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Extraction of Cellulases Produced through Solid-State Fermentation by *Trichoderma reesei* CCT-2768 Using Green Coconut Fibers Pretreated by Steam Explosion Combined with Alkali

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Abstract: The industrial processing of coconut to produce valuable foods, such as water and milk, generates large volumes of waste, especially the fruit shell. Despite this, material can be used in bioprocess applications, e.g., the production of enzymes, its recalcitrance hinders the cultivation of microorganisms, and low productivity is usually achieved. In this study, the production of cellulolytic enzymes through solid-state fermentation (SSF) and their extraction was investigated using the green coconut fiber pretreated by steam explosion, followed by alkali. The fungus *Trichoderma reesei* CCT-2768 was cultivated, using an experimental design, to study the effect of the water activity and the amount of biomass in the reactor. The combination of the pretreatment strategies yielded more porous biomass, with less hemicellulose (5.38%, compared to 10.15% of the raw biomass) and more cellulose (47.77% and 33.96% in the pretreated and raw biomasses, respectively). The water activity significantly affected the production of cellulases, with maximum activity yielded at the highest investigated value (0.995). Lastly, the extraction of the enzymes from the cultivation medium was studied, and a 9 g/L NaCl solution recovered the highest CMCase and FPase activities (5.19 and 1.19 U/g, respectively). This study provides an important contribution to the valorization of the coconut residue through (i) the application of the steam explosion technology to optimize the production of cellulases using the SSF technology and (ii) their extraction using different solvents.

Keywords: green coconut shell; solid-state fermentation; steam explosion; cellulase; enzyme recovery

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1. Introduction

The global demand for coconut products, such as coconut water, oil, milk, and meat, is on the rise. More than 93 countries dedicate over 12 million hectares to this fruit culture, especially in the tropical and subtropical regions [1]. In Brazil, for example, almost 2.5 million tons of coconut are produced every year, representing the fourth largest production, and occupying the seventh largest area in the world [2]. However, approximately 80% of the net weight of the fruit is formed by the shell, which is generally discarded in the environment [3,4] and can cause a series of problems in the regions of extraction, processing, and commerce [5]. Alternatively, the residue can be used in bioprocesses, such as the production of enzymes [6,7] and cellulosic ethanol [3,8–12].

The use of lignocellulosic biomasses, such as the green coconut residues, to produce cellulosic ethanol is strongly dependent on the pretreatment step, as it reduces the recalcitrance of the biomass, removing part of the hemicellulose and lignin. These compounds act by blocking the accessibility of the enzymes to the cellulosic fibers, as well as promoting the non-productive adsorption of cellulases [13,14]. Recently, studies have focused on the use

of pretreatment methods for other applications besides the production of ethanol. The goal is to improve the interaction between the microorganism and the cellulose, increasing the yield of the bioproduct, e.g., cellulolytic enzymes [7,15]. This has been confirmed in several studies using different materials: carnauba straw [15], sugarcane bagasse [16], wheat bran, rice hulls [17], rice straw [18], olive pomace [19], and soybean hulls [20].

Presently, a variety of pretreatment methods have been developed and studied, most of them involving the use of high temperatures and chemicals (bases, acids, organic solvents, oxidizing agents, etc.) [13]. When pretreated by steam explosion, the biomass is subjected to heat by the injection of pressurized steam, leading to the depolymerization of lignin and hemicellulose. Later, when the material is depressurized, the fibrils of cellulose are exploded, reducing the crystallinity, and increasing the superficial area available for the microorganisms [21]. On the other hand, the alkali pretreatment causes the dissolution of the lignin and hemicellulose and the saponification of the intermolecular ester bonds. In addition, the degree of polymerization of the compounds is modified and the cellulose fibrils swell, yielding changes in the porosity, superficial area, and crystallinity of the material [13,14].

The most common systems used to produce cellulolytic enzymes are submerged fermentation (SF) and solid-state fermentation (SSF). The main difference between them is the presence of free water. While in the first the biomass is completely surrounded in liquid in such a way that the solids are dissolved or submerged, in the latter, the only water available is associated with the solid matrix or in a thin layer adsorbed by the particles [22,23]. The filamentous fungi are the most capable microorganisms to grow in the SSF conditions because of (i) their natural ability to grow on solid surfaces with low water activity, and (ii) their growth morphology as hyphae, which allows them to penetrate deeper in the substrate. Furthermore, these microorganisms yield higher concentrations of enzymes, higher productivity, present lower sterilization demand, and lower downstream cost [24,25]. Typically, commercial cellulase preparations are manufactured using fungal strains, such as *Trichoderma reesei* and *Aspergillus niger*, through SF [15,26]. The cellulase extract of *T. reesei* contains approximately 80% of exoglucanases, and some of the best strains can produce tens of grams per liter of this enzyme [27].

Cellulases are among the most used enzymes, with industrial applications in fields such as pulp and paper, textile, food and feed, beverages, detergent, and, more recently, biofuels. The market size of these biocatalysts is expected to reach a figure of approximately USD 15 billion by 2027, according to an estimation made in 2019. Furthermore, few companies control the production of cellulases, marketing their cocktail with hidden formulations and under exclusive licensors [28]. The use of cheap substrates, e.g., lignocellulosic wastes, to produce cellulases through a cost-effective process, such as SSF, might make the enzyme less expensive, positively affecting the economics of biofuels [29].

Thus, the present study aims to evaluate the production of cellulolytic enzymes through the SSF of the green coconut fiber, pretreated by a steam explosion followed by alkali, using the filamentous fungus *T. reesei* CCT-2768. To the best of the authors' knowledge, this is the first study applying this pretreatment strategy to coconut fiber to improve cellulase production through SSF. A 2^2 rotatable central composite design (RCCD) was carried out to determine the best cultivation conditions regarding the water activity and the content of lignocellulosic material per reactor. Moreover, the recovery of the enzymatic activity after the fermentation has been investigated by comparing several extraction solvents.

2. Materials and Methods

2.1. Green Coconut Fiber

The green coconut residues were collected in a coconut water stand located in Parnamirim (Rio Grande do Norte, Brazil) (5°53′09.3″ S 35°11′58.3″ W), with a visual selection of the epicarp and mesocarp fractions. The material was transported to the laboratory, where it was cut into small pieces (6–7 cm), washed with tap water to remove dirties and

other residual compounds, dried at 50 °C in an oven with forced air circulation (Marconi, MA-035, São Paulo, Brazil) for 72 h, milled (Wiley mill, TE-680, Tecnal, São Paulo, Brazil), and sieved to pass a 48-mesh sieve. The final powder was stored in plastic containers at room temperature.

2.2. Steam Explosion

The coconut powder was pretreated by a steam explosion following the methodology described by the work presented in [30]. Accordingly, 500.0 g of the biomass was inserted in the reactor (14 L, UpControl, Caxias do Sul, Brazil), and steam was injected until the temperature of 210 °C was reached. Then, the system was kept at this temperature for 10 min, resulting in a severity factor (R_0) of 4.24, calculated using Equation (1), presented by [31].

$$R_0 = \log[\int_0^t exp\left(\frac{T-100}{14.75}\right)dt], \quad (1)$$

where t represents the pretreatment residence time (in min), T is the temperature (°C), 100 °C is the reference temperature, and 14.75 corresponds to an empiric factor related to the activation energy. At the end of the residence time, the material was exploded to atmospheric pressure, and the solids were collected using a cloth filter and thoroughly washed with tap water until neutral pH. Then, the material was dried at 50 °C for 24 h and stored in plastic containers.

2.3. Alkaline Pretreatment

After the steam explosion, the material was subjected to a second pretreatment using sodium hydroxide. Thus, 500.0 g of steam-exploded biomass was mixed with a 1.0 M NaOH solution at a 1:5 (w/v) ratio. The mixture was taken to an autoclave and kept at 121 °C for 30 min. The liquor and the cellulose-rich solids were separated using a cloth filter. The liquor was discarded, and the solid material was washed with tap water until neutral pH, dried (50 °C, 24 h), and stored in plastic containers until use.

2.4. Chemical Characterization of the Biomass

The raw and pretreated materials were characterized regarding their moisture content, ashes [32], extractives [33], cellulose, hemicellulose, and Klason lignin [34]. The determination of the degradation products of the acid hydrolysis of cellulose and hemicellulose was carried out in an HPLC (Shimadzu Corporation, Tokyo, Japan) using the column Shim-pack SCR-101H (Shimadzu Co., Tokyo, Japan) and the RID 10A detector for the sugars and organic acids, and the column Shim-Pack CLC-ODS (Shimadzu Co., Japan) and the detector SPD-10AV for furfural and hydroxymethylfurfural. The first column was operated at 50 °C and eluted using 5.0 mM H_2SO_4 at a rate of 0.6 mL/min. The second column was set at 35 °C and used a gradient of acetonitrile and acetic acid (1.0% v/v) for elution at a constant flow rate of 0.6 mL/min [35].

2.5. Scanning Electron Microscopy

The surfaces of the raw and pretreated coconut fibers were observed using scanning electron microscopy (SEM), in a Hitachi TM3000 (Tokyo, Japan) microscope, with a focus on the areas of degradation of the fiber.

2.6. Microorganism

The filamentous fungus *Trichoderma reesei* CCT-2768 was obtained from the Tropical Cultures Collection of the André Tosello Foundation (Campinas, Brazil). The fungus was grown in PDA medium at 30 °C for 5 days and then kept in the refrigerator (4 °C) for up to 3 months. To obtain a concentrated spore suspension, the spores were transferred from the plates to a medium of corncobs and peptone [36]. After 5 days at 30 °C, the medium was flooded with a sterile Tween 80 solution (0.5% v/v), mixed with a glass rod, and the

suspension was collected. The spore concentration was determined using a Neubauer chamber in an optical microscope (BX 51, OLYMPUS, Tokyo, Japan).

2.7. Solid-State Fermentation

A 2^2 RCCD was carried out using as independent variables the water activity and the amount of lignocellulosic material. In a 125-mL Erlenmeyer flask, a determined amount of pretreated coconut fiber was added and acted as the only source of carbon during the fermentation. The system was sterilized (121 °C, 15 min), and then, a corresponding amount of saline solution was added (KH_2PO_4 5.0 g/L; $(NH_4)_2SO_4$ 5.0 g/L; $MgSO_4.7H_2O$ 1.0 g/L; NaCl 1.0 g/L; $FeSO_4$. $7H_2O$ 5.0 g/L; $MnSO_4$ 1.6 mg/L; $ZnSO_4.7H_2O$ 3.45 mg/L; $CoCl_2.6H_2O$ 2.0 mg/L). The mass of fiber and the water activity followed the values and the experimental planning described in Tables 1 and 2, respectively. The water activity was determined before the fermentation as a function of the biomass humidity, using the AquaLab PRE (METER Group, Pullman, WA, USA).

Table 1. Factors and codified levels for the 2^2 RCCD with triplicate at the central point to evaluate the production of cellulases through SSF using green coconut fiber and *T. reesei* CCT-2768.

Factors	Codified Levels				
	$-\alpha$	−1	0	+1	$+\alpha$
Water activity	0.80	0.83	0.90	0.97	1.00
Mass of fiber [1] (g)	2.68	3.50	5.25	7.00	8.32

[1] Dry weight.

Table 2. Matrix of the 2^2 RCCD with triplicate at the central point to evaluate the production of cellulases through SSF using green coconut fiber and *T. reesei* CCT-2768.

Title 1	Water Activity	Mass of Fiber (g)
1	−1	−1
2	−1	+1
3	+1	−1
4	+1	+1
5	$-\alpha$	0
6	$+\alpha$	0
7	0	$-\alpha$
8	0	$+\alpha$
9 [1]	0	0
10 [1]	0	0
11 [1]	0	0

[1] Central point.

Then, the wet material was inoculated with the spore suspension in a concentration of 1.0×10^7 spores per g of dry substrate. The flasks were closed with a cotton plug and placed in an incubator at 30 °C. The cultivation lasted for 168 h (7 days), with manual agitation every 24 h. For each condition, 7 identical flasks were prepared and, every 24 h, one was withdrawn.

2.8. Enzyme Extraction

The raw enzymatic extract was obtained by pouring 5.0 mL of sodium acetate buffer (200.0 mM, pH 5.0) per g of the initial dry substrate into the fermented medium. The mixture was homogenized using a glass rod, agitated at 160 rpm for 30 min (room temperature), and then clarified using filter paper and manual pressing, followed by centrifugation (11,750× *g*, 10 min) [15]. The supernatant containing the enzymes was used in the enzymatic activity determination.

Alternatively, other extraction solutions were studied based on the literature, namely, NaCl (9.0 g/L) [37], $Ca(OH)_2$ (3.0 mM) [38], Tween 80 (0.01% *v*/*v*) [39], and glycerol

(10% v/v) [40]. The extraction methodology was the same used with the sodium acetate buffer.

2.9. Enzymatic Activity

The cellulase activities using filter paper and carboxymethylcellulose (FPase and CMCase, respectively) were determined in triplicate according to [41]. The released sugars were measured by the DNS method [42]. One unit of FPase or CMCase (UI) was determined as the amount of enzyme that releases 1.0 µmol of reducing sugar per minute in the conditions used.

2.10. Statistical Analyses

The results were analyzed using the software Statistica 7.0 (Statsoft Inc., Tulsa, OK, USA), and compared using the Tukey test at a 95% confidence level. All results are presented as average ± standard deviation. The graphs were obtained using the software OriginPro 2016 (OriginLab Corp., Northampton, MA, USA) and Microsoft© Office Excel©. The experimental planning was designed and analyzed in Statistica 7.0 (Statsoft, Tulsa, OK, USA).

3. Results and Discussion

3.1. Biomass Composition and Pretreatment

The visual changes caused by the pretreatments in the green coconut fibers are presented in Figure 1. It can be observed that, compared to the raw material (Figure 1a), the steam explosion (Figure 1b) darkened the fibers, with some long fibers remaining light brown. After the alkali pretreatment, the biomass turned completely dark, more homogeneously.

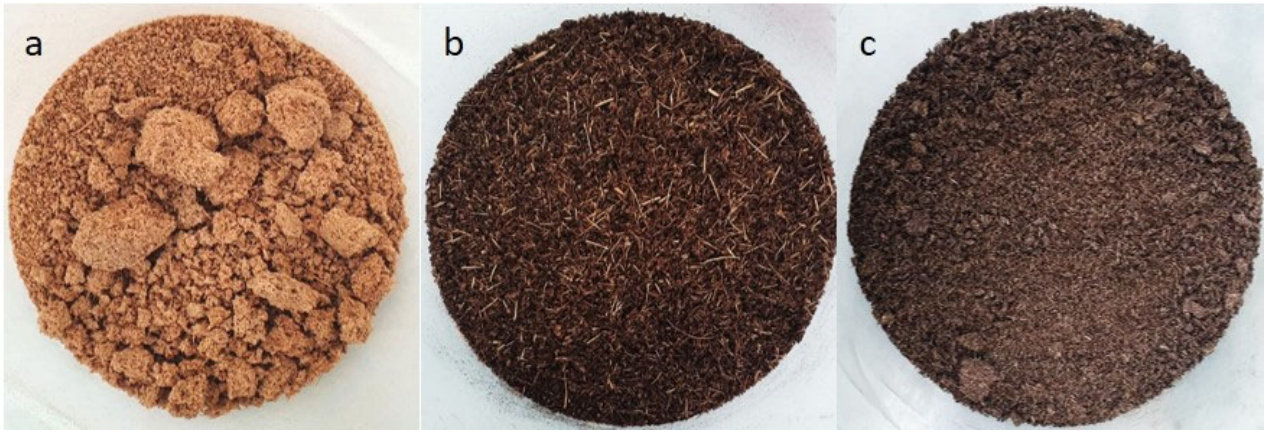

Figure 1. Green coconut fiber: (**a**) raw, (**b**) pretreated by steam explosion, and (**c**) pretreated by a steam explosion followed by alkali.

Moreover, the raw and pretreated biomasses were chemically characterized, in terms of moisture, ashes, extractives, cellulose, hemicellulose, and lignin contents. The results are summarized in Table 3. The raw fiber was determined to contain 3.51% of ashes, 25.04% of extractives, 33.96% of cellulose, 10.16% of hemicellulose, and 21.32% of lignin, all on a dry basis. The values are close to other studies that used green coconut fibers, such as [43] (38.0% holocellulose, 39.9% lignin), [3] (25.6% cellulose, 23.5% hemicellulose, 32.2% lignin), [10] (28.8% cellulose, 29.2% hemicellulose, 30.5% lignin), and [30] (27.7% cellulose, 15.5% hemicellulose, 32.6% lignin).

The steam explosion pretreatment was used to modify the physicochemical characteristics of the biomass. The pretreatment significantly reduced the content of extractives, as these compounds are mostly soluble in water. Consequently, the composition of the macromolecules cellulose and lignin was improved. Furthermore, comparing the relative

content of the macromolecules, the cellulose-to-hemicellulose ratio increased from 3.34 to 4.53 g.g^{-1}, and the cellulose-to-lignin ratio decreased from 1.59 to 1.29. The results confirm the action of the steam explosion pretreatment on the removal of the hemicellulose fraction.

Table 3. Chemical composition (in % *w*/*w*) of the green coconut fibers: raw, pretreated by the steam explosion (SE), and pretreated by a steam explosion followed by alkali (SEA). For the same row, numbers followed by the same letter are statistically similar (95% confidence interval).

	Raw	**SE**	**SEA**
Moisture	10.32 ± 0.10 ^{a}	7.69 ± 0.19 ^{b}	5.65 ± 0.03 ^{c}
Ashes	3.51 ± 0.06 ^{a}	1.32 ± 0.06 ^{b}	1.42 ± 0.03 ^{b}
Extractives	25.04 ± 0.27 ^{a}	3.74 ± 0.05 ^{b}	3.08 ± 0.02 ^{b}
Cellulose	33.96 ± 1.80 ^{c}	42.16 ± 0.29 ^{b}	47.77 ± 1.73 ^{a}
Hemicellulose	10.16 ± 0.13 ^{a}	9.32 ± 0.21 ^{a}	5.38 ± 0.22 ^{b}
Klason Lignin	21.32 ± 0.33 ^{b}	32.68 ± 0.60 ^{a}	34.82 ± 1.48 ^{a}

Using the hydrothermal pretreatment of the green coconut fiber (121 °C, 1 h, 10% *w*/*v* solid load), Nogueira et al. (2019) [3] varied the cellulose-to-hemicellulose ratio from 1.69 to 1.70, and cellulose-to-lignin ratio from 1.07 to 1.04. The mild conditions used (R_0 = 2.40) may have not been able to cause effective changes in the biomass composition. Ribeiro et al. (2019) [30] studied the steam explosion of the green coconut fiber at three different severity factors (R_0), 2.17, 3.21, and 4.23. Again, mild conditions have not yielded significant changes in the biomass composition, being the highest R_0 the only one that significantly enriched the material in cellulose. The authors also observed that the lignin was not removed by the steam explosion pretreatment and, therefore, opted for the use of an alkali pretreatment in sequence.

After the alkali pretreatment, the cellulose-to-hemicellulose ratio further increased to 8.98 g.g^{-1}, and the cellulose-to-lignin ratio increased to 1.39, which suggests the pretreatment efficiently removed both hemicellulose and lignin. These changes have possibly made cellulose more exposed and accessible to enzymes and microbes.

3.2. Scanning Electron Microscopy

Figure 2 presents the images observed by SEM of the green coconut fibers before and after the pretreatments. It can be seen in Figure 2a,b how the fibers are organized in the raw biomass. After the steam explosion (Figure 2c,d), the structure presents important modifications, the fibers are less organized and present more pores. This is a typical result of the steam explosion pretreatment, which promotes the instantaneous decompression of the system, causing a violent conversion of the water, present inside the biomass in the liquid state at high temperature and pressure, into vapor. Lastly, Figure 2e,f show the effect of the sequential alkali pretreatment on the steam-exploded biomass. The material, although still presenting a fibrous structure, is more open, and presents much more pores and available surfaces.

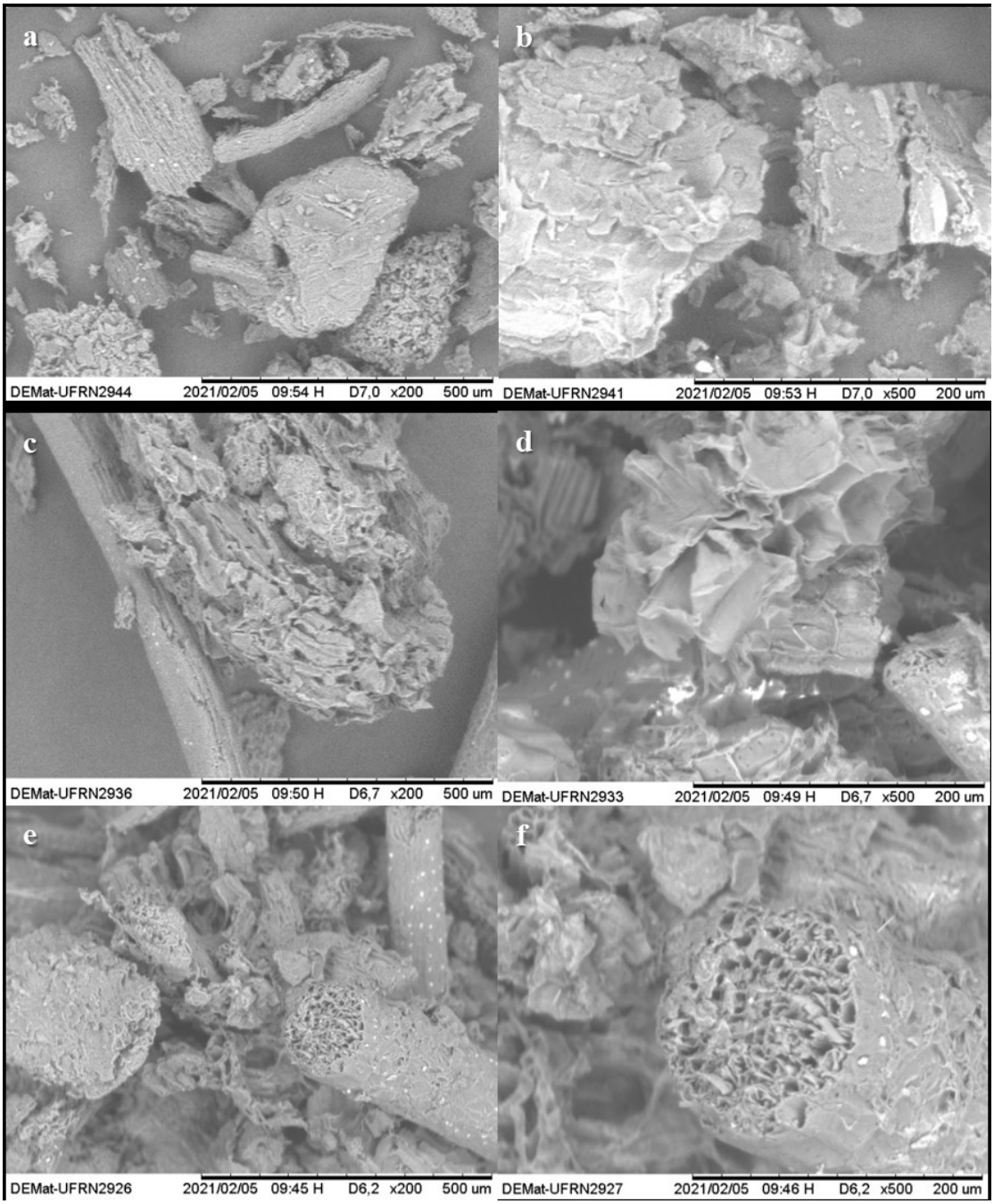

Figure 2. SEM images of the green coconut fiber: raw at the magnification of 200× (**a**) and 500× (**b**); pretreated by a steam explosion at 200× (**c**) and 500× (**d**), and pretreated by a steam explosion followed by alkali at 200× (**e**) and 500× (**f**).

3.3. Production of Cellulases

The biomass after the steam explosion and alkali pretreatment was chosen to be used in the solid-state fermentation to produce cellulolytic enzymes using *Trichoderma reesei* CCT-2768 because of the higher content of cellulose and higher fragmentation of the fibers. These characteristics might improve the accessibility of the fungus to the cellulosic fibers, which can result in a more pronounced enzyme induction mechanism.

The effect of the initial water activity and the mass of the substrate was evaluated using an experimental design. Each condition was evaluated for seven days (168 h) regarding the production of FPase and CMCase. Areeshi (2022) has reviewed the importance of water activity on microbial cultivation in SSF [29]. Generally, an increase in the moisture content

causes an increase in enzyme production up to some level, when the addition of more water hinders the process. The optimum condition is achieved when the water added does not create a barrier to the diffusion of nutrients while efficiently solubilizing the compounds and causing the swelling of the substrate. Additionally, the amount of biomass added to the reactor may impact its performance as it affects diffusion of heat and mass, and agitation.

For this investigation, besides the pretreated material, the raw material was studied using the conditions of the central point for comparison. Figure 3 presents the time course for the enzymatic activity.

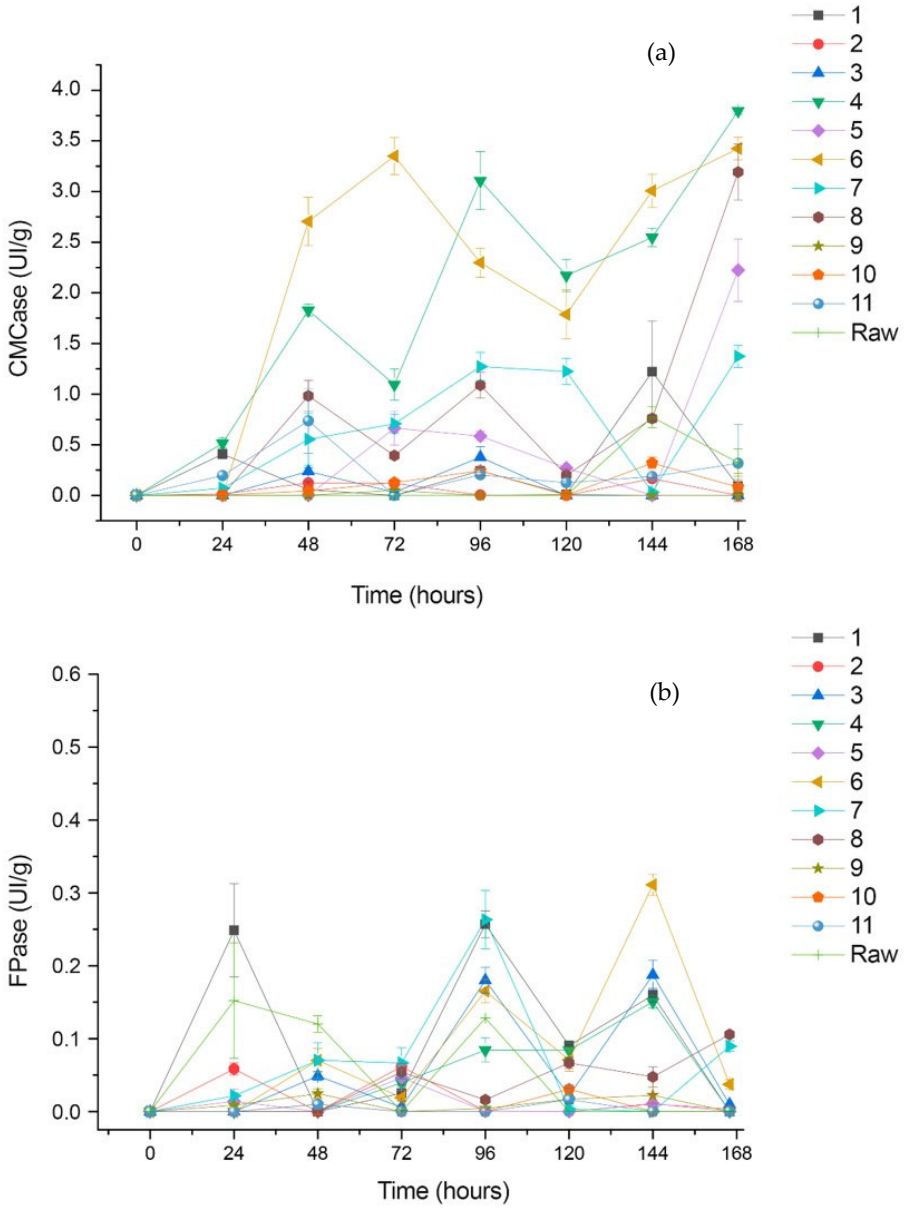

Figure 3. (**a**) CMCase and (**b**) FPase activities (UI/g) of the 2^2 RCCD with triplicate at the central point using the green coconut fiber pretreated by a steam explosion followed by alkali and the raw biomass (water activity of 0.90 and 5.25 g of material per flask).

The use of raw biomass yielded CMCase activity only on the sixth day of cultivation (0.77 ± 0.10 UI/g), and the maximum FPase activity was observed on the second day (0.15 ± 0.08 UI/g). The low production of the enzyme can be explained by the low cellulose content of the biomass, which is not enough to induce the cellulase expression, and by

the presence of lignin, which strongly adsorbs the cellulases, reducing the yield of the extraction operation [30].

Usually, longer fermentation times are necessary to produce endocellulases because the fibers need to be exposed to the action of other enzymes, e.g., xylanases. However, longer cultivations also promote the production of proteases that inactivate the target enzymes [19]. In a pretreatment step, removing the compounds that cover the cellulose may favor the production of cellulases earlier. In runs number 4 and 6, the CMCase reached a maximum at 168 h, yielding 3.80 ± 0.03 and 3.42 ± 0.11 UI/g, respectively. These runs correspond to the water activity of 0.97 and mass of the substrate of 7.00 g (run 4) and water activity of 1.00 and the mass of the substrate of 5.25 g (run 6). On the seventh day, run numbers 5, 7, and 8 also presented their highest CMCase values (2.22 ± 0.31, 1.37 ± 0.01, and 3.12 ± 0.28 UI/g, respectively).

For the FPase production, an oscillating behavior was observed (Figure 3b). Some peaks were observed at 24 h (for run number 1 and the raw biomass), 96 h (runs 1, 3, 6, 7, and raw biomass), and 144 h (run 1, 3, 4, and 6). The maximum FPase activities registered (UI/g) were 0.31 ± 0.01 (run 6, 144 h), 0.26 ± 0.04 (run 7, 96 h), 0.24 ± 0.02 (run 1, 96 h), and 0.24 ± 0.06 (run 1, 24 h).

Using wheat bran, rice bran, and rice husk, Heng and Hamzah (2022) observed maximum cellulase activity by *Trichoderma harzianum* between the seventh and ninth days of cultivation in SSF [44]. Maximum CMCase activity was observed for wheat bran on the 7th day (20.8 and 17.7 UI/g, for the CMCase and FPase, respectively). Cultivation of *Trichoderma reesei* RUT C30 on alkali-pretreated olive pomace yielded maximum FPase production after 24 days (1.28 UI/g) without any nutrient supplementation [45].

Considering runs #4 and #6 as the most promising conditions, as they yielded good CMCase results at 96 and 144 h, and their FPase activities peaked at 144 h, the sixth day was chosen as the best production day (144 h). An ANOVA was performed with the data related to this day, which are detailed in Table 4. The Pareto charts are presented in Figure 4, indicating the effect of the independent variables (water activity and mass of substrate) on the activities of CMCase (Figure 4a) and FPase (Figure 4b). For the CMCase, both the linear and quadratic terms of the water activity were statistically significant (confidence level at 95%). Additionally, the linear effect of the mass of substrate, and the synergic interaction between both independent variables were also significant. For the FPase, the linear and quadratic terms related to the water activity are significant, as well as the effect of the synergic term.

Table 4. 2^2 RCCD with triplicate at the central point for the production of cellulases by *T. reesei* CCT-2768 in SSF after 144 h of fermentation using pretreated green coconut fiber as substrate.

Run	Water Activity (X_1)	Mass of Substrate (X_2)	CMCase (U/g)	FPase (U/g)
1	−1	−1	1.221	0.160
2	−1	+1	0.165	0.010
3	+1	−1	0.000	0.187
4	+1	+1	2.546	0.151
5	$-\alpha$	0	0.000	0.011
6	$+\alpha$	0	3.008	0.311
7	0	$-\alpha$	0.033	0.000
8	0	$+\alpha$	0.761	0.047
9 [1]	0	0	0.000	0.022
10 [1]	0	0	0.320	0.000
11 [1]	0	0	0.186	0.000

[1] Central point.

The aeration during an SSF significantly affects microbial growth and the productivity of enzymes. The conduction of an SSF in an Erlenmeyer flask simulates the conditions found in tray bioreactors, where aeration is challenging. Besides providing the oxygen necessary for aerobic fermentation, aeration also removes both CO_2 and metabolic heat [46].

In this study, by increasing the mass of coconut fiber in the flask, the problems of aeration caused by a thick layer of solids were evaluated, and the effect was determined as significant for the CMCase.

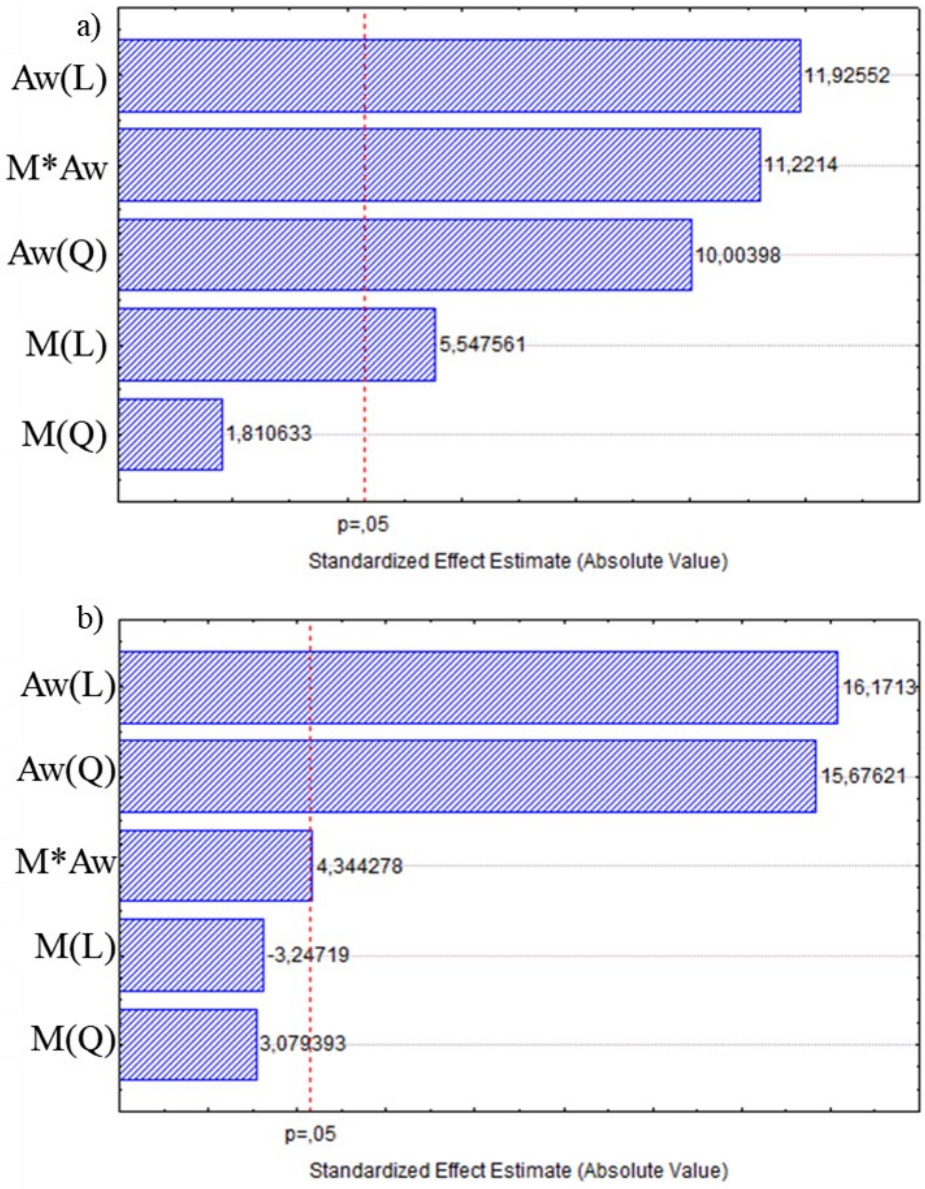

Figure 4. Pareto charts of *t*-values (absolute values) for the coefficients of the model for cellulase production by *T. reesi* CCT-2768 on the 6th day of SSF using green coconut fiber pretreated with a steam explosion followed by alkali. (**a**) CMCase and (**b**) FPase. Aw = water activity; M = mass of fiber; (L) = linear; (Q) = quadratic.

Equations (2) and (3) represent the model developed for the CMCase and FPase, respectively. The ANOVA results are summarized in Table 5 and indicate that the CMCase mathematical model (Equation (2)) is significant and predictive, with the regression coefficient $R^2 = 0.89$. The FPase model (Equation (3)) is significant, but not predictive, with an $R^2 = 0.82$. In both Equations, X_1 and X_2 represent the codified levels of water activity and mass of substrate, respectively.

$$CMCase(UI/g) = 0.169 + 0.677X_1 + 0.315X_2 + 0.676X_1^2 + 0.122X_2^2 + 0.901X_1X_2, \quad (2)$$

$$FPase(UI/g) = 0.007 + 0.074X_1 - 0.015X_2 + 0.086X_1^2 + 0.017X_2^2 + 0.028X_1X_2, \quad (3)$$

Table 5. ANOVA results for the models of CMCase and FPase activities after 6 days of SSF using the alkali-pretreated, steam-exploded green coconut fiber.

Enzymatic Activity	Source of Variation	Quadratic Sum	Degrees of Freedom	Quadratic Mean	F	R [2]
CMCase	Regression	10.280	5	2.0600	7.9200 [1]	0.8870
	Residue	1.3100	5	0.2600	-	
	Lack of fit	1.2600	3	0.4200	14.0000 [2]	
	Pure error	0.0500	2	0.0300	-	
	Total	11.5900	10	-	-	
FPase	Regression	0.0900	5	0.0300	10.3448 [1]	0.8182
	Residue	0.0200	5	0.0029	-	
	Lack of fit	0.0197	3	0.0066	33.000 [2]	
	Pure error	0.0003	2	0.0002		
	Total	0.11	10	-	-	

[1] Regression/Residue: $F_{tab}(0.95;5;5) = 5.05$. [2] Lack of fit/Pure error: $F_{tab}(0.95;3;2) = 19.16$.

Both CMCase and FPase productions were favored by the increase in water activity. Generally, SSF studies use the moisture content of the medium between 50 and 70%, depending on the specific capacity of the material to retain water, which is represented by the water activity [47–50]. Pessoa et al. (2021) [51] evaluated the effect of moisture and pH on the production of endoglucanases through SSF using *T. reesei* CCT-2768 applied to three different lignocellulosic substrates (green coconut fiber, sugarcane bagasse, and cashew apple bagasse). It was observed that the best moisture varied among the three substrates, being 80% (1.55 U/g), 60% (2.29 U/g), and 70% (1.46 U/g), respectively. Lodha et al. (2020) [24], in an attempt to optimize cellulase production from the fungal co-culture of *T. reesei* NCIM 1186 and *Penicillium citrinum* NCIM 768 under solid-state fermentation using untreated and pretreated wheat bran, concluded that the highest yield occurred on the sixth day of cultivation in the FSS with hydrothermally pretreated wheat bran, with an activity of 6.71 FPU/g, when the initial moisture content was 70% and pH 5.0, incubated at 30 °C. In this study, the best water activity (Aw = 0.995) corresponds to a moisture content of 95.6%.

3.4. Enzyme Extraction

Pandey and Negi (2020) [52] highlighted how SSF for cellulase production investigations has focused on upstream operations. However, efficient recovery of the enzymes is essential for obtaining high cellulase yields. Therefore, four extraction solvents were chosen to be compared with the sodium acetate buffer previously used. They consist of aqueous solutions of sodium chloride (0.9% *w*/*v*); calcium hydroxide (3.0 mM); Tween 80 (0.01% *v*/*v*); and glycerol (10% *v*/*v*) all applied after 144 h of fermentation at the conditions of the run number 6. The CMCase and FPase activities recovered are presented in Figure 5.

It can be observed that the average recovered CMCase activity was improved using any alternative extraction solvent, and the Tween 80 was the only one that reduced the FPase. The NaCl solution significantly increased the CMCase from 3.12 ± 0.28 to 5.19 ± 0.05 UI/g. $Ca(OH)_2$, Tween 80, and glycerol yielded endoglucanase activities of 4.66 ± 0.16, 4.73 ± 0.03, and 3.29 ± 0.09, respectively, but they are all statistically equal to the acetate buffer result. The maximum FPase extracted was also using the NaCl solution (1.19 ± 0.09 UI/g), which was significantly similar to the glycerol (0.48 ± 0.21 UI/g), and different from $Ca(OH)_2$, Tween 80, and acetate buffer (0.41 ± 0.06 UI/g, 0.32 ± 0.17 UI/g, and 0.32 ± 0.01 UI/g, respectively).

The use of salts to extract enzymes in SSF consists of one of the most common methods used both in laboratories and industries [53]. Besides the advantage of being a cheap reagent, the salt acts by stabilizing the protein structure and, therefore, the enzymatic activity [52]. However, at high salt concentrations, the increased ionic strength favors the hydrophobic interactions between the enzyme and the solid matrix, which may hinder cellulase desorption [54,55].

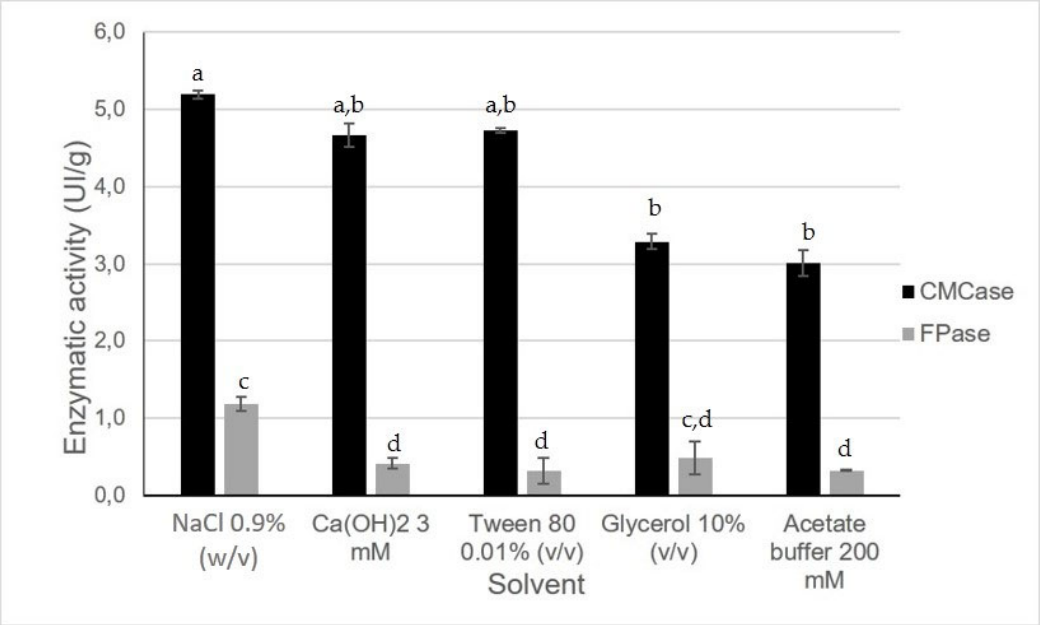

Figure 5. Effect of the extraction solvent on the recovered cellulase activity after 6-day SSF using the green coconut fiber pretreated by a steam explosion followed by alkali. The same letter on the top of the column indicates the values are not statistically different (95% confidence level).

In a study to optimize the desorption of cellulolytic enzymes from Avicel, Otter et al. (1989) [38] observed that a NaOH solution (pH 10.0) was favored by the addition of 0.005% Triton X-100 or Tween 80. Alternatively, the use of glycerol at the same pH yielded similar results, but at a concentration of 20% (*v*/*v*). More recently, Marín et al. (2019) [56] have observed a higher cellulase recovery using distilled water, compared to citrate buffer (pH 4.8), after SSF of orange peel, apple pomace, and rice fiber using compost as inoculum.

Regarding the practical application of cellulolytic cocktails in the hydrolysis of lignocellulosic biomasses, the effect of specific cultivation conditions on cellulase activity must be considered [57]. After the acid or alkali pre-treatment of the biomass, the pH has to be adjusted by the addition of salts able to neutralize the medium. At this point, salt-tolerant cellulase can eliminate or reduce the adverse impacts of salt on enzyme activity [58].

4. Conclusions

The combination of the steam explosion with the alkali pretreatment efficiently removed the hemicellulose of the green coconut fiber, reducing the content from 10.15 to 5.38%. The images obtained from SEM also revealed a more porous structure of the fibers, which favored the induction of cellulase production by *T. reesei* CCT-2768. The SSF of the pretreated biomass yielded 3.0 U/g of CMCase and 0.31 U/g of FPase, using a water activity of 0.995, 5.25 g of dry biomass (in 125-mL flasks), and an acetate buffer for extraction. Moreover, water activity significantly affected cellulase production. Lastly, the use of a 9.0 g/L NaCl solution has increased the recovery of cellulase to 5.19 and 1.19 U/g of CMCase and FPase, respectively.

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