volume), time, temperature, nitrogen source and enzyme activity. The observations that support the model adequate quality are the large and uniform values of R^2 presented in Table 1, which include a small residue average and a residual histogram close to a normal plot (Supplementary Materials Figure S1). However, the large residual standard deviation resulted from some large residues (Table 1), and the central point triplet standard deviation (10.199) indicated that model estimation was possibly subject to moderate estimation errors. We conclude that, although possibly subjected to estimations errors compatible with the residues' standard deviation, the resulting ANN is adequate to qualitatively model the experiment, especially because it is a simple model (for an ANN) with a large R^2, which indicates a high generalization capability.

It is important to observe that this dataset is particularly complex because the maximum enzymatic activity is an outlier (essay 21, Table 1). Observing the Pearson correlation coefficients between each input and the output provided in Supplementary Materials Table S3, a small correlation was observed for all inputs, although, while excluding essay 21, a significant positive correlation was observed between the nitrogen source and enzymatic activity. In Table 1, the maximum enzyme activity is obtained for the minimum nitrogen source.

The tridimensional perspectives of the ANN experiment model are shown in Supplementary Materials Figure S2. Each perspective was obtained by varying two inputs and fixing the other two to the value of essay 21. The 3D graphics show how the ANN improved the model complexity compared to the quadratic and linear models and justified why it is a powerful tool to better represent enzyme activity experiments. Moreover, it was observed that the enzyme activity resulting from essay 21 was close to the maximum possible for the model interval, while the distance to the maximum for all perspectives was compatible with the provided standard deviations. A significant increase was not expected in the enzyme activity according to the variations in the inputs in the interval of the Table 1 essays, and the optimal condition for the enzyme activity was a moisture level of 4 mL, a fermentation time of 2 days, a temperature of 35 °C and a nitrogen source of 0.5%.

Sethi et al. [35] showed that the use of an inorganic nitrogen source positively influences pectinase biosynthesis, and ammonium persulfate was the best choice followed by sodium nitrate and urea. The organic nitrogen substrates utilized as peptone also increased the pectinase production during fungi growth. The authors encourage the use of this resource as an effective and low-cost supplementation. In the present study, one of the nitrogen sources used was the cocoa seed peel, which is also an agroindustrial waste that was used with the jackfruit waste as a low-cost substrate that increased the production of the pectinase.

The interaction between the variables substrate, carbon and nitrogen source and moisture is important to evaluate the production of enzymes in solid-state fermentation (SSF) [36]. The variables time and temperature of incubation positively influence the production of enzymes during the growth and development of the fungus, as demonstrated in the work with *P. roqueforti* using jackfruit waste in SSF to produce endoglucanases and xylanases, while water activity did not influence the production of these enzymes [37].

3.3. Physicochemical Characterization of Pectinase in the Extract

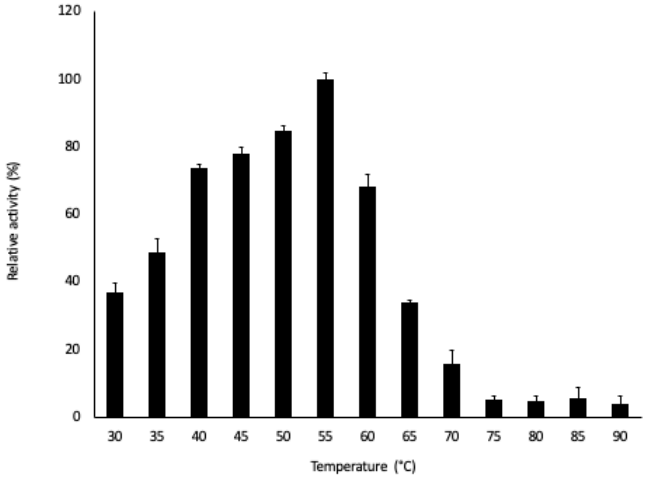
According to Figure 3a, *P. rolfsii* CCMB-714 pectinases showed the best activity between 40 and 55 °C; at temperatures above the optimum, the activity declined. Similar studies were also observed for *Aspergillus aculeatus* pectinase, which had an optimal temperature of 40 °C [34] and *Schizophyllum commune* at 45 °C [38]. According to Figure 3a, *P. rolfsii* CCMB-714 pectinases showed the best activity between 40 and 55 °C, and at temperatures above the optimum, the activity declined. However, the pectinases showed thermal stability at 30–50 °C with 80% relative activity until 60 min of incubation (Figure 3c). Similar studies were also observed for *Aspergillus aculeatus* pectinase, which had an optimal temperature of 40 °C [34]; *Schizophyllum commune*, which had an optimal temperature of 45 °C [38]; and *Bacillus subtilis*, where the optimum temperature was 50 °C and it was stable at this temperature for up to 60 min [26]. The characteristics of *P. rolfsii* pectinase were better than those found for *Geotrichum candidum* AA15, which showed optimal activity at 35 °C and a low thermo stability, probably due to enzyme denaturation [29].

These results demonstrate the importance of the characterization of enzymes and that these pectinases can be efficiently applied in processes that demand higher temperatures, such as in the food-processing industry, as their thermal action characteristics would allow the preservation of the beneficial properties of foods [39].

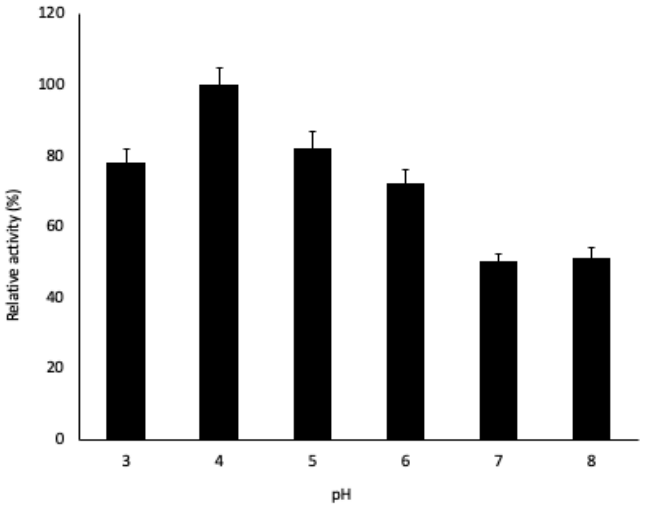
The evaluation of the enzyme against different pHs (3.0 to 8.0) showed that it can be considered an acid pectinase, as its best activities were in the pH range between 3.0 and 5.0 (Figure 3b). Similar results were found for polygalacturonase from *A. fumigatus*, which had its optimal activity at pH 5.0 [40]. Acid pectinases are versatile and have important industrial applications, such as in the beverage industry [41,42] and in the biofuel industry [43], as they have properties for the saccharification of biomass.

The effect of ions and reagents on pectinase activity was analyzed (Supplementary Materials Table S4). Among the ions used, Na_2CO_3, $NiSO_4$, $ZnSO_4$, $MgCl_2$, $CaCl_2$, $LiSO_4$ and $MnSO_4$ had no significant effect on pectinase activity at both concentra-

tions. $CuSO_4$ increased pectinase activity by 17% at a 1 mM concentration and strongly inhibited it at a 5 mM concentration, which reduced the enzymatic activity by 43.2%.



(**a**)



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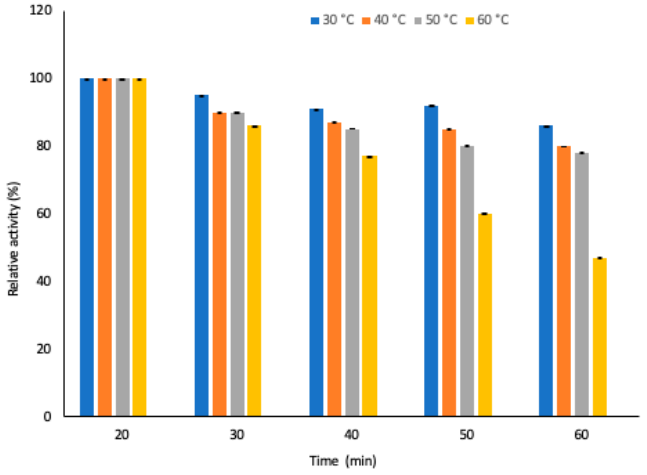


(**c**)

Figure 3. Effects of temperature (**a**), pH (**b**) and temperature stability (**c**) on pectinase activity produced by *P. rolfsii* CCMB-714.

In the present study, EDTA was the only reagent that positively affected pectinase activity, and at a concentration of 5 mM, the increase was twofold. Considering the chelating nature of the reagent [44], the result indicates that the pectinase obtained was not a metalloenzyme; that is, it did not depend on the presence of ions for catalysis, which thus corroborated the findings obtained from the analysis of the effects of ions. Additionally, the reagent had a significant positive effect on pectinase efficiency, possibly due to its ability to remove the cross-bridges of the divalent ions present in pectin polymers, which thus modified the structure and altered its accessibility for enzyme action [45].

β-mercaptoethanol, a disulfide-bridge reducing agent [40], decreased the pectinase activity by up to 20%, which suggested that the disulfide bonds present in the structure were few or not found in vulnerable regions of the protein. On the other hand, SDS at a concentration of 5 mM drastically reduced the activity (96%); however, at a concentration of 1 mM, the activity remained stable. SDS is an anionic surfactant that denatures proteins even at millimolar concentrations, and the conformational transitions caused vary according to the used surfactant concentrations [45].

3.4. Application of Pectinase

3.4.1. Saccharification of Agricultural Wastes

All the residues tested had a chemical constitution rich in pectin. Previous studies have shown that mangosteen peels are a source of pectin with a high degree of purity, with approximately 75.98% polygalacturonic acid [46]. A passion fruit peel is known as an important source of pectin with potential for extraction for industrial purposes [47,48]. Studies on the constitution of cocoa husks indicate that cellulose, hemicellulose and pectic substances are the main polysaccharides present [49]. In this way, the characteristics of the residues added to the physicochemical characteristics of the enzymatic extract indicated a promising performance in the saccharification processes, without the need for the pretreatment of the raw material. After analyzing the action of the enzymatic extract produced by *P. rolfsii* CCMB-714 with different agroindustrial wastes, it was observed that there was the production of reducing sugars in the residues evaluated at different times (Figure 4a). These results showed that the passion fruit peel was the most promising waste for the production of reducing sugars for the two tested times, 24 and 48 h (48.39 ± 0.98 and 45.18 ± 2.46 μmol/mL, respectively). In addition, it was observed that the highest production occurred up to 24 h of incubation, after which there was no significant increase in the production of reducing sugars. Therefore, in the optimization process, the maximum incubation time was stipulated in this interval.

According to the CCRD, the 22 matrices performed to evaluate the influence of temperature and incubation time on the saccharification of passion fruit peels showed that the highest experimental response was obtained with experiment 8 (126.55 μmol/mL of reducing sugars). The results obtained were fitted to a quadratic model that did not have its statistically nonsignificant terms (p-value > 0.10) removed since this caused a reduction in R^2 from 0.8032 to 0.6656 and in R^2_{adj} from 0.6065 to 0.5820 (Supplementary Materials Table S6). Thus, the complete model was submitted to an ANOVA, and the regression was approved with 90% reliability (F_{cal} = 4.08 and p-value = 0.0744), and the lack of fit was not considered statistically significant (F_{cal} = 0.31 and p-value = 0.8200).

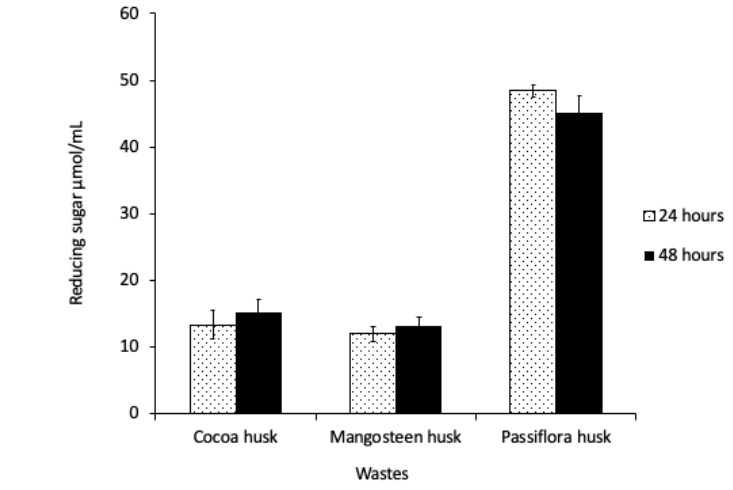
The obtained contour curve is shown in Figure 4b, in which it is possible to observe a region of greater responses above experiment 8, as mentioned above, which was carried out at temperature level 0 (45 °C) and a higher time level (22 h). Under these conditions, the theoretical value provided by the model was 117.14 μmol/mL, which represents an 8% deviation from the value of 126.55 μmol/mL. However, experiment 8 was performed in triplicate, and the obtained value was 84.84 ± 3.24 μmol/mL, which represents a deviation of approximately ~28% from the theoretical value, probably due to inherent experimental variations. Thus, two new temperatures were selected (40 and 50 °C) with the same time of 22 h, which were performed in triplicate together with experiment 8 (45 °C/22 h). The means obtained were evaluated with the Tukey test (95% reliability),

which revealed no statistically significant difference between the three conditions. This result agreed with the region with the highest responses over the evaluated temperature range (30 to 60 °C), as seen in Figure 4b. The equation for the generated coded model was:

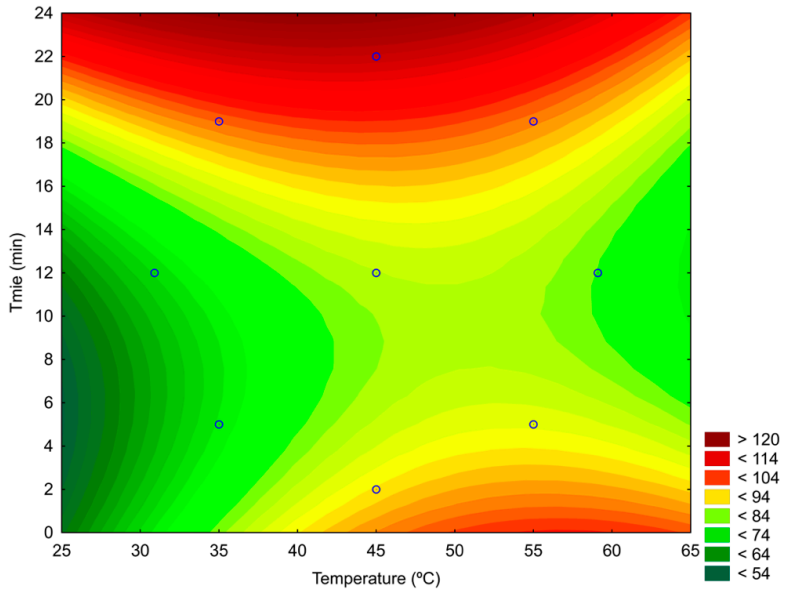
$$\text{RS}\ (\mu\text{mol/mL}) = 85.871 + 3.396\cdot(\text{T}) - 4.500\cdot(\text{T}^2) + 8.800\cdot(\text{t}) + 9.486\cdot(\text{t}^2) - 4.182\cdot(\text{T})\cdot(\text{t})\,//\,\text{R}^2 = 0.8032;\ \text{R}^2_{\text{adj}} = 0.6065$$

where RS = reducing sugar, T = temperature (°C) and t = time (h).

In a similar study, using a multienzymes extract of *Aspergillus niger* with high pectinase activity, this enzyme was shown to play a key role in the saccharification of agave biomass. The enzyme acted on the pectic structure of the plant and allowed a better coordination between the various enzymes that destroy the structure of lignocellulosic biomass and thus release significant amounts of reducing sugars under mild conditions at 35 °C and pH 5.0 [17]. Parkhey et al. [50] highlight the importance of identifying and optimizing the parameters of the saccharification process as well as the statistical tools involved to establish an efficient and sustainable bioconversion process.

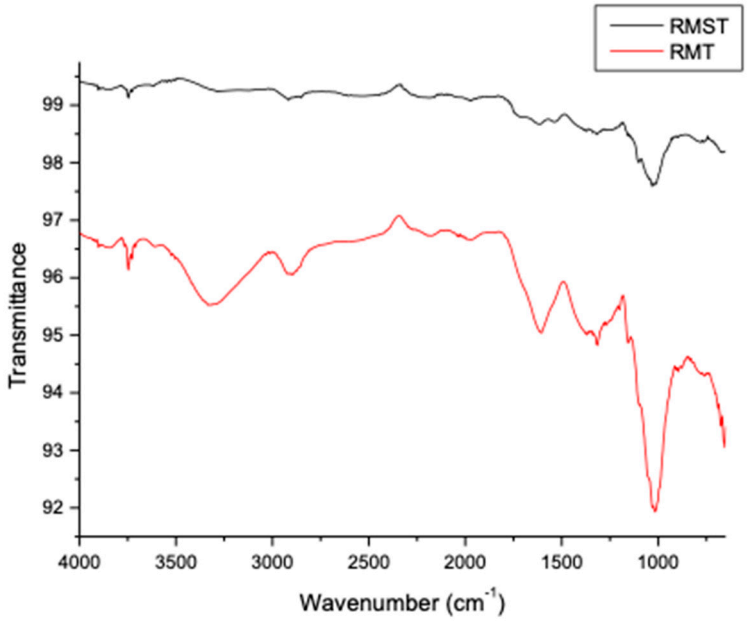


(**a**)



(**b**)

Figure 4. *Cont.*



(**c**)

Figure 4. Enzymatic saccharification of agroindustrial residues. (**a**) Cocoa, mangosteen and passion fruit husks expressed as produced reducing sugars produced; (**b**) contour curve for the coded factors temperature (T, °C) and time (t, h) on the reducing sugars' (RS, µmol/mL) response to passion fruit peel saccharification by *P. rolfsii* CCMB-714 pectinases; and (**c**) comparative spectrum by FTIR spectroscopy of passion fruit residue without enzymatic treatment (RMST3—upper profile in black) and with enzymatic treatment (RMT—lower profile in red).

The infrared spectra of the passion fruit peel samples without and with treatment with the enzymatic extract (Figure 4c) showed significant differences, which indicated the effectiveness of the action of the enzymes in the extract. Between 3600–3905 cm^{-1}, bands could be observed that were possibly attributed to the molecular vibrations of hydroxyl groups (O-H), which are found in carbohydrates, carboxylic acids, protein species, lignin, cellulose and water molecules. This difference between the reduced spectra in the residue treated with the enzymatic extract suggests a degradation of the cellulose and hemicellulose present in the plant biomass [17].

It is important to highlight the appearance of a strong spectral band at 3324.29 cm^{-1} in the samples of passion fruit peels treated with the enzymatic extract and their nonexistence in the spectrum of the untreated passion fruit peels, which thus indicates the modification of the sample after the action of the enzymes. This wavenumber value and the characteristic broad band are usually attributed to the O-H stretch that is generally observed between 3500 and 3000 cm^{-1}. Several bands of weak and medium intensity in the range of 3000–2850 cm^{-1}, attributed to the C-H stretching of aliphatic compounds, were also observed. Wavenumber values in the region of 2500–2000 cm^{-1} are attributed to triple bond stretching, with CC bonds absorbing between 2300 and 2050 cm^{-1} and CN nitrile groups absorbing between 2300 and 2200 cm^{-1}. The wave number values of 1975 cm^{-1}, 1612.14 cm^{-1} and 1533.57 cm^{-1} for the untreated passion fruit residues and 1969.29 cm^{-1} and 1606.43 cm^{-1} for the passion fruit residues with enzymatic extract treatment were due to C=C and C=O stretching.

Wavenumber values in the range of 1500–600 cm^{-1} do not allow for the assigning of individualized absorption bands relative to the infrared spectrum. This region is called the fingerprint region because many similar molecules produce different absorption patterns at these frequencies. Here, again, we see differences between the untreated and enzyme-treated passion fruit peels.

Although the wave number values were located in these spectral bands for the passion fruit peel residues without and with enzymatic treatment, the values assigned were different in each case; particularly, there were prominent changes observed between 3500 and 3000 cm^{-1} that were attributed to the O-H stretching. These results showed the structural and/or conformational changes caused by the action of the enzymes and the importance of the involvement of pectinases that degrade pectic substances and hydrolyze glycosidic bonds along the carbon chain and thus contribute to the saccharification process.

3.4.2. Orange Juice Clarification

Additionally, the extract produced by *P. rolfsii* was applied in the clarification of orange juice. Through a CCDR matrix, the best conditions of time, extract volume and temperature were evaluated to reduce the absorbance values. Considering the absorbance of the untreated juice, the greatest reduction was observed with assay 4 (Table 2). With the data obtained, it was possible to perform the coefficient analysis of the quadratic model (data not shown), and the nonstatistically significant terms (p-value > 0.15) were removed from the model because despite the decrease in R^2 (from 0.9264 to 0.9043), an increase in R^2_{adj} was observed (from 0.8310 to 0.8724) (Supplementary Materials Table S7). The reduced model was then analyzed with an ANOVA. The regression was approved with 95% reliability (F_{cal} = 28.41 and p-value < 0.0001), and the lack of fit was not statistically significant (F_{cal} = 0.53 and p = 0.7958). The adjusted coded model was:

$$abs = 0.1332 - 0.0459 \cdot (V) + 0.0262 \cdot (V^2) + 0.0159 \cdot (T) + 0.0147 \cdot (t) \cdot (T) // R^2 = 0.9042;\ R^2_{adj} = 0.8724$$

where abs = absorbance, T = temperature (°C) and t = time (h).

Table 2. Matrix of the central composite rotational design (CCDR 2^3) for the coded and uncoded variables: time (min), volume of enzymatic extract (mL) and temperature (°C) and the absorbance response (read at 600 nm) from the clarification of orange juice by *P. rolfsii* CCMB-714 pectinases.

	Variables			Responses	
Experiment	**Time (Minutes)**	**Enzyme Extract Volume (mL)**	**Temperature (°C)**	**Absorbance Predict**	**Absorbance Experimental**
1	−1 (80)	−1 (4)	−1 (30)	0.187	0.181
2	1 (120)	−1 (4)	−1 (30)	0.182	0.189
3	−1 (80)	1 (8)	−1 (30)	0.119	0.123
4	1 (120)	1 (8)	−1 (30)	0.084	0.081
5	−1 (80)	−1 (4)	1 (50)	0.197	0.215
6	1 (120)	−1 (4)	1 (50)	0.252	0.263
7	−1 (80)	1 (8)	1 (50)	0.113	0.121
8	1 (120)	1 (8)	1 (50)	0.113	0.157
9	−1.68 (66.4)	0 (6)	0 (40)	0.136	0.113
10	1.68 (133.6)	0 (6)	0 (40)	0.120	0.123
11	0 (100)	−1.68 (2.64)	0 (40)	0.285	0.275
12	0 (100)	1.68 (9.36)	0 (40)	0.137	0.120
13	0 (100)	0 (6)	−1.68 (23.2)	0.131	0.114
14	0 (100)	0 (6)	1.68 (56.8)	0.108	0.135
15	0 (100)	0 (6)	0 (40)	0.162	0.117
16	0 (100)	0 (6)	0 (40)	0.136	0.165
17	0 (100)	0 (6)	0 (40)	0.136	0.130

In this way, it was possible to generate the contour curves, in which the regions with the lowest responses indicate the best conditions for the clarification of orange juice. The analysis made it possible to identify (within the analyzed ranges) the coded conditions of time = +1.68 (uncoded value = 133.6 min), extract volume = +1.0 (uncoded value = 8.0 mL) and temperature = −1.68 (uncoded value = 23.2 °C), which resulted in a theoretical absorbance value of 0.0451 (Supplementary Materials Figure S3a–c). Under these same conditions, the orange juice was clarified in triplicate; we obtained an absorbance value of

0.113 ± 0.013, which represents a deviation of 60% from the value predicted by the model. However, in comparison with the control (juice without clarification), the experimental value represents a reduction of 55% of the absorbance value, which evidences the efficiency of the enzymatic extract for the clarification process. Similar results were found for the application of pectinases from *G. candidum* AA15, in which an orange juice clarification of 61% with 4% (*v*/*v*) pectinase occurred for 180 min at 30 °C [29]. In addition, the use of enzymatic crude extract for juice clarification has been extensively studied [41] due to the ease of obtaining the extract and lower cost, as it does not require enzyme purification steps [30,51].

4. Conclusions

P. rolfsii CCMB-714, for first time, was shown to effectively produce pectinase via solid-state fermentation by using agroindustrial wastes, especially jackfruit seeds. The use of statistical tools showed an increase in the production of pectinase by up to 44% when compared to the initial conditions. In addition, the physicochemical characteristics of the crude enzyme, being an acid pectinase and having an optimal temperature between 40 and 50 °C, and with thermal stability, may favor its use in the food and beverage industries, such as the clarification of fruit juices, as well as for the obtention of easily fermentable sugars for biofuel production.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/fermentation9100917/s1, Figure S1. Histogram of residuals and approximation of normal probability density function of pectinase activity produced by *P. rolfsii* CCMB-714; Figure S2. Tridimensional perspectives of the ANN experiment model. Each perspective was obtained by varying two inputs and fixing the other two to the value of experiment 21. (a) T = 35/FN = 0.5; (b) t = 32/FN 0.5; (c) t = 2/T = 35; (d) V = 4/FN 0.5; (e) V = 4/T = 35; and (f) V = 4/t = 2. Asterisks provide a comparison between experiment 21 enzyme activity (*) and ANN model estimation. Moisture (M), time (t), temperature (T), nitrogen (FN) and enzymatic activity (EA, U/g); Figure S3. Contour curves for the relationships between the coded factors: (a) volume of enzymatic extract (V, mL) versus time (t, min), (b) temperature (T, °C) versus t (time, min) and (c) T (°C) versus V (mL) for the absorbance response (abs) from orange juice clarification by *P. rolfsii* CCMB-714 pectinases. Table S1: Mixing Matrix using nitrogen sources: cocoa seed peel (CSP), yeast extract (YE) and ammonium phosphate (AP) as a supplement to the substrate (5 g) of jackfruit seed meal at a final concentration of 1% (*w*/*w*) for production of pectinases (PEC, U/g) by *P. rolfsii* CCMB-714; Table S2: Results obtained for ANOVA showing the evaluated mathematical models and the quadratic used to generate the surface using nitrogen sources: cocoa seed peel (CSP), yeast extract (YE) and ammonium phosphate (AP) as a supplement to the substrate (5 g) of jackfruit seed meal at a final concentration of 1% (*w*/*w*) for production of pectinases (PEC, U/g) by *P. rolfsii* CCMB-714; Table S3: Pearson correlation coefficients between each input and output. Second line (*) computed without 21st experiment. The correlation was between moisture (M), time (t), temperature (T), nitrogen source (NS) and the enzyme activity (EA, U/g); Table S4: Analysis of the effect of ions and reagents (1 and 5 mM) on the pectinase activity produced by *P. rolfsii* CCMB–714, values expressed as relative activity (%); Table S5: Matrix of the central composite rotational design (CCDR 2^2) for the coded and uncoded independent variables: temperature (°C) and time (h), and the reducing sugar response (μmol/mL) from the saccharification of passion fruit peel by *P. rolfsii* CCMB-714 pectinases; Table S6: Table of the coefficient model chosen according to statistical significance by the *t* test and *p* value. The test evaluated the influence of temperature and incubation time on the saccharification of passion fruit peel by pectinase from *P. rolfsii* CCMB-714; Table S7: Table of coefficient model chosen according to statistical significance by the *t* test and *p* value. The test evaluated the time, volume of enzymatic extract, temperature and the absorbance response (read at 600 nm) from the clarification of orange juice by *P. rolfsii* CCMB-714 pectinases.

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A.P.T.U., A.M.d.C., L.C.S., E.A.-O., E.G.P.d.S., T.P.d.C. and K.M.M. All authors have read and agreed to the published version of the manuscript.

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