

## Article

# Bioethanol Production from Chemically Obtained Cassava Hydrolysates: Effect of Nature of the Acid

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## Abstract

Ethanol production from corn and sugarcane is well established; however, reliance on these feedstocks raises concerns related to food security, highlighting the need to explore alternative raw materials such as cassava. This study evaluates bioethanol production from different cassava root fractions—peeled cassava, starch, and peel—assessed individually within a unified experimental framework. Chemical hydrolysis using HCl or H<sub>2</sub>SO<sub>4</sub> was employed as a cost-effective alternative to enzymatic saccharification. Hydrolysis efficiencies above 95% were achieved for both cassava and starch, regardless of the acid used, although HCl promoted higher glucose release in all samples. Chemical hydrolysis induced noticeable increases in surface roughness and porosity across all fractions. Fermentation efficiencies for cassava ( $93.1 \pm 1.1\%$  vs.  $77.8 \pm 4.9\%$ ) and peel ( $85.7 \pm 2.8\%$  vs.  $69.8 \pm 7.4\%$ ) were higher when HCl was used during hydrolysis, whereas starch showed no significant differences between acids. In contrast, the highest ethanol yields on a dry raw material basis were obtained from cassava hydrolyzed with H<sub>2</sub>SO<sub>4</sub> (up to  $0.37 \pm 0.002$  g/g), corresponding to a 73% ethanol recovery efficiency. The highest volumetric ethanol production rates were achieved with HCl hydrolysates, reaching up to 1.85 g/L/h for peel-derived hydrolysates.

**Keywords:** cassava; acid hydrolysis; bioethanol; alcoholic fermentation

## 1. Introduction

Amid a global landscape marked by the urgency of reducing greenhouse gas emissions and mitigating climate change derived from the use of fossil fuels, biofuels have emerged as a sustainable and promising alternative. Among them, bioethanol stands out as one of the leaders in the transition toward a more sustainable energy matrix due to its potential to partially or totally replace fossil fuels while addressing crucial global environmental, social, and economic challenges/concerns [1,2].

Bioethanol is an alternative fuel to fossil fuels because it is a renewable resource that contributes positively to climate change and reduces the negative environmental impacts generated by the use of petroleum [2]. This biofuel was first introduced in the United States at the beginning of the 20th century [3]. Bioethanol is the leading biofuel, with a global production of approximately 113 billion liters by 2023 (<https://ethanolrfa.org/markets-and-statistics/annual-ethanol-production>, accessed on 25 December 2025). The USA and



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Brazil are the most prominent countries, contributing 52% and 28% of global production, respectively [4].

First-generation bioethanol is obtained mainly from raw materials rich in sucrose or starch, such as sugarcane or edible grains, respectively [4,5]. Starch is the second most abundant carbon source in plants. It is a reserve polymer made up of glucose monomeric units linked by  $\alpha$ -1,4 glycosidic bonds. It consists of two polymers: amylose (linear) and amylopectin (branched) [6]. The production of bioethanol from starch requires liquefaction and saccharification prior to alcoholic fermentation [5]. These steps involve the use of commercial enzymes; however, their large-scale application is limited because the enzymes are cost-prohibitive and cannot be reused. In this sense, chemical hydrolysis (with inorganic acids) has attracted attention because it is fast and easy to implement and scale up and does not require the addition of commercial enzymes [4]. Acid hydrolysis is an effective and rapid approach for starch saccharification; however, saccharification efficiency is highly dependent on acid type, concentration, temperature, and reaction time, requiring careful optimization to minimize sugar degradation and the formation of inhibitory byproducts [2,4,7].

Cassava (*Manihot esculenta*), unlike other crops, can be grown in areas with low soil fertility (high phosphorus fixation, erosion, high aluminum content, among others) [8]. In addition, it is resistant to adverse environmental conditions. It tolerates a wide range of rainfall, and its mature roots can maintain their nutritional value for a long time without water [9]. On the other hand, unlike other cereals, cassava roots have a low protein content, and the protein they do have is of poor quality. In addition, they contain cyanide in a range of 75–1000 mg/kg, depending on the variety, the age of the plant, and growing conditions [9,10]. It also contains many compounds that are toxic to humans, so before using it as food, cassava must undergo a detoxification process such as peeling, cooking, or drying (never eat it raw) [10]. Furthermore, cassava has a higher yield per unit area compared to other crops [11]. From a socioeconomic perspective, cassava is characterized as a staple crop for subsistence and local markets in Africa, Asia, and Latin America; unlike maize, it is not fully integrated into global commodity markets, and therefore its price is considered to be less susceptible to volatility in global financial markets [12]. Finally, from a regional perspective, cassava is recognized for its high productivity on marginal lands. Its mature roots can retain their nutritional value for long periods without water. This characteristic enables its cultivation in soils unsuitable for intensive cereal farming, thereby optimizing land use without displacing primary food crops [13]. These agronomic and market characteristics suggest that cassava-based bioethanol production may mitigate the food-versus-fuel debate commonly associated with feedstocks such as maize or sugarcane, particularly when cultivation occurs on marginal lands or when non-edible fractions of the root are utilized [12]. This has motivated its use as a source of biofuels.

To assess the valorization potential of cassava, it is necessary to understand the distribution of its main components. The root consists of three fractions: the edible pulp, the peel (approximately 15–20% of the fresh weight), and the bagasse, a fibrous residue from starch extraction [10]. Given the global production of about 333 million tons in 2023—led by Nigeria, the Democratic Republic of the Congo, and Thailand, with Brazil and Paraguay as major producers in Latin America (<https://ourworldindata.org/grapher/cassava-production>, accessed on 27 December 2025)—industrial processing generates large volumes of residues [8]. The characterization and conversion of these by-products into value-added bioproducts can enhance the production chain while reducing environmental impacts [8,9].

To date, the production of bioethanol from cassava flour [14] and starch [11,15] has been reported. In both cases, pretreatment and enzymatic saccharification are consistently

applied. However, most published studies rely on commercial cassava flour or purified starch, which limits the evaluation of ethanol yields and process performance on a raw biomass basis. In contrast, fewer studies have explored chemical hydrolysis as an alternative to enzymatic saccharification, despite its potential to reduce enzyme-related costs. Although most work has focused on these two fractions of cassava roots [16,17], there is little on the production of bioethanol (second generation) from cassava peel, a lignocellulosic residue made up of three main polymers: cellulose, hemicellulose, and lignin, which, like starch, requires pretreatment followed by saccharification for its use.

In contrast to previous studies, the present work focuses on raw cassava root biomass and its individual fractions, including peeled cassava, starch, and peel. The objective of this study was to evaluate bioethanol production from different fractions of raw cassava roots using chemical hydrolysis as a simple and scalable alternative to enzymatic saccharification. The effect of chemical hydrolysis using two mineral acids (HCl and H<sub>2</sub>SO<sub>4</sub>) on the production of fermentable sugars was evaluated. In addition, the morphological and structural characteristics of the raw materials before and after chemical hydrolysis were systematically analyzed to gain insight into the mechanisms governing the hydrolysis process. Finally, the fermentable sugars were subsequently converted into ethanol, and fermentation efficiency was determined as the percentage of the theoretical ethanol yield. By evaluating the edible and non-edible fractions of cassava under a unified experimental framework, this study provides a comparative perspective on the valorization of raw cassava biomass through chemical hydrolysis, including lignocellulosic residues that are often excluded from cassava-based bioethanol research.

## 2. Materials and Methods

### 2.1. Biomass and Yeast for Fermentation

The cassava root was obtained from the Mexico City wholesale market. The specific cassava variety and cultivation conditions were not identified, as the roots corresponded to commercially available material intended for human consumption. It was ensured to be in optimal condition, that is, free of foreign bodies and/or contamination. The cassava was subsequently washed (with tap water) to remove traces of soil and other impurities. Three fractions of the biomass were obtained: peel, peeled cassava (hereafter referred to as cassava), and starch.

Using a homemade peeler, the cassava peel was removed and sun-dried for two days. Once the peel was dry, it was pulverized in a vibrating micro-ball mill (Pulverisette 0, Fritsch GmbH, Idar-Oberstein, Germany) until a powder was obtained and stored at room temperature for later use. For peeled cassava (cassava), the tuber was cut into thin slices and left to dry for two days in a solar dryer, then pulverized and stored. To extract the starch, the cassava was sliced and blended with tap water (0.5 kg of fresh cassava per liter of water). The mixture was then filtered using a homemade strainer and a sky blanket. The liquid fraction was left to settle in a glass container until the starch sedimentation was observed. The supernatant was removed by decantation, and the starch was dried using an indirect solar dehydrator (MiNego10, SAECSA, Puebla, Mexico) for two days and stored.

The commercial yeast *Saccharomyces cerevisiae* (Tradipan<sup>®</sup>, SAFMEX, Toluca, Mexico) was used as fermentation inoculum. All reagents used were ACS grade or equivalent.

### 2.2. Gelatinization—Acid Hydrolysis

Before acid hydrolysis, a starch gelatinization process was carried out to disrupt the starch structure and decrease its crystallinity [18]. This process was performed on the three cassava fractions, since starch will be present in all of them. 50 mL of a 50 g/L suspension was prepared for each biomass, and H<sub>2</sub>SO<sub>4</sub> or HCl was added to reach a final

concentration of 0.08 M or 0.25 M, respectively. These acid concentrations were chosen based on preliminary experiments in which each acid was evaluated independently at 0.08, 0.125, and 0.25 M, while maintaining all other gelatinization and hydrolysis conditions constantly. Subsequently, the suspension was heated at 90 °C for one hour.

Once gelatinization is complete, all samples are immediately autoclaved (121 °C, 15 psi, 60 min) (CVq-280, ECOSHEL, Denver, CO, USA) to hydrolyze the biomass [19]. At the end of the process, the samples are filtered to determine the biomass weight loss, and the liquid fraction is characterized in terms of reducing sugars (predominantly glucose) and released glucose. In peel-derived hydrolysates, other reducing sugars such as xylose, galactose, and mannose are also expected due to the presence of hemicellulose in this type of biomass. All tests were performed in duplicate.

### 2.3. Alcoholic Fermentation

Before fermentation, *S. cerevisiae* yeast was activated by inoculating 15 g/L yeast into liquid culture medium for 24 h at 30 °C, under constant stirring (150 rpm). The liquid medium contained the following components (g/L): glucose, 10; yeast extract, 4;  $\text{KH}_2\text{PO}_4$ , 2;  $(\text{NH}_4)_2\text{SO}_4$ , 3;  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 1 [11]. Commercial gentamicin (AMSA Laboratories, Mexico City, Mexico, 80 mg/2 mL) was added at 0.01 g/L to prevent bacterial growth, without adversely affecting the growth or fermentative activity of *S. cerevisiae* under the condition evaluated [20]. The hydrolysate was neutralized using potassium carbonate before fermentation.

For alcoholic fermentation, 120 mL glass bottles with a working volume of 50 mL were used. The mineral medium described above was used for all experiments, replacing glucose with hydrolysate until a final glucose concentration of 7 g/L was obtained. The use of the same initial glucose concentration in all experiments was necessary to ensure a fair comparison among the different hydrolysates. Fermentation was carried out using *S. cerevisiae*, inoculated at 10% (v/v), as the ethanol-producing microorganism. The initial pH was adjusted to 5 with 5N HCl or 5N NaOH solutions. The glass bottles were sealed with butyl rubber stoppers and aluminum seals, purged with  $\text{N}_2$  gas for 30 s, and subsequently incubated at 30 °C (9025E, ECOSHEL, Denver, CO, USA) [5]. Samples were taken twice a day for five days (biogas was purged during each sample collection) and immediately filtered through nylon membranes (0.22  $\mu\text{m}$ ) and stored under refrigeration for subsequent characterization of the fermentation broth (determination of ethanol produced, residual glucose, and reducing sugars). For the determination of reducing sugars and residual glucose, the filtered samples were previously neutralized with potassium carbonate ( $\text{K}_2\text{CO}_3$ ), while the ethanol concentration was determined directly from the filtered samples. All tests were performed in duplicate.

### 2.4. Analytical Techniques

#### 2.4.1. Characterization of Biomass and Hydrolysates

The moisture content, total solids (TS), and volatile solids (VS) of the three cassava fractions, before and after acid hydrolysis, were determined according to standard methods [21].

The surface morphology and elemental composition of the three cassava fractions (before and after acid hydrolysis) were analyzed by scanning electron microscopy (SEM) coupled to an energy dispersive spectroscopy (EDS) detector at 20 kV (S-5500 SEM, Hitachi High-Tech Corporation, Hitachinaka City, Japan) [22]. The structure and functional groups of the studied biomasses were analyzed in a Fourier transform infrared spectrometer (FTIR) with a DTGS detector equipped with a single attenuated total reflectance (ATR) cell

containing a germanium crystal (incident angle of 45°) (Spectrum 100 FTIR, PerkinElmer, Waltham, MA, USA) [23]. The samples were analyzed within the range of 4000 to 500 cm<sup>-1</sup>.

Reducing sugars were quantified by the 3,5-dinitrosalicylic acid (DNS, Sigma-Aldrich, St. Louis, MO, USA; CAS 609-99-4) method, using D-glucose (CAS 50-99-7, PQM Fermont, Monterrey, Mexico) as a standard [24]. Glucose was quantified by liquid chromatography using a Dionex UltiMate 3000 HPLC (Thermo Scientific™, Waltham, MA, USA). The equipment consisted of a quaternary pump (LPG-3400SD), a vacuum micro-degasser (series 200), an autosampler (WPS-3000TSL), a thermostatted column compartment (TCC-3000SD), and a refractive index detector (RefractoMax 520), all from Thermo Fisher Scientific™ (Waltham, MA, USA). Glucose separation and quantification were performed using a Hi-Plex H column (Part No. PL1170-6830, 7.7 × 300 mm × 8 µm, Agilent Technologies, Inc., Santa Clara, CA, USA) using 0.0025 M H<sub>2</sub>SO<sub>4</sub> as the mobile phase at a flow rate of 0.4 mL/min. The column and RI detector temperatures were 35 and 40 °C, respectively. The injection volume was 20 µL. Chromeleon™ 7.2 software (Thermo Scientific™, Waltham, MA, USA) was used for data processing.

#### 2.4.2. Determination of Ethanol

Ethanol was determined using a gas chromatograph (Agilent 7890B, Agilent Technologies, Inc., Santa Clara, CA, USA) equipped with a flame ionization detector (FID) and a BD-ALC1 column (Part No. 123-9134, 30 m × 0.32 mm × 1.8 µm, Agilent Technologies Inc., Santa Clara, CA, USA). The injector and detector temperatures were maintained at 250 and 280 °C, respectively. The initial column temperature was 35 °C; it was then increased at a rate of 2 °C/min until it reached 38 °C, and subsequently, the temperature was increased at a rate of 10 °C/min until it reached 75 °C. N<sub>2</sub> gas was used as the carrier gas at a flow rate of 6.25 mL/min. The H<sub>2</sub> and air flow rates were 30 and 300 mL/min, respectively. An injection volume of 1 µL was used. ACS grade ethanol (CAS 64-17-5, PQM Fermont, Monterrey, Mexico) was used as a standard.

#### 2.5. Data Analysis

The overall hydrolysis efficiency was determined based on solids removal (Equation (1)):

$$\%OHE = \frac{W_f - W_i}{W_i} \times 100 \quad (1)$$

where  $W_f$  and  $W_i$  are the biomass weight before and after hydrolysis.

While the saccharification efficiency was calculated based on Equation (2):

$$\%Saccharification = \frac{\text{Glucose released or reducing sugars (g/L)}}{\text{Biomass used (g/L)} \times 1.11 \left( \frac{\text{g glucose}}{\text{g sugar}} \right)} \times 100 \quad (2)$$

The factor of 1.11 g<sub>glucose</sub>/g<sub>sugar</sub> is a value determined by the stoichiometry of the hydrolysis reaction of starch and cellulose, which are the predominant polysaccharides in the different biomasses used.

To estimate the maximum volumetric ethanol production rate ( $R_{p,max}$ ), the production kinetics were fitted to the modified Gompertz equation (Equation (3)):

$$y(t) = y_0 + y_{max} \exp \left[ -\exp \left( \frac{2.71828 R_{p,max} (\lambda - t)}{y_{max}} + 1 \right) \right] \quad (3)$$

where  $y(t)$  is the ethanol produced at time  $t$  (in g/L),  $y_{max}$  is the maximum concentration of ethanol produced (in g/L),  $R_{p,max}$  is the maximum volumetric rate of ethanol production (in g/L/d),  $\lambda$  is the lag phase time (in d), and  $t$  is the incubation time (in d). Nonlinear

regression was performed using GraphPad Prism version 8.0.2 for Windows (GraphPad Software, Boston, MA, USA, [www.graphpad.com](http://www.graphpad.com)).

The apparent ethanol yield ( $Y'_{p/s}$ ) was determined based on the changes in the ethanol produced ( $\Delta p$ , in g/L) and the glucose consumed ( $\Delta s$ , in g/L), according to the following equation:

$$Y'_{p/s} = -\frac{\Delta p}{\Delta s} \quad (4)$$

The efficiency (Ef) of the fermentation process was calculated based on the relationship between  $Y'_{p/s}$  and the theoretical yield ( $Y_{p/s}$ ) (Equation (5)). The latter was calculated based on the reaction stoichiometry of glucose fermentation, resulting in a value of 0.511 g ethanol/g<sub>glucose</sub>.

$$\%Efficiency = \frac{Y'_{p/s}}{Y_{p/s}} \quad (5)$$

### Statistical Analysis

All experimental results are presented as mean  $\pm$  standard deviation. Statistical comparisons were performed considering each biomass-acid combination as an independent treatment (cassava-H<sub>2</sub>SO<sub>4</sub>, cassava-HCl, peel-H<sub>2</sub>SO<sub>4</sub>, peel-HCl, starch-H<sub>2</sub>SO<sub>4</sub>, and starch-HCl). Statistically significant differences among treatments were determined using a one-way ANOVA followed by a Tukey-Kramer test implemented in R (version 4.5.1) using RStudio (version 2025.09.1+401; Posit Software, Boston, MA, USA) [25] ( $\alpha < 0.05$ ). All experiments were performed in duplicate.

## 3. Results

### 3.1. Characteristics and Composition of the Substrates Used

Cassava roots contain high carbohydrate content, mainly starch, whose condensed formula is (C<sub>6</sub>H<sub>10</sub>O<sub>5</sub>)<sub>n</sub> [26]. Elemental analysis of both starch and cassava (peeled cassava) shows that it resembles the composition of a complex carbohydrate, such as starch, where the C/N ratio is 1.2 (Table 1, Figure S1). Deviations of the C/N ratio from the theoretical value suggest the presence of proteins in these samples, as previously reported [10,27], which have a higher C/N ratio than carbohydrates (C<sub>5</sub>H<sub>7</sub>O<sub>2</sub>N) [25]. On the other hand, for cassava peel, the C/N ratio is very different (Table 1, Figure S1), which suggests that there are other components, in addition to carbohydrates, that are more reduced, such as lignin, for which the condensed formula C<sub>9</sub>H<sub>10.2</sub>O<sub>3.4</sub> and C/N ratio of 2.65 have been reported [28] and which has been previously reported to be abundant in cassava peel [29]. In addition to C and O as primary elements, potassium was found in cassava, while a wide variety of metals and metalloids were found in the peel (Table 1). This is consistent with what was previously reported by Morgan and Choc [10] and Scaria et al. [27], who report that most of the minerals are present in the cassava peel.

**Table 1.** Elemental composition and recovery yields of cassava fiber, peel, and starch.

| Parameter                     | Cassava             | Peel                             | Starch              |
|-------------------------------|---------------------|----------------------------------|---------------------|
| Elemental analysis (atomic %) | 58.24 $\pm$ 0.10% C | 61.36 $\pm$ 0.69% C              |                     |
|                               | 40.73 $\pm$ 0.99% O | 32.86 $\pm$ 4.83% O              | 57.97 $\pm$ 0.01% C |
|                               | 0.88% K             | 6.97% B                          | 42.03 $\pm$ 0.01% O |
|                               |                     | K, Al, Si, Fe, Ca, Mg, P, S < 1% |                     |
| C/N ratio                     | 1.41                | 1.86                             | 1.37                |
| Yield (gTS/g dry matter)      | 0.891 $\pm$ 0.020   | 0.109 $\pm$ 0.001                | 0.559 $\pm$ 0.010   |
| Yield (gST/g raw matter)      | 0.338 $\pm$ 0.003   | 0.041 $\pm$ 0.001                | 0.205 $\pm$ 0.001   |

Although carbohydrates (mainly starch) constitute around 75% of the dry weight of cassava root, in the present work, only an extraction yield of 20.5 g of starch per 100 g of fresh cassava was achieved (Table 1). Dorantes-Fuertes et al. [30] recently carried out a detailed discussion of the different starch extraction methods from tubers, finding that for cassava, extraction yield values between 204 and 234 kg of starch per 1000 kg of processed cassava, using the wet method, are typically reported. However, one way to improve starch recovery is to use non-conventional extraction methods, such as dry extraction [30]. Furthermore, these methods allow obtaining starch with a lower fiber content, which is more refractory to acid hydrolysis than starch. On the other hand, the mean humidity of cassava was estimated at  $61 \pm 3\%$ , and losses of 5% were reported during the processing of the raw material, both the peel and the cassava.

Figure 1 shows the FTIR spectra associated with cassava, cassava peel, and starch. Cassava contains a high starch content, so both this fraction and the starch exhibit the characteristic spectrum of these molecules, with a broad band associated with hydroxyl groups ( $3000\text{--}3600\text{ cm}^{-1}$ ) [31]. This same curvature is observed in the peel since it contains structural carbohydrates such as cellulose and hemicellulose. Also, bands associated with  $\text{-CH}$  and  $\text{-CH}_2$  bonds (between  $2850$  and  $2950\text{ cm}^{-1}$ ) can be observed in all spectra, as well as absorbed water ( $1650\text{ cm}^{-1}$ ). The pattern obtained in the range  $900\text{--}1700\text{ cm}^{-1}$  is characteristic of glucose [23], which is the monomeric unit of both starch and structural carbohydrates, which are abundant in the three samples evaluated [32]. In addition, the vibration of the glycosidic bond ( $\text{C-O-C}$ ,  $1157\text{--}1164\text{ cm}^{-1}$ ) is observed [32]. Although cassava has proteins, the low content of these makes the vibration of the amide bond ( $1640\text{ cm}^{-1}$ ) not visible. In the case of cassava peel, additional absorption bands were observed in the  $1510\text{--}1600\text{ cm}^{-1}$  region, associated with aromatic ring vibrations, and bands near  $1450\text{ cm}^{-1}$  and  $1230\text{--}1270\text{ cm}^{-1}$  [33], related to lignin structures. While these bands may partially overlap with the signals from other biomass components, they are more evident in the peel samples, supporting the lignocellulosic nature of this fraction.

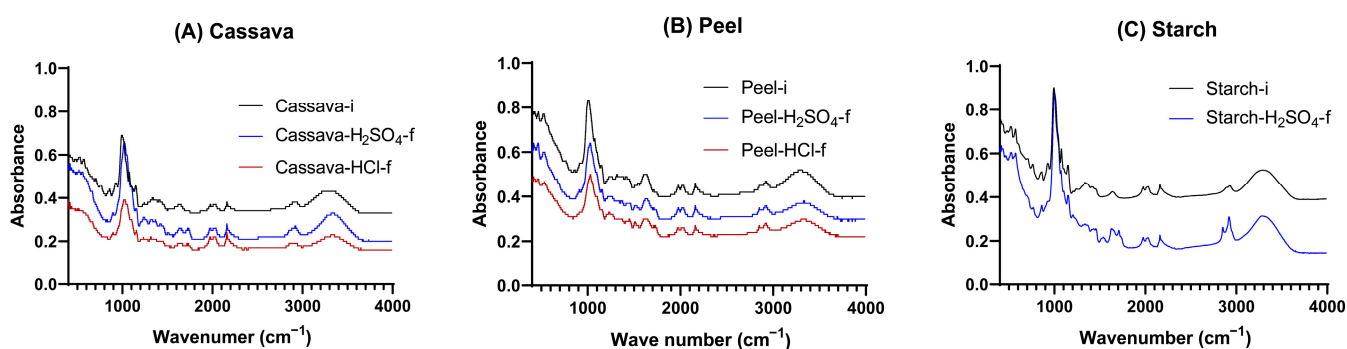


**Figure 1.** FTIR spectra of cassava fractions before and after acid hydrolysis. The label “i” represents the initial (untreated) material, whereas “f” corresponds to the hydrolyzed sample. Each spectrum is associated with the type of inorganic acid employed during hydrolysis.

### 3.2. Acid Hydrolysis of the Different Fractions of Cassava

The overall hydrolysis efficiency (OHE) for both cassava and starch was higher than that of the peel (Figure 2a); this could be because the peel contains lignin, a compound refractory to acid hydrolysis that delays the acid hydrolysis of polysaccharides [34], since in lignocellulosic biomass, lignin coats both cellulose and hemicellulose. When the hydrolysis efficiency is evaluated in terms of the release of reducing sugar (Figure 2b), it is lower than the OHE. This can be explained because part of the carbohydrates (and other components of the substrates) remain as oligomers, so the hydrolysis of the macromolecules is not complete, which is evident in Figure 2c. The saccharification efficiency for cassava starch

obtained in the present work falls within the range of values reported in the literature for enzymatic hydrolysis [11,35], which is widely recognized as an efficient method for starch conversion. Acid hydrolysis, however, represents a simpler and faster alternative and may reduce costs associated with the use of commercial enzymes. Concerning the peel, previous works have reported higher saccharification efficiencies using enzymes [36,37]. Although the methodology used was efficient in the hydrolysis of starch and cassava, it was not efficient in hydrolyzing the peel. This is due to the refractory nature of the fibrous material it contains, and appropriate conditions for this type of waste should be investigated. Saccharification efficiency is governed by various factors, such as the nature and concentration of the substrate, acid concentration, reaction time, and temperature, as well as potential interactions among these variables; therefore, the design of experiments (based on a response surface methodology or Taguchi design) represents a suitable strategy for multi-factor optimization of saccharification efficiency. However, the present study focused on the comparative evaluation of cassava fractions and hydrolysis conditions to establish baseline performance and identify process limitations rather than performing process optimization.

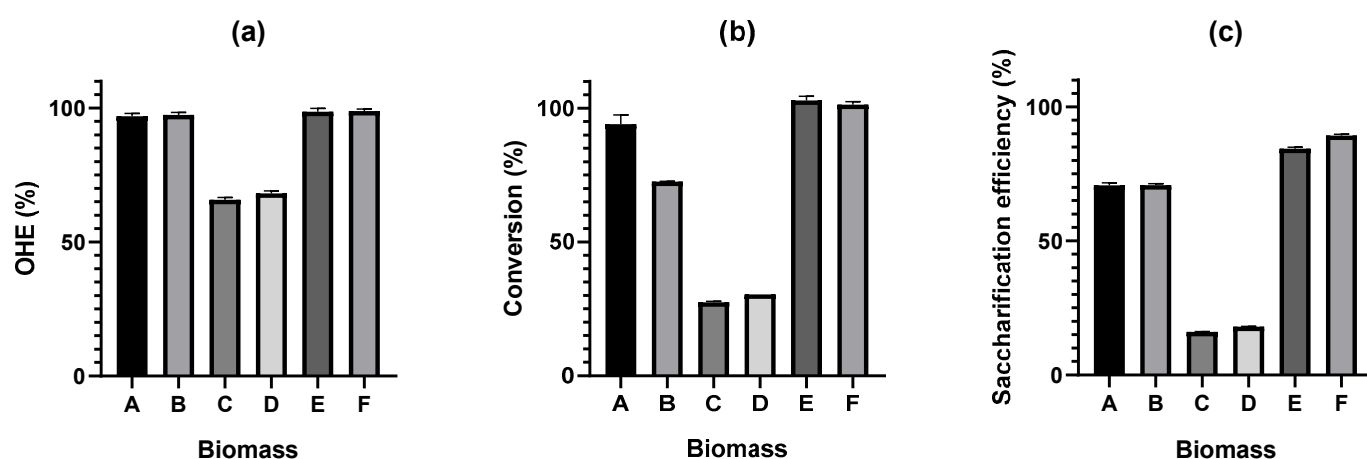


**Figure 2.** Hydrolysis (a) and saccharification (b,c) efficiencies of cassava fiber, peel, and starch pretreatment with acid. (A) and (B) cassava; (C) and (D) peel; (E) and (F) starch. (A), (C), and (E) were hydrolyzed with  $H_2SO_4$ ; while (B), (D), and (F) were hydrolyzed with HCl.

SEM was used to examine the morphological changes in the substrates subjected to acid hydrolysis (Figure 3). Prior to hydrolysis, cassava exhibited spherical granules (Figure 3A), which disappeared after treatment, giving rise to flat and amorphous structures (Figure 3B,C). On the other hand, before hydrolysis, more amorphous structures (which could presumably be fibrous material) and some starch granules are observed in the peel (Figure 3D); after hydrolysis, it is observed that the starch granules disappeared and the amorphous structures became more porous and rough (Figure 3E,F). The same effect as in cassava can be observed in the starch samples (Figure 3G,H), only that, in this case, the starch granules are well defined before hydrolysis, according to what was previously reported by Weligama Thuppahige et al. [38]. These observations are consistent with previous work that has hydrolyzed starch [39,40] and cassava peel [41] with dilute acids.

IR spectroscopy is sensitive to structural alterations occurring within starch. Absorbance ratios of 1045/1022 and 1022/995 are commonly used to measure short-range order in starch. A deconvolution of the IR spectra presented in Figure 2 was performed, and the results are presented in Table 2 and Figure S2. The 1046/1022 ratio is typically used to quantitatively assess the level of organization of crystalline regions in starch, while the 1022/995 ratio is used to estimate the proportion of amorphous to ordered structure

within carbohydrates [42]. In this sense, hydrolysis of cassava with acid (either HCl or  $\text{H}_2\text{SO}_4$ ) increases crystallinity (a higher 1047/1022 ratio) along with the amorphous regions within carbohydrates (a higher 1022/995 ratio) of the residue. This indicates that the amorphous regions are more susceptible to acid hydrolysis. The increase in crystallinity could be associated with the interaction between starch, proteins, and cassava fiber. This interaction is promoted at high temperatures and leads to a reorganization of the starch, as suggested by Chávez-Murillo et al. [43], who observed a similar behavior in legume flours. Although starch is the main component in cassava (without peel), its behavior was different, decreasing both crystallinity and the proportion of amorphous regions within the starch granule, probably due to the removal of proteins and fiber from this sample. These results are consistent with those previously reported by Mendez-Montecalvo et al. [44], who hydrolyzed achira starch with HCl. On the other hand, crystallinity in the peel increased when  $\text{H}_2\text{SO}_4$  was used, but HCl had little effect on it; meanwhile, the proportion of amorphous regions increased with HCl but was little affected when  $\text{H}_2\text{SO}_4$  was used. The ratios 1045/1022 and 1022/995 for cassava peel are generally not reported in the literature, since these ratios are mainly defined for starch, and the peel contains lignin in addition to carbohydrates. Therefore, these absorbance ratios should be interpreted as preliminary indicators of structural changes in cassava peel during acid hydrolysis rather than definitive measures of crystallinity. Additional structural analyses, such as X-ray diffraction, could provide complementary confirmation of the crystallinity variations suggested by the FTIR results.

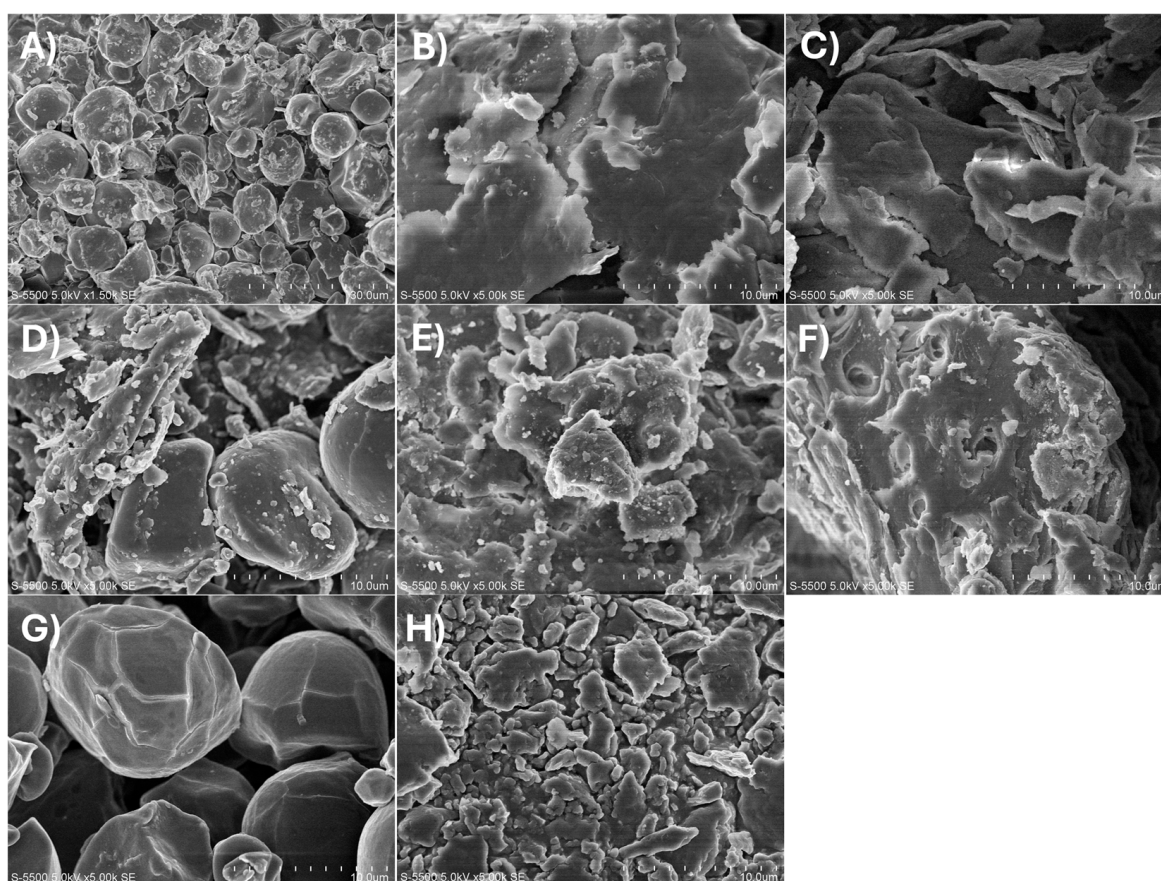


**Figure 3.** SEM micrographs of the different substrates used, before and after hydrolysis. (A,D,G) are the cassava, peel, and starch samples before hydrolysis, respectively; (B,E,H) are the same samples hydrolyzed with  $\text{H}_2\text{SO}_4$ , and (C,F) are the corresponding cassava and peel samples hydrolyzed with HCl.

**Table 2.** IR absorbance ratio of cassava, peel, and starch before and after acid hydrolysis.

| Sample                                    | IR Ratio  |          |
|-------------------------------------------|-----------|----------|
|                                           | 1047/1022 | 1022/995 |
| Cassava-i                                 | 0.782     | 0.923    |
| Cassava-H <sub>2</sub> SO <sub>4</sub> -f | 1.261     | 1.334    |
| Cassava-HCl-f                             | 1.077     | 1.374    |
| Peel-i                                    | 1.122     | 1.033    |
| Peel-H <sub>2</sub> SO <sub>4</sub> -f    | 1.210     | 1.088    |
| Peel-HCl-f                                | 1.060     | 1.212    |
| Starch-i                                  | 0.905     | 1.040    |
| Starch-H <sub>2</sub> SO <sub>4</sub> -f  | 0.859     | 0.829    |

The letters “i” and “f” refer to the sample at the beginning and end of hydrolysis.

### 3.3. Ethanol Production from Cassava Hydrolysates

Evaluating cassava fractions separately reveals compositional and structural differences that significantly affect hydrolysis and fermentation performance. Since agro-industrial cassava processing generates pulp, peel, and bagasse as distinct streams, this approach is relevant for integrated biorefinery strategies aimed at maximizing the valorization of each fraction. Prior to fermentation, the hydrolysates exhibited different reducing sugar concentrations depending on the biomass fraction and acid used, ranging from  $7.17 \pm 0.24$  to  $11.94 \pm 0.18$  g/L. Cassava hydrolysates contained  $9.31 \pm 0.39$  g/L (H<sub>2</sub>SO<sub>4</sub>) and  $7.17 \pm 0.24$  g/L (HCl), peel hydrolysates showed the highest values ( $11.94 \pm 0.18$  and  $11.78 \pm 0.00$  g/L for H<sub>2</sub>SO<sub>4</sub> and HCl, respectively), and starch hydrolysates presented intermediate concentrations ( $8.36 \pm 0.23$  and  $7.95 \pm 0.18$  g/L). As detailed in Table S2, glucose represented the major fraction of reducing sugars in all cases. To ensure comparable fermentation conditions, all hydrolysates were adjusted to the same initial glucose concentration prior to inoculation.

Acid hydrolysates were fermented in the presence of *S. cerevisiae* yeast. Figure 4 shows the time course of ethanol production, and Table 3 summarizes the kinetic parameters (yields and volumetric productivities). Table S1 summarizes the value of the constants obtained using the modified Gompertz model for all the treatments evaluated. The lag phase time ( $\lambda$ ) obtained from the modified Gompertz model provides insight into the adaptation of the yeast to the different cassava hydrolysates. In most cases,  $\lambda$  values were close to zero, indicating rapid microbial adaptation and good fermentability of the hydrolysates. This suggests that the hydrolysis and subsequent neutralization steps did not generate inhibitory conditions that significantly delayed the onset of ethanol production. Slightly higher  $\lambda$  values were observed for cassava and peel hydrolysates obtained with HCl, which may be associated with differences in ionic composition or the presence of trace inhibitory compounds. Although inhibitory compounds such as furfural, HMF, and organic acids may be generated during acid hydrolysis [6], their concentrations were not quantified in this study; nevertheless, the short or negligible lag phases and high fermentation efficiencies observed indicate that their effect on yeast performance was not significant under the conditions evaluated.

The ethanol yield based on glucose ( $Y'_{p/s}$ ; glucose-based yield) for almost all hydrolysates was equal to or greater than the theoretical yield, with the highest values obtained for the husk hydrolysate (regardless of the type of acid used). This is because this yield considers glucose as the sole substrate; however, the hydrolysates may contain other reducing sugars that can be fermented by *S. cerevisiae* yeast, such as maltose (derived from starch hydrolysis) or galactose and mannose (products of hemicellulose hydrolysis) [45]. The percentage of glucose in the different hydrolysates is presented in Table S2, with its lowest contribution in the peel hydrolysate, which could explain the high  $Y'_{p/s}$  values

for this hydrolysate. When the yield is corrected and reducing sugars are considered as a fermentation substrate, the reducing-sugar-based yield ( $Y'_{p/s}^*$ ) values are lower than the theoretical value, finding process efficiencies in the range of  $69.8 \pm 7.4\%$  to  $93.1 \pm 1.1\%$ . However, the Tukey HSD test revealed that there is only a significant difference between the peel hydrolyzed with  $H_2SO_4$  and cassava hydrolyzed with HCl (Table S3). Finally, the yield was determined based on the whole cassava on a dry basis ( $Y'_{p/s}^{**}$ ; dry-basis yield). From a practical perspective,  $Y'_{p/s}^{**}$  (dry-basis yield) represents the most economically relevant parameter because it relates ethanol production directly to raw biomass. Hydrolysates from cassava (peeled cassava) showed higher  $Y'_{p/s}^{**}$  values than the other hydrolysates, regardless of the acid used, and no statistically significant differences were detected between acids for this biomass (Table S3). These higher  $Y'_{p/s}^{**}$  values may be explained by the fact that peeled cassava represents the majority of the whole tuber, while in the case of starch, losses during extraction decrease the  $Y'_{p/s}^{**}$  value. The peel represents a smaller portion of the entire tuber (10.9%), which results in lower  $Y'_{p/s}^{**}$  values. Under the experimental conditions evaluated, no statistically significant difference was detected in  $Y'_{p/s}^{**}$  when different acids were used for the same biomass.

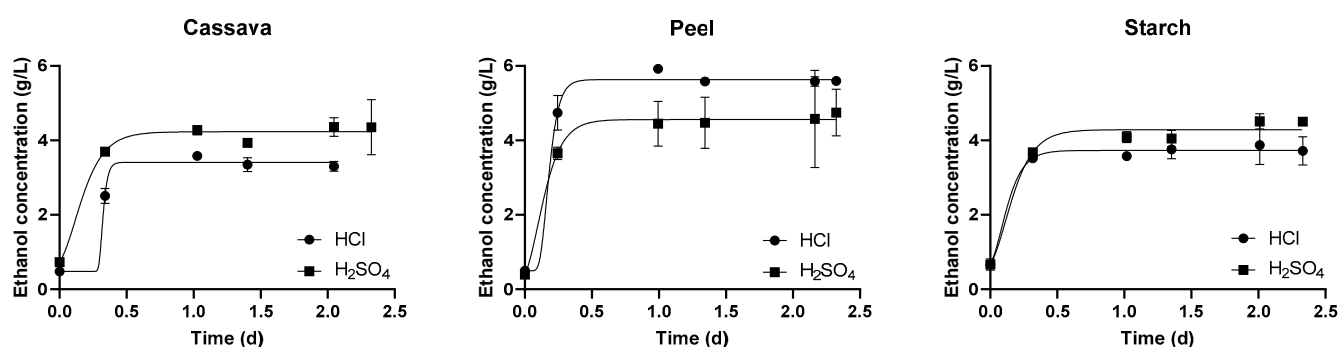


**Figure 4.** Time course of ethanol production from cassava, peel, and starch hydrolysates obtained with HCl or  $H_2SO_4$ . The solid lines correspond to the fit to the modified Gompertz model.

**Table 3.** Comparison of the kinetic parameters of batch ethanol production from cassava, peel, and starch hydrolysates.

| Hydrolysate        | $Y_{p/s}'$ (g/g) | Efficiency (%)   | $Y'_{p/s}^*$ (g/g) | Efficiency* (%) | $Y'_{p/s}^{**}$ (g/g) | $R_{p,max}$ (g/L/h) |
|--------------------|------------------|------------------|--------------------|-----------------|-----------------------|---------------------|
| Cassava- $H_2SO_4$ | $0.53 \pm 0.03$  | $103.5 \pm 6.6$  | $0.40 \pm 0.03$    | $77.8 \pm 4.9$  | $0.37 \pm 0.02$       | $0.47 \pm 0.07$     |
| Cassava-HCl        | $0.49 \pm 0.01$  | $95.4 \pm 1.2$   | $0.48 \pm 0.01$    | $93.1 \pm 1.1$  | $0.34 \pm 0.01$       | $0.85 \pm 0.09$     |
| Peel- $H_2SO_4$    | $0.61 \pm 0.06$  | $119.0 \pm 12.6$ | $0.36 \pm 0.04$    | $69.8 \pm 7.4$  | $0.01 \pm 0.00$       | $0.67 \pm 0.01$     |
| Peel-HCl           | $0.74 \pm 0.02$  | $144.2 \pm 4.7$  | $0.44 \pm 0.01$    | $85.7 \pm 2.8$  | $0.02 \pm 0.00$       | $1.85 \pm 0.33$     |
| Starch- $H_2SO_4$  | $0.54 \pm 0.02$  | $105.0 \pm 3.1$  | $0.44 \pm 0.01$    | $86.1 \pm 2.6$  | $0.28 \pm 0.01$       | $0.50 \pm 0.07$     |
| Starch-HCl         | $0.47 \pm 0.05$  | $92.8 \pm 8.8$   | $0.42 \pm 0.04$    | $81.7 \pm 7.8$  | $0.26 \pm 0.02$       | $0.57 \pm 0.06$     |

$Y'_{p/s}$ : ethanol yield based on glucose consumption (glucose-based yield).  $Y'_{p/s}^*$ : ethanol yield calculated from reducing sugars (reducing-sugar-based yield).  $Y'_{p/s}^{**}$ : ethanol yield calculated on a dry raw biomass basis (dry-basis yield, whole cassava tuber). Efficiency\* was calculated based on reducing sugars.

The  $Y'_{p/s}^*$  values obtained from cassava starch were higher than those reported by Krajang et al. [11] ( $0.38 \pm 0.00$ – $0.43 \pm 0.01$  g/g). However, in the work as mentioned above, hydrolysis and fermentation were carried out in a single stage. On the other hand, when commercial cassava flour is used, fermentation efficiencies of up to 93.5% have been achieved through a one-stage enzymatic saccharification and fermentation system [15]. The high fermentation efficiency could be attributed to the high activity of the enzyme cocktail (genetically engineered glucoamylase and commercial  $\alpha$ -amylase) used for the hydrolysis of cassava flour. This stage is the bottleneck in these fermentation processes.

While enzymatic hydrolysis, including processes using genetically modified enzymes, can achieve high saccharification efficiencies, enzyme procurement represents a significant contribution to the overall cost of bioethanol production. Conversely, chemical hydrolysis avoids the cost associated with commercial enzymes and can simplify process operation, although sometimes at the expense of slightly lower conversion efficiencies. Therefore, choosing between enzymatic and chemical hydrolysis may involve a trade-off between process efficiency and operational cost, especially in small-scale or resource-constrained production scenarios.

Regarding cassava peel, fermentation yields (considering the  $Y'_{p/s}^*$  value) are higher than those obtained from hydrolysates obtained by thermohydrolysis (0.40 g/g) and enzymatic hydrolysis (0.24 g/g) [17]. The low yields obtained in enzymatic hydrolysates could be associated with the presence of cellobiose (indicating incomplete saccharification), a reducing sugar derived from cellulose hydrolysis that cannot be assimilated by *S. cerevisiae*.

Although the mass yield  $Y'_{p/s}$  is a kinetically relevant parameter in fermentation processes, from a practical and economic perspective, the parameter  $Y'_{p/s}^{**}$ , which is expressed on a raw biomass basis, is more representative. However, this value is rarely reported in the literature, as most published studies rely on cassava flour or commercial starch or use cassava peel without specifying the proportion that this fraction represents relative to the whole cassava root, thereby hindering direct comparison. Aruwajoye et al. [16] reported  $Y'_{p/s}^{**}$  values of up to 0.329 g/g of peel, which are significantly higher than those obtained in the present study (0.01–0.02 g/g). Similarly, Kouteu Nanssou et al. [17] reported a value of 0.026 g/g of peel. Although these yields are higher than those observed in this work, the actual yields expressed per gram of whole cassava are considerably lower.

The final ethanol concentration in the fermentation broth is a key parameter, particularly from a downstream processing perspective, as it largely determines the costs associated with ethanol recovery and final use. Although the ethanol concentrations obtained in the present study (between 2.93 and 5.32 g/L) are lower than those reported in the literature—where values of up to 365.1 [14] g/L and 80.85 g/L [11] have been achieved at laboratory and pilot scales, respectively—these studies consistently indicate that the main bottleneck of the process lies in the hydrolysis step. In this context, our results highlight an opportunity to enhance fermentable sugar (or glucose) concentrations through the optimization of acid hydrolysis. A promising approach could be the application of the Grey–Taguchi method, which enables multi-objective optimization with a reduced number of experimental runs, for instance by maximizing fermentable sugar release while simultaneously minimizing the formation of inhibitory compounds.

Another parameter of great importance in fermentation processes is the maximum volumetric productivity of ethanol production ( $R_{p,max}$ ), which is obtained with the highest values for the peel hydrolysate with HCl (Table S3). It is important to note that the hydrolysates obtained with HCl yielded higher  $R_{p,max}$  values compared to those obtained with  $H_2SO_4$ . This difference may be associated with variations in the ionic composition of the hydrolysates, including the potential influence of sulfate ions. Although sulfates have been reported to negatively affect sugar consumption rates and ethanol production by *S. cerevisiae* [46], sulfate concentration and glucose consumption kinetics were not directly measured in this study. Therefore, the contribution of sulfate cannot be conclusively established, and other factors such as acid concentration, residual ionic strength, and pH adjustment during neutralization may also influence fermentation performance. The highest  $R_{p,max}$  value obtained (peel hydrolysate with HCl) is higher than that reported with enzymatic hydrolysates of cassava starch [11]. These have been described as the most efficient hydrolysis methods because they do not generate byproducts that can be inhibitory, as in the case of acid hydrolysis. However, sterile conditions were not used in the work as

mentioned above, which could have led to competition for fermentable sugars between the yeast *S. cerevisiae* and bacteria that could have contaminated the fermentation broth. To prevent bacterial growth, a broad-spectrum antibiotic (gentamicin) was added in the present work, which was reflected in increased ethanol productivity. Although antibiotics were used in this study under laboratory conditions, contamination control in industrial bioethanol production is typically achieved without antibiotics. Instead, strategies such as substrate pasteurization, low pH operation, high yeast inoculum, and rigorous cleaning and sterilization procedures, including clean-in-place (CIP) and sterilization-in-place (SIP) systems, are commonly employed to suppress bacterial growth.

In this regard, in recent years, a discontinuous process for ethanol production from previously sterilized cassava flour hydrolysates (enzymatic via) coupled with a membrane was reported [14]. The membrane allowed the recovery of ethanol from the fermenter (avoiding the inhibitory effect it can have on yeast) and the reuse of enzymes. The authors reported  $R_{p,max}$  values of up to 2.63 g/L/h. These results suggest that to improve the performance of alcoholic fermentation, competition for substrate by bacteria and continuous ethanol removal must be avoided. However, in the present work, the inhibitory effect of ethanol should not have occurred due to the low concentrations of ethanol in the fermentation broth.

As previously described, ethanol production from cassava hydrolysates, including flour, starch, and peel, has been reported. However, most studies focus on determining the kinetic parameters of ethanol production (mainly  $Y_{p/s}$  and  $R_{p,max}$ ) [1,17]. In this regard, the present work not only evaluated the performance of the fermentation process but also studied the hydrolysis process more comprehensively to better understand the mechanisms governing this stage from a morphological (through microscopy) and molecular (through FTIR studies) perspective. However, an experimental design is needed to maximize the hydrolysis of the different substrates used, as well as the fermentation process.

#### 4. Conclusions

This work demonstrates the use of inorganic acids to hydrolyze different cassava root fractions: peeled cassava, starch, and peel. High overall hydrolysis efficiencies were achieved for both cassava and starch (greater than 95%). In contrast, lower efficiencies obtained for the peel were associated with the presence of fibrous material in this biomass. However, HCl improved the saccharification of all samples evaluated. The hydrolysates obtained via the acid process were suitable for alcoholic fermentation, as demonstrated by the high yields achieved for cassava and starch, ranging from 0.40 to 0.48 g<sub>ethanol</sub>/g<sub>reducing sugar</sub>. However, the highest dry-basis yields of the feedstock were greater in the case of peeled cassava, up to  $0.37 \pm 0.02$  g/g TS. On the other hand, the use of HCl in hydrolysis improved volumetric bioethanol production productivity in all samples tested. The results suggest that the process can be maximized through an experimental design in both the hydrolytic and fermentation stages.

**Supplementary Materials:** The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/pr14050766/s1>, Figure S1: Elemental composition of cassava (A and A'), peel (B and B'), and starch (C and C') determined by EDS analysis. Panels A/A', B/B', and C/C' correspond to independent replicate measurements of the same sample; Figure S2: Deconvoluted FTIR peaks at 1047, 1022, and 995 cm<sup>-1</sup> of cassava, peel, and starch before and after acid hydrolysis. The letters "i" and "f" indicate the initial and final samples; the type of acid used is also indicated; Table S1: Kinetic parameters of the modified Gompertz model for ethanol production with cassava, peel, and starch hydrolysates; Table S2: Contribution of glucose to reducing sugars in different hydrolysates; Table S3: Pairwise comparison *p*-values of  $Y'_{p/s}$ ,  $Y'_{p/s}^*$ ,  $Y'_{p/s}^{**}$ , and  $R_{p,max}$  obtained under different treatments using Tukey's HSD.

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## Abbreviations

The following abbreviations are used in this manuscript:

|                                           |                                               |
|-------------------------------------------|-----------------------------------------------|
| $\lambda$                                 | Lag phase time                                |
| $(\text{NH}_4)_2\text{SO}_4$              | Ammonium sulfate                              |
| ACS grade                                 | American Chemical Society grade               |
| ATR                                       | Attenuated total reflectance                  |
| CIP                                       | Clean-in-place                                |
| DNS                                       | 3,5-dinitrosalicylic acid                     |
| EDS                                       | Energy dispersive spectroscopy                |
| FID                                       | Flame ionization detector                     |
| FTIR                                      | Fourier transform infrared spectrometer       |
| $\text{H}_2$                              | Hydrogen                                      |
| $\text{H}_2\text{SO}_4$                   | Sulfuric acid                                 |
| HCl                                       | Hydrochloric acid                             |
| $\text{KH}_2\text{PO}_4$                  | Monopotassium phosphate                       |
| $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ | Magnesium sulfate heptahydrate                |
| $\text{N}_2$                              | Nitrogen                                      |
| NaOH                                      | Sodium hydroxide                              |
| OHE                                       | Overall hydrolysis efficiency                 |
| RI                                        | Refractive index                              |
| $R_{p,\max}$                              | Maximum volumetric rate of ethanol production |
| SEM                                       | Scanning electron microscopy                  |
| SIP                                       | Sterilization-in-place                        |
| TS                                        | Total solids                                  |
| VS                                        | Volatile solids                               |
| $y_{\max}$                                | Maximum concentration of ethanol produced     |
| $Y'_{p/s}$                                | Apparent ethanol yield                        |
| $Y_{p/s}$                                 | Theoretical yield                             |

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