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# Bench-Scale Second-Generation Bioethanol Production from Bleached *Pinus taeda* Kraft Pulp

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## Abstract

The production of second-generation bioethanol from lignocellulosic biomass requires efficient enzymatic hydrolysis and fermentation processes that remain effective at industrially relevant solids loadings. In this study, bleached *Pinus taeda* kraft pulp was evaluated as a model substrate for bioethanol production at bench scale (4 L reactor) under high-consistency conditions (12.5–13.9% solids). Three process configurations were compared: separate hydrolysis and fermentation (SHF), simultaneous saccharification and fermentation (SSF), and pre-hydrolysis followed by simultaneous saccharification and fermentation (pSSF). Enzymatic hydrolysis in the SHF and SSF configurations stabilized between 54% and 58%, indicating that hydrolysis was the main process bottleneck under the evaluated conditions. In contrast, *Saccharomyces cerevisiae* efficiently fermented the available glucose, achieving nearly complete conversion of glucose. Among the evaluated strategies, pSSF showed the highest ethanol yield and volumetric productivity, achieving an ethanol yield of 61.8% and a productivity of 0.61 g $L^{-1}$ $h^{-1}$. While laboratory-scale SSF experiments conducted at 2% solids achieved complete conversion, the ethanol yield decreased to approximately 58% at the bench scale, highlighting the impact of high-solids operation on process performance. The lower performance observed at high solids may be associated with factors commonly reported during scale-up, including increased slurry viscosity, reduced mixing efficiency, limited enzyme accessibility, and mass-transfer constraints. Overall, the results manifest the need to enhance hydrolysis performance through improved reactor design, more effective mixing strategies, and optimized high-solids processing to facilitate the scale-up of lignocellulosic bioethanol production.

**Keywords:** second-generation bioethanol; enzymatic hydrolysis; simultaneous saccharification and fermentation; kraft pulp; bench-scale bioreactor

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## 1. Introduction

There is a global tendency to replace fossil fuels with renewable energy sources to reduce greenhouse gas emissions and mitigate climate change. Bioethanol has emerged as a relevant alternative, with applications ranging from transportation fuel to an intermediate for the production of bioplastics such as bio-based polyethylene [1–3].

Lignocellulosic biomass has emerged as a promising renewable feedstock for second-generation (2G) bioethanol production because it enables the valorization of agricultural, forestry, and agro-industrial residues without competing directly with food

resources [4,5]. The efficient conversion of these materials into fermentable sugars depends on the integration of pretreatment, enzymatic hydrolysis, and fermentation, as well as on the intrinsic characteristics of the biomass and the operating conditions employed [6–8].

Among these stages, enzymatic saccharification is particularly critical because it governs the release of glucose and other fermentable sugars from cellulose and hemicellulose. Operational variables such as pH, temperature, enzyme loading, solids concentration, and mixing strongly affect hydrolysis efficiency and process economics. In addition, non-productive adsorption onto lignin remains a relevant limitation [9–12]. Recent studies have also emphasized the need to tailor enzymatic systems to specific feedstocks and to develop reactor configurations capable of handling high-solids slurries under industrially relevant conditions [13–15].

Different process configurations have been proposed to address these limitations. In the separate hydrolysis and fermentation (SHF) approach, hydrolysis and fermentation occur in independent stages, allowing each step to operate at its optimal temperature. Simultaneous saccharification and fermentation (SSF) integrates both processes in a single reactor, reducing end-product inhibition because fermenting microorganisms consume sugars as they are produced [16,17]. Pre-hydrolysis simultaneous saccharification and fermentation (pSSF) represents an intermediate strategy that introduces a short hydrolysis step before fermentation to increase sugar availability [18].

Fermentation is commonly carried out using *Saccharomyces cerevisiae*, the microorganism most widely used in industrial ethanol production for its high efficiency in converting glucose to bioethanol. This yeast is categorized as GRAS (Generally Recognized as Safe), shows high productivity and ethanol tolerance, and functions effectively within an acidic pH range.

Laboratory experience indicates that it is possible to obtain good yields of bioethanol from pine sawdust [16,17]. Laboratory-scale experiments typically employ Erlenmeyer flasks or small reactors operating at low consistency (1–2%), conditions that facilitate mixing and mass transfer but do not accurately represent industrial processes. As solids loading increases, the rheological properties of the system change significantly, leading to higher viscosity and mixing limitations [19]. These factors affect the uniform distribution of the enzymes and heat [20].

Bench-scale reactors serve as an intermediate platform for evaluating process performance under more realistic operating conditions. These studies help identify limitations related to scale, such as restrictions in mass transfer, enzyme inhibition, and the impacts of high-solids loading on the efficiency of hydrolysis and fermentation. Although high-total-solids operation presents challenges related to agitation and mixing, it also offers important advantages, including reduced capital and production costs. Additionally, downstream product separation requires lower energy to reduce water content.

The recent literature shows a sustained interest in maintaining saccharification efficiency during scale-up under high-solids conditions, which is currently one of the most active and technically important areas in lignocellulosic bioethanol research. Although many enzymatic hydrolysis systems perform satisfactorily at laboratory scale using relatively low solids contents (1–5%) and small reactor volumes, their performance often decreases significantly during scale-up due to poor mixing, non-Newtonian slurry rheology, dead zones, mass-transfer limitations, insufficient bulk flow, enzyme inhibition, and the excessive power demand required for agitation. Therefore, advancing the understanding and optimization of enzymatic hydrolysis under high-solids and scale-up conditions remains essential for the development of economically viable and industrially scalable second-generation bioethanol processes.

Bleached kraft pulp has undergone several delignification and bleaching stages to remove lignin, resulting in a material with high cellulose content. For this reason, it represents a suitable model substrate for studying enzymatic hydrolysis and fermentation processes. In this work, bleached kraft pulp was subjected to enzymatic hydrolysis and fermentation at the bench scale to compare with laboratory results and analyze the influence of solids consistency in a stirred reactor.

The objective of this work is to evaluate different hydrolysis–fermentation strategies for second-generation bioethanol production at the bench scale using bleached *Pinus taeda* kraft pulp. Its novelty lies in the bench-scale evaluation of second-generation bioethanol production from high-consistency bleached kraft pulp. By comparing SHF, SSF, and pSSF configurations, the study identifies differences in hydrolysis and ethanol production performance under high-solids bench-scale conditions relevant for process scale-up.

## 2. Materials and Methods

### *2.1. Raw Material*

Bleached *Pinus taeda* kraft pulp was used as a highly delignified lignocellulosic substrate. It was provided by ARAUCO Argentina (Puerto Esperanza, Misiones, Argentina). The pulp was supplied as dried sheets. After measuring humidity, 120 g (oven-dry basis) was weighed (for the bench-scale tests), resuspended at 2% consistency using a pulper (Paper Fiber Pulp Disintegrator; Testing Machines Inc. (TMI), New Castle, DE, USA), and finally dewatered by spin-drying to a 30% dry content to eliminate excess water. The material was weighed again to calculate the water content in the resuspended pulp.

The chemical composition of the pulp (substrate) was characterized according to NREL (National Renewable Energy Laboratory) standards in the USA to determine the structural carbohydrates and lignin (NREL/TP-510-42618). HPLC (Waters Corp., Milford, MA, USA) was performed using an Aminex HPX-87H column (Bio-Rad Laboratories Inc., Hercules, CA, USA) with a refractive index detector, with $H_2SO_4$ 5 mM as the eluent, at 0.6 mL $min^{-1}$ and 60 °C. HPLC analyses were performed using an Empower 2 software system (Waters Corporation, Milford, MA, USA).

The pulp composition was 84.3% glucans and 15.7 xylans plus mannans, with traces of lignin.

### *2.2. Enzymes and Microorganism*

In the experiment, the enzymatic complex used for hydrolysis was Cellic® CTec3 HS, donated by Novozymes (São Pablo, Brasil), and the cellobiase obtained from *Aspergillus niger*, referred to as β-glucosidases, purchased from Sigma-Aldrich (Buenos Aires, Argentina).

The cellulase activity was determined in terms of "filter paper units" (FPU) according to the NREL/TP-510-42628 standard [21]. The activity of cellobiase was determined by its capacity to hydrolyze 4-nitrophenol β-D-glucopyranoside (p-NPG) to 4-nitrophenol (p-NP). This method involves adding 0.5 mL of various enzyme dilutions to 2 mL of 1 mmol $L^{-1}$ p-NPG solution. The mixture was incubated for 30 min at 50 °C, and the reaction was stopped by adding 2.5 mL of $Na_2CO_3$. Finally, the absorbance was measured at 400 nm and expressed in IU $mL^{-1}$ [22].

The filter paper activity of Cellic® CTec3 HS (donated by Novozymes, São Pablo, Brasil) was 247 FPU $mL^{-1}$, and that of β-glucosidases was 300 IU $mL^{-1}$.

The yeast *Saccharomyces cerevisiae* IMR 1181 (SC 1181) employed for fermentation was donated by QUIMOBI (Center for Research in Biological Organic Chemistry) from the National Technological University (UTN), Argentina.

### 2.3. Pre-Inoculum and Inoculum

YPD medium (Yeast Extract–Peptone–Dextrose) was prepared with the composition of 10 g $L^{-1}$ yeast extract, 20 g $L^{-1}$ peptone, and 20 g $L^{-1}$ dextrose according to the NREL (TP-510-42630) standard for simultaneous saccharification and fermentation (SSF). It was supplemented with the following nutrients: 5 g $L^{-1}$ $KH_2PO_4$, 1.5 g $L^{-1}$ $NH_4Cl$, and 0.65 g $L^{-1}$ $MgSO_4 \cdot 7H_2O$ [23]. The medium was sterilized in an autoclave at 121 °C for 30 min. A pre-inoculum was prepared to promote cell growth by transferring 4 mL of *Saccharomyces cerevisiae* IMR 1181 (SC 1181), previously stored in an Eppendorf tube, into 12 mL of YPD medium and incubating until the optical density reached 0.5–0.9. The culture was then scaled up to a final volume of 90–110 mL (inoculum) and further incubated until the optical density reached 0.7–0.8. Both the pre-inoculum and inoculum stages were conducted in an orbital shaker at 180 rpm and 35 °C (±0.1 °C). The pre-inoculum stage required 17.5–22 h, and the inoculum stage required 21–24.5 h.

To quantify yeast concentration, 1 mL of sample plus 9 mL of NaCl solution (at 0.85% *w*/*v*) was used to measure the optical density at 600 nm (UV-1800 Spectrophotometer, Shimadzu Corporation, Kyoto, Japan). The reference was the culture medium without yeast.

### 2.4. Equipment

The bench-scale experiments were conducted in a 4 L stainless-steel reactor (Bench scale equipment Co., subsidiary of Chemineer, Dayton, OH, USA) equipped with a five-blade high-shear impeller, positioned centrally within the vessel for high-shear agitation, and a heating system with a PT-100 temperature controller (NOVUS, Model N1050-PRRR-485, Canoas, Brasil) with a sensitivity of 0.1 °C.

### 2.5. Bench-Reactor Assays

In all bench-scale experiments, the initial solids consistency was adjusted to achieve the same final working volume of 960 mL. The liquid-to-solid ratio (LSR) was 7:1 for experiments conducted at 12.5% consistency and 6.2:1 for those conducted at 13.9% consistency. The stirred-tank reactor experiments were performed at 12.5–13.9% consistency to evaluate process performance under more concentrated and technologically relevant conditions.

The solids content was expressed as pulp consistency, defined as the ratio of the dry mass of solids to the total mass of the suspension, expressed as a percentage (Equation (1)).

$$\text{Consistency}(\%) = \frac{\text{dry weight of pulp (g)}}{\text{dry weight of pulp (g)} + \text{liquid (g)}} \times 100 \quad (1)$$

The different trials accomplished at the bench scale are summarized in Figure 1.

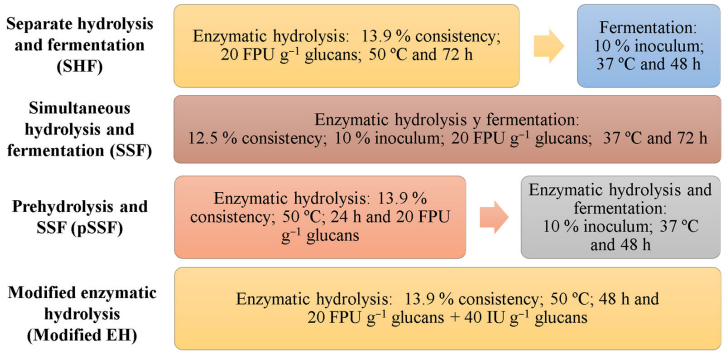



**Figure 1.** Experimental design and process configurations evaluated for bioethanol production from bleached *Pinus taeda* kraft pulp. SHF: separate hydrolysis and fermentation; SSF: simultaneous

saccharification and fermentation; pSSF: pre-hydrolysis followed by simultaneous saccharification and fermentation. Laboratory-scale assays were conducted in 200 mL Erlenmeyer flasks with a working volume of 100 mL at a solids loading of 2% and an agitation rate of 130 rpm. Bench-scale experiments were conducted in a 4 L stirred-tank reactor, with a final working volume of 960 mL and a solids concentration of 12.5–13.9%.

### *2.6. Different Trials at the Bench Scale*

#### 2.6.1. SSF (Simultaneous Saccharification and Fermentation)

At the bench scale, the resuspended pulp (120 g, dry basis) was subjected to simultaneous saccharification and fermentation with *Saccharomyces cerevisiae*. The initial consistency was 12.5%, working at 37 °C—the optimal temperature determined in a previous article [17]—with an initial agitation (5 min) of 420 rpm and then 200 rpm until the end. The reactor was fitted with a gas trap to prevent oxygen from entering the system and to allow the release of carbon dioxide produced by the microorganisms.

For this purpose, 420 mL of water (considering water in the pulp and inoculum volume), 420 mL of buffer solution (0.1 M sodium citrate, pH 5), 8.20 mL of Cellic® CTec3 HS (equivalent to 20 FPU g $^{-1}$ glucans), 96 mL of inoculum (10%), 0.3 mL of Tween 80 surfactant, 0.5 g $L^{-1}$ of yeast extract, and 1 g $L^{-1}$ of peptone were added. Before starting the process, the resuspended pulp, buffer solution, and water were autoclaved for 30 min at 121 °C. Samples were taken at time zero and then at 10, 24, 48, and 72 h to quantify glucose and ethanol.

At the laboratory scale, simultaneous saccharification and fermentation (SSF) assays were performed using 200 mL Erlenmeyer flasks with a working volume of 100 mL. The flasks were placed on an orbital shaker operating at 130 rpm and a consistency of 2%. All other conditions were consistent with those used in the 4 L stirred-tank reactor. A solids loading of 2% was selected for the flask assays because suspensions with consistencies of 12–14% could not be mixed effectively under orbital agitation due to severe rheological limitations. Experiments were performed in duplicate.

#### 2.6.2. Separate Hydrolysis and Fermentation (SHF)

First, the resuspended pulp was subjected to enzymatic saccharification, followed by fermentation. For this purpose, 120 g (dry basis) of resuspended pulp was enzymatically hydrolyzed for 72 h at 50 °C and 420 rpm, with an initial solids consistency of 13.9%. After hydrolysis, the slurry was frozen for approximately 48 h until further use. Before fermentation, it was thawed in a thermostatic water bath at 37 °C and subsequently fermented for 48 h at 37 °C and 200 rpm using *Saccharomyces cerevisiae*. Both stages were conducted in a 4 L stainless-steel stirred-tank reactor.

For the saccharification stage, 420 mL of water (including the water contained in the pulp and the volume corresponding to the inoculum added in the subsequent fermentation step), 420 mL of 0.1 M sodium citrate buffer (pH 5.0), 8.20 mL of Cellic® CTec3 HS (equivalent to 20 FPU g $^{-1}$ glucans), and 0.3 mL of Tween 80 were added to the reactor. Samples were collected at 3, 16, 24, 48, and 72 h to determine glucose concentration.

For the fermentation stage, 96 mL of yeast inoculum, 0.5 g $L^{-1}$ of yeast extract, and 1 g $L^{-1}$ peptone were added. The reactor was fitted with a gas trap to prevent oxygen from entering the system and to allow the release of carbon dioxide produced by the microorganisms. Samples were collected at 0, 8, 24, and 48 h for ethanol quantification.

2.6.3. Pre-Hydrolysis and Then SSF (pSSF)

The same quantity of resuspended pulp used in the previous test was enzymatically hydrolyzed for 24 h at 50 °C and 420 rpm, with an initial consistency of 13.9%. Samples were taken from the pre-saccharification process at 0, 16, 20, and 24 h to quantify glucose. Then, it was fermented for 48 h at 37 °C and 200 rpm with *Saccharomyces cerevisiae*. Both stages were conducted in a 4 L stainless-steel stirred-tank reactor. For the saccharification stage, 420 mL of water (including the water contained in the pulp and the volume corresponding to the inoculum added in the subsequent fermentation step), 420 mL of 0.1 M sodium citrate buffer (pH 5.0), 8.20 mL of Cellic® CTec3 HS (equivalent to 20 FPU $g^{-1}$ glucans), and 0.3 mL of Tween 80 were added to the reactor.

Before the addition of 96 mL of yeast inoculum, 0.5 g $L^{-1}$ of yeast extract, and 1 g $L^{-1}$ of peptone, the reactor temperature and agitation speed were adjusted to the previously described fermentation conditions (37 °C and 200 rpm). The reactor was fitted with a gas trap to prevent oxygen from entering the system and to allow the release of carbon dioxide produced by the microorganisms. Samples were taken at times 0, 2, 16, 20, 24, 40, and 48 h to quantify ethanol.

2.6.4. Enzyme Hydrolysis with Complex and Additional Cellobiase (Modified EH)

As in the SHF experiment, the resuspended pulp underwent saccharification for 48 h. A key difference was that, in this experiment, both Cellic® CTec3 HS and β-glucosidases were used. To achieve this, 120 g (dry basis) of resuspended pulp was enzymatically hydrolyzed for 48 h at 50 °C and 420 rpm, with an initial consistency of 13.9%. This took place in a 4 L stainless-steel stirred-tank reactor. For the first stage, 323 mL of water (including water in the pulp), 420 mL of buffer solution (0.1 M sodium citrate, pH 5), 8.2 mL of Cellic® CTec3 HS (equivalent to 20 FPU $g^{-1}$ glucans), 13.5 mL of cellobiase (equivalent to 40 IU $g^{-1}$ glucans), and 0.3 mL of Tween 80 were added. Samples were taken from the saccharification process at time 0, 1.30, 15.30, 24, 39, and 48 h to quantify glucose.

*2.7. Characterization of the Products*

Glucose and bioethanol concentrations were quantified using high-performance liquid chromatography (HPLC) (Waters Corp., Milford, MA, USA) with an Aminex-HPX-87H column (Bio-Rad Laboratories Inc., Hercules, CA, USA). The conditions were as follows: 5 mM $H_2SO_4$ as the eluent, a flow rate of 0.6 mL $min^{-1}$, a temperature of 60 °C, and a refractive index detector.

For the hydrolysis alone, the enzymatic hydrolysis yield (EH Yield (%)) was calculated according to Equation (2):

$$\text{EH Yield } (\%) = \frac{\text{glucose (g)} \times 0.9}{\text{glucans in the material (g)}} \times 100 \quad (2)$$

When both processes occur simultaneously, the glucose produced at a given time was estimated by adding the measured glucose concentration to the ethanol concentration, converted to glucose equivalents using a factor of 1.96.

Ethanol yield (%) was calculated as the percentage of the theoretical ethanol that could be produced from the glucose contained in the original kraft bleached pulp, assuming complete conversion of glucan to glucose and subsequent fermentation to ethanol (Equation (3)).

$$\text{Ethanol Yield } (\%) = \frac{\text{produced ethanol } (\text{g L}^{-1})}{\text{theoretical ethanol } (\text{g L}^{-1})} \times 100 \quad (3)$$

where theoretical ethanol was calculated as 0.51 × the glucose theoretically obtainable from the glucan content of the original pulp sample (g $L^{-1}$).

Overall productivity was calculated according to Equation (4) using the maximum ethanol concentration reached during the experiment and the processing time for each process configuration (e.g., the combined hydrolysis and fermentation time for the SHF process).

$$\text{Productivity (g L}^{-1}\text{ h}^{-1}) = \frac{\text{ethanol produced (g L}^{-1})}{\text{time (h)}} \quad (4)$$

## 3. Results and Discussion

In all instances, a sample of the inoculum was taken to check for the presence of glucose or ethanol at the initial time (t = 0).

### *3.1. Separate Hydrolysis and Fermentation (SHF)*

In this assay, the resuspended kraft pulp was first subjected to enzymatic hydrolysis using the enzymatic complex Cellic® CTec3 HS at 50 °C with maximum agitation (420 rpm) for the entire processing duration of 72 h. The advantage of this method is that hydrolysis and fermentation yields can be analyzed separately. Glucose concentration stabilizes after 24 h without reaching the maximum possible value (variation less than 5% between 24, 48, and 72 h, Figure 2). Hsieh et al. [13] state that high glucose concentrations inhibit cellulase activity and enzyme binding. Therefore, the product inhibition by glucose may explain this behavior.

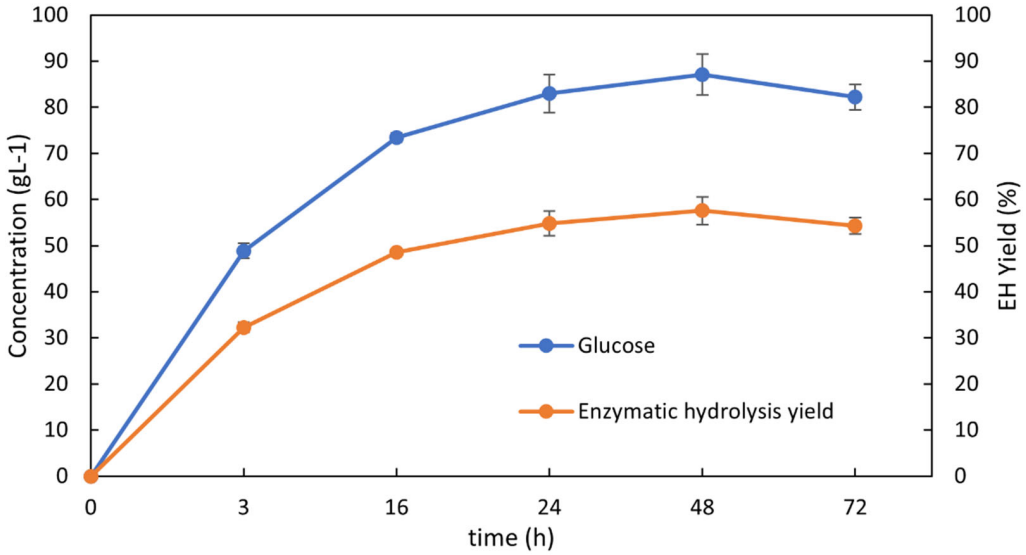



**Figure 2.** Enzymatic hydrolysis profile of bleached *Pinus taeda* kraft pulp in the separated hydrolysis and fermentation (SHF) experiments in the stirred-tank reactor. Glucose concentration is expressed in g $L^{-1}$ as a function of hydrolysis time. Experiments were performed in duplicate at 13.9% solids consistency, 50 °C, pH 5.0 (using 0.1 M sodium citrate buffer), and 420 rpm with Cellic® CTec3 HS at a dosage of 20 FPU $g^{-1}$ glucan. Error bars represent standard deviations (number of replicates n = 2).

In the fermentation stage, as shown in Figure 3, all the glucose is consumed within the first 24 h. This indicates that high glucose concentrations do not limit fermentation.

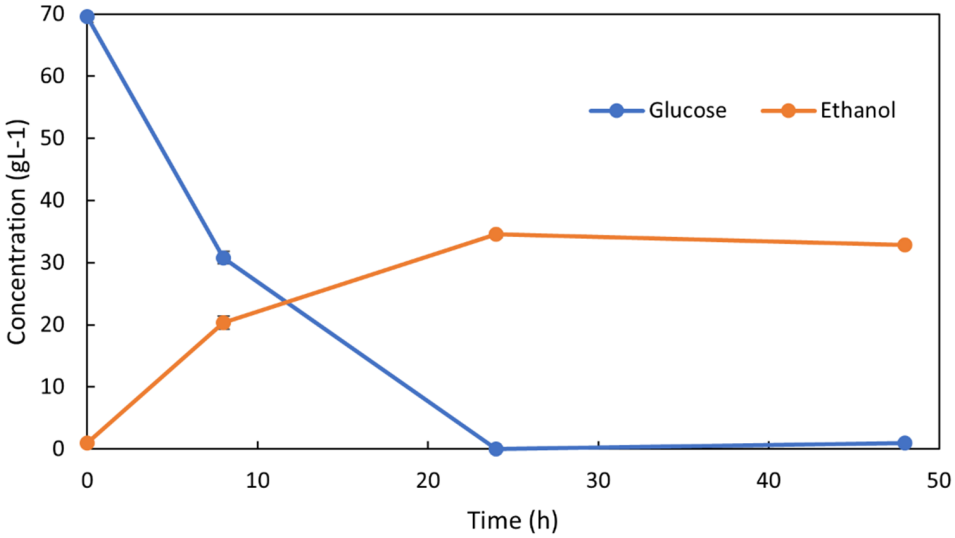



**Figure 3.** Evolution of glucose and ethanol concentrations during the fermentation stage of the SHF (separate hydrolysis and fermentation) process using bleached *Pinus taeda* kraft pulp. The hydrolysate was obtained after 72 h of enzymatic hydrolysis at 50 °C, pH 5.0, and 420 rpm using Cellic® CTec3 HS (20 FPU $g^{-1}$ glucan). Fermentation was subsequently conducted with *Saccharomyces cerevisiae* in a 4 L stirred-tank reactor utilizing a 960 mL working volume at 37 °C and 200 rpm. Glucose and ethanol concentrations are expressed in g $L^{-1}$ as a function of fermentation time. Error bars represent standard deviations (n = 2).

### *3.2. Simultaneous Saccharification and Fermentation (SSF)*

Laboratory-scale and bench-scale experiments were initiated simultaneously under similar conditions. In these assays, resuspended kraft pulp, the enzymatic complex Cellic® CTec3 HS, and the yeast were simultaneously added at the beginning of the process. The temperature was set at 37 °C, which is below the optimum for enzymatic hydrolysis but above the optimal range for yeast fermentation (30–32 °C). The temperature effect was analyzed in a previous work [17].

In the laboratory experiments, the ethanol yield approached 100% at the end of the process (72 h) (Figure 4). However, at higher consistency, the maximum ethanol yield for the reactor reached approximately 58% after 72 h processing time, with a theoretical ethanol concentration of 68 g $L^{-1}$. Romaní et al. [24], working with SSF at high solids loading, found that, for most experiments, the highest ethanol concentration was obtained at 72 h. Considering the tendency of the resulting concentrations to stabilize, the stated processing time was sufficient.

At the bench scale, the pH at the end of fermentation, regardless of the strategy used, ranged from 4.20 to 4.33. Meanwhile, the pH at the end of the saccharification step was 4.62. Therefore, the decrease observed during hydrolysis was moderate, while the lower final pH in the simultaneous configurations likely reflects additional acidification associated with the fermentation stage and $CO_2$ production.

At the bench scale, the initial sample showed glucose accumulation with no ethanol formation, suggesting that enzymatic hydrolysis predominated during the early stage of the process and yeast had not yet reached its maximum activity. This behavior is consistent with a short adaptation phase of the microorganisms to the reaction medium. The glucose concentration reached its maximum at 10 h and decreased rapidly thereafter, indicating that the glucose released by hydrolysis was continuously consumed by *Saccharomyces cerevisiae* during fermentation. Glucose did not accumulate after this point, and ethanol production continued to increase. These results indicate that the conversion of cellulose to glucose was the primary limiting step under the evaluated conditions.

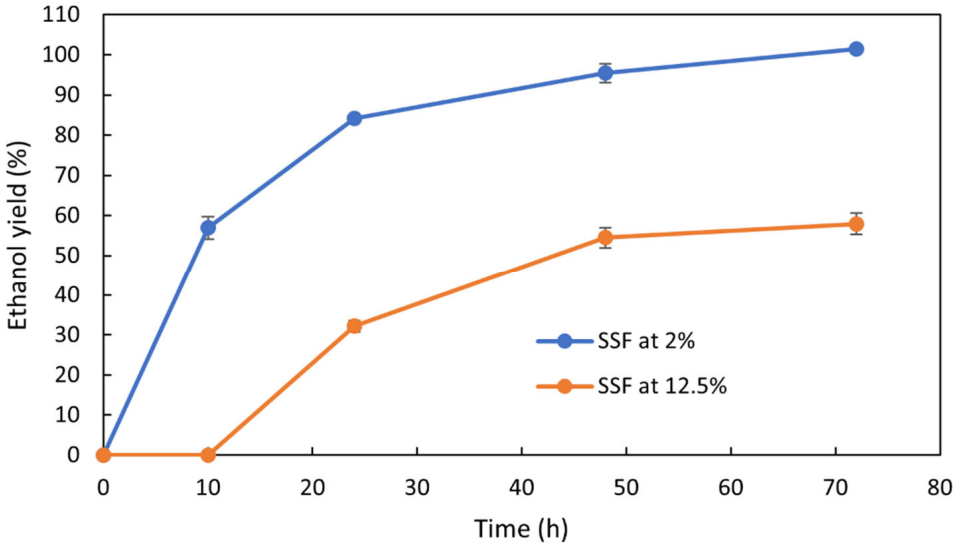



**Figure 4.** Comparison of ethanol yield during the SSF (simultaneous saccharification and fermentation) experiments at laboratory- and bench-scale using bleached *Pinus taeda* kraft pulp. Enzymatic hydrolysis was performed with Cellic® CTec3 HS at pH 5.0, and *Saccharomyces cerevisiae* was added at the beginning of the simultaneous process. Error bars represent standard deviations (n = 2).

### *3.3. Pre-Hydrolysis and Then SSF (pSSF)*

Given that the SSF identified enzymatic hydrolysis as the bottleneck in the system, a 24 h pre-saccharification step was implemented. This pre-saccharification step, often referred to as liquefaction, is expected to improve mass transfer and reduce mixing limitations during SSF. A broad range of pre-saccharification times tested have been reported (1–4 h [25], 12 h [18], 24 h [26]). The selected duration for the previous hydrolysis step was 24 h, based on the saccharification results in the SHF process, as seen in Figure 2.

An enzymatic hydrolysis yield of 54.0% was obtained, as expected, at the end of the 24 h pre-hydrolysis step (previous samples yielded 50.4% at 16 h and 52.0% at 20 h). The hydrolysis rate approached a plateau during this period.

After the saccharification stage, yeast was added. As shown in Figure 5, all the available glucose is fermented within the first 16 h. As fermentation proceeds, continued glucose consumption by the microorganisms results in an increase in ethanol concentration. However, the fermentation occurs so fast that glucose levels remain practically at zero.

After 48 h of simultaneous hydrolysis and fermentation, an ethanol yield of 62% (61.81%) was obtained. The pSSF process involved 24 h of pre-hydrolysis, followed by 48 h of simultaneous saccharification and fermentation. When comparing this to the SSF process over 72 h, the ethanol yield increased by approximately 4 percentage points in the pSSF method. In this strategy, it is worth noting that the minimum concentration required to meet the industrial production requirements of 40 g $L^{-1}$ of ethanol [27] is achieved.

The final ethanol yield was similar to that reported by Guigou et al. [28]. They used *Eucalyptus grandis* sawdust subjected to autohydrolysis and soda pulping followed by a 24 h pre-hydrolysis step and 48 h SSF in a laboratory reactor, obtaining an ethanol potential yield of 64%.

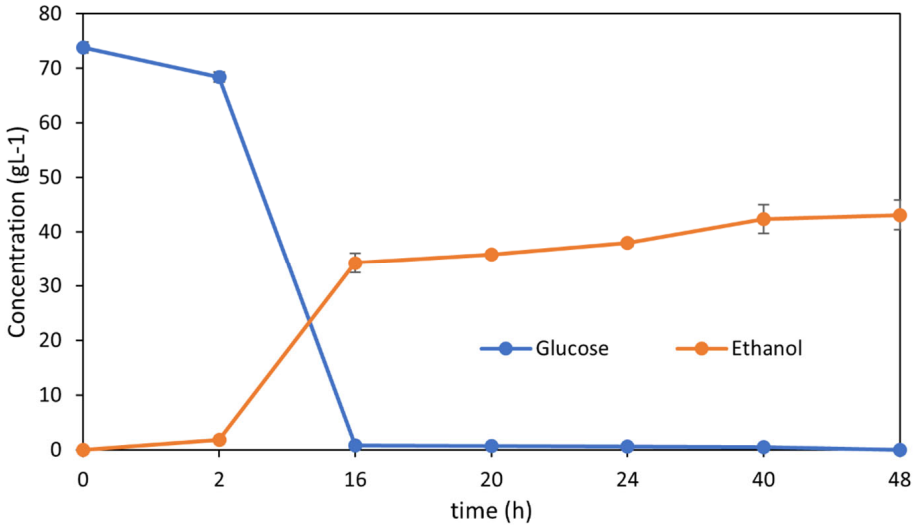



**Figure 5.** Evolution of glucose and ethanol concentrations during the pSSF (pre-hydrolysis followed by simultaneous saccharification and fermentation) experiments after yeast addition. Bleached *Pinus taeda* kraft pulp was subjected to a 24 h pre-hydrolysis step before inoculation with *Saccharomyces cerevisiae*. The simultaneous hydrolysis and fermentation stage was conducted in a 4 L stirred-tank reactor with a working volume of 960 mL at 37 °C and 200 rpm. Glucose and ethanol concentrations are expressed in g $L^{-1}$ as a function of process time after yeast addition. Error bars represent standard deviations (n = 2).

### *3.4. Enzyme Hydrolysis with Complex and Additional Cellobiase (Modified EH)*

As mentioned earlier, this assay involved complementing the enzymatic complex (Cellic® CTec3 HS) with β-glucosidases. The objective was to enhance the enzymatic hydrolysis yield, as the presence of cellobiase would improve cellulase activity by reducing its potential inhibition by products [29].

The enzymatic hydrolysis yield was lower than that obtained by the complex alone (53.8% at the end of a processing time of 48 h) (Figure 6).

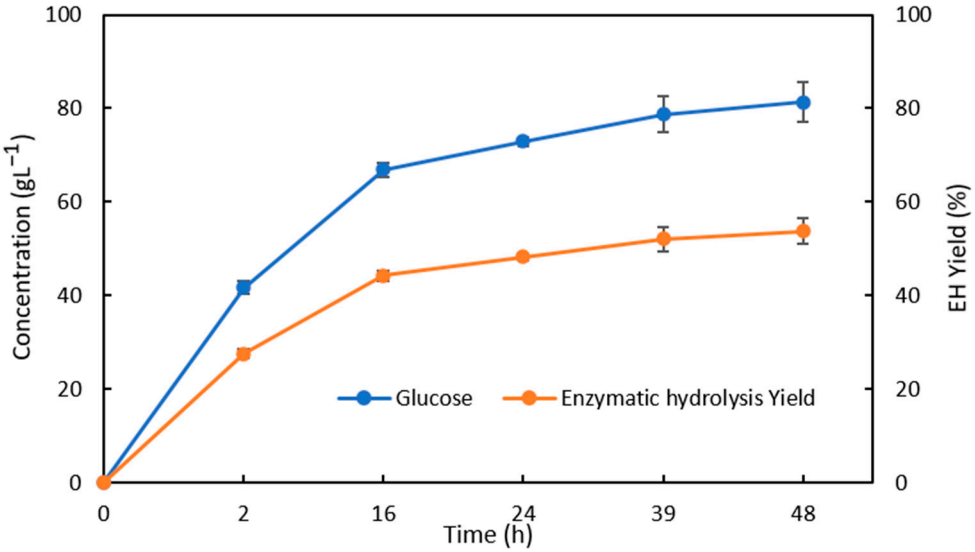



**Figure 6.** Effect of additional β-glucosidase supplementation on enzymatic hydrolysis of bleached *Pinus taeda* kraft pulp. Hydrolysis was conducted at 50 °C, pH 5.0, 420 rpm and a solids consistency of 13.9%. Cellic® CTec3 HS was used as the base enzyme preparation, and the supplemented treatment received additional β-glucosidase from *Aspergillus niger*. Glucose concentration is expressed in g $L^{-1}$ as a function of hydrolysis time, and enzymatic hydrolysis (EH) yield as a percentage. Error bars represent standard deviations (n = 2).

The absence of a substantial change in hydrolysis yield after supplementation with additional β-glucosidase suggests that β-glucosidase deficiency may not have been the only factor limiting hydrolysis under the conditions tested; however, because cellobiose was not quantified, the contribution of cellobiose accumulation could not be determined.

### *3.5. All Strategies Comparison*

Figure 7 shows a comparison of the evaluated processes. Table 1 summarizes the key time points analyzed.

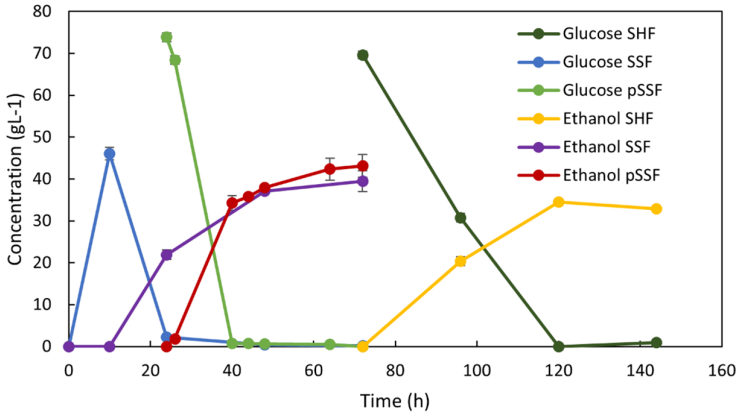



**Figure 7.** Evolution of glucose and ethanol concentrations during the fermentation process for the SHF, SSF, and pSSF configurations using bleached *Pinus taeda* kraft pulp. Data are presented as a function of total process time, including hydrolysis and fermentation stages. SHF: separate hydrolysis and fermentation; SSF: simultaneous saccharification and fermentation; pSSF: pre-hydrolysis followed by simultaneous saccharification and fermentation. Glucose and ethanol concentrations are expressed in g $L^{-1}$. Error bars represent standard deviations (n = 2).

**Table 1.** Comparison of hydrolysis and ethanol production performance for SHF, SSF, and pSSF configurations using bleached *Pinus taeda* kraft pulp. EH Yield (%): enzymatic hydrolysis yield; SHF: separate hydrolysis and fermentation; SSF: simultaneous saccharification and fermentation; pSSF: pre-hydrolysis followed by simultaneous saccharification and fermentation. Productivity was calculated using the time required to reach the maximum ethanol concentration for each process configuration.

| Process | Hydrolysis Strategy | EH Yield (%) at 48 h | Maximum EH Yield (%) | Maximum Ethanol Yield (%) | Productivity (g $L^{-1}$ $h^{-1}$) |
|---|---|---|---|---|---|
| SSF | Simultaneous saccharification and fermentation | 54.8 (a) | 58.1 (72 h) (a) | 57.9 (72 h) | 0.55 (72 h) |
| SHF | Separate hydrolysis and fermentation | 57.6 | 57.6 (48 h EH) | 51.3 (72 h EH + 24 h fermentation) | 0.36 (96 h) |
| pSSF | 24 h pre-hydrolysis + SSF | 57.4 (a) | 54.0 (24 h pre-hydrolysis) 64.6 (24 h EH + 48 h fermentation) (a) | 61.8 (24 h EH + 48 h fermentation) | 0.61 (72 h) |
| Modified EH | Hydrolysis with cellulase + cellobiase supplementation | 53.8 | 53.8 (48 h) | – | – |

(a) Estimated as mentioned in Section 2.7.

The data show that, regardless of the strategy, the process stabilizes. Operating at the optimal temperature for the enzymatic complex significantly accelerates the saccharification process. Simultaneous saccharification and fermentation (SSF) takes twice as long to achieve the same yields as enzymatic hydrolysis alone.

Previous studies have demonstrated that pSSF can attain high ethanol productivity in lignocellulosic systems. However, the results depend strongly on feedstock type, pretreat-

ment, solids loading, and process configuration. A direct comparison of the reported pSSF conditions and performances is presented in Table 2.

**Table 2.** Comparative performance of lignocellulosic ethanol production using pre-saccharification and simultaneous saccharification and fermentation (pSSF).

| Feedstock | Solids Loading (%) | Enzyme Loading | Pre-Hydrolysis (h) | SSF/ Fermentation (h) | Ethanol Yield (%)/Concentration | Productivity (g $L^{-1}$ $h^{-1}$) | Scale | Reference |
|---|---|---|---|---|---|---|---|---|
| Bleached *Pinus taeda* kraft pulp | 13.9 | 20 FPU $g^{-1}$ glucan | 24 | 48 | 61.8% | 0.61 | 4 L reactor | This work |
| Sugarcane bagasse *(native)* | 12.0 | Commercial cellulase | 12 | 48 | ~65.0–68.0% | 0.63 | Laboratory scale | [18] |
| *Eucalyptus* sawdust (autohydrolysis + soda pulping) | 7.5 | 15 FPU $g^{-1}$ glucan | 24 | 48 | 64.0% | n.r. | Laboratory scale | [28] |
| *Eucalyptus globulus* bark | 17.5 | Commercial cellulase | 48 | 24 | ~60.0–62.0% | 0.52 | Laboratory scale | [26] |
| OrganoCat-pretreated vine shoots (cellulose-rich pulp) | 15% initial + 5% added after 24 h (20% final, fed-batch) | 45 FPU $g^{-1}$ cellulose (Cellic® CTec2) | 48 | 48 | 70% of theoretical/ 33 g $L^{-1}$ | n.r. | Laboratory scale | [30] |

n.r. = not reported.
Enzyme loading is expressed on a glucan basis or biomass basis according to the original reference.

Comparison with previous studies shows that the pSSF strategy evaluated in this work performs competitively under relatively demanding conditions. The process achieved an ethanol yield of 61.8% and a productivity of 0.61 g $L^{-1}$ $h^{-1}$ at a solids loading of 13.9% in a 4 L stirred reactor using bleached *Pinus taeda* kraft pulp. These values are comparable to those reported for sugarcane bagasse by Neves et al. (2016) (65–68% yield and 0.63 g $L^{-1}$ $h^{-1}$ productivity at 12% solids) [18] and higher than the productivity reported for *Eucalyptus globulus* bark by Gomes et al. (2021) (0.52 g $L^{-1}$ $h^{-1}$ at 17.5% solids) [26]. Although Guigou et al. (2019) obtained a slightly higher ethanol yield from eucalyptus sawdust, their process was conducted at a lower solids loading (7.5%) [28]. In addition, the present study required only 24 h of pre-hydrolysis, which is substantially shorter than the 48 h reported by Gomes et al. (2021) [26]. Lara-Serrano et al. (2025) obtained an ethanol concentration of 33 g $L^{-1}$, corresponding to approximately 70% of the theoretical pSSF yield, from OrganoCat-pretreated vine-shoot pulp using a fed-batch pSSF configuration with a final solids loading of 20% and 45 FPU $g^{-1}$ cellulose; however, volumetric productivity was not reported, making direct comparison more difficult [30].

Overall, the results indicate that pSSF can provide competitive ethanol productivity while operating at relatively high solids loading and with a reduced pre-hydrolysis time. The results indicate that the 24 h pre-hydrolysis step provided a moderate increase in saccharification and improved process operability, likely associated with slurry liquefaction, enhanced enzyme accessibility, reduced viscosity, and mitigation of mass-transfer limitations.

### *3.6. Scale-Up Implications and Reactor Limitations*

As mentioned previously, most operating conditions were maintained unchanged during the scale-up, including enzyme dosage, inoculum percentage, temperature, Tween 80 concentration, and nutrient supplementation. As a result, the differences observed in

hydrolysis and ethanol yields are mainly attributed to the higher solids loading and the operational limitations associated with the larger reactor volume. Table 3 compares the main characteristics and results obtained at laboratory and bench scales of the SSF strategy.

**Table 3.** Operating conditions for laboratory-scale and bench-scale experiments of the SSF strategy. Flask experiments were conducted in 200 mL Erlenmeyer flasks with a 100 mL working volume at 2% solids consistency and 130 rpm. Reactor experiments were performed in a 4 L stirred-tank reactor with a 960 mL working volume at solids consistencies of 12.5%. Temperature, pH, enzyme loading, agitation, inoculum, and nutrient conditions are reported for each process configuration.

| Scale | Laboratory | Bench Scale |
|---|---|---|
| Reactor volume (L) | 0.2 | 4 |
| Working volume (mL) | 100 | ~1000 |
| Consistency (%) | 2 | 12.5 |
| Rpm | 130 | 420 (5 min), then 200 |
| Agitation mode | Orbital shaker | Mechanical impeller |
| Maximum ethanol yield (%) | 100 | 57.9 |

Laboratory-scale assays were designed to determine the maximum achievable bioethanol yield under ideal and unconstrained conditions and were therefore used in this study as control experiments. Reactions were conducted in 200 mL Erlenmeyer flasks at a solids loading of 2% using an orbital shaker, since higher solids concentrations result in severe rheological limitations that hinder effective mixing and mass transfer. These low-solids conditions were selected to maximize enzyme accessibility and ensure homogeneous mixing throughout the reaction medium. Nevertheless, such conditions are not representative of industrial practice, where economically viable lignocellulosic ethanol production requires operation at substantially higher solids loadings in mechanically stirred reactors. Consequently, future work will focus on high-solids hydrolysis to bridge the gap between laboratory screening and industrial-scale application.

Although complete conversion was achieved at the laboratory scale (2% solids), ethanol yield decreased to approximately 58% at the bench scale (12.5% solids). The lower ethanol yields obtained in the 4 L reactor compared with the laboratory-scale experiments may be due, in part, to the higher concentrations of intermediate and end products, which may inhibit cellulase activity. This effect is likely associated with the higher solids loading. At high consistency, the fiber network increases slurry viscosity and reduces mixing efficiency, which may limit enzyme–substrate contact and decrease hydrolysis rates. These effects become increasingly relevant during scale-up and have been identified as major bottlenecks in industrial lignocellulosic ethanol production. Kristensen et al. [31] analyze the composition of substrate, product inhibition, water concentration, and cellulase adsorption, emphasizing the difficulty in interconnecting the factors affecting enzymatic yield; they conclude that the solid effect could be explained by the inhibition of enzyme adsorption by hydrolysis products (glucose and cellobiose).

It is relevant that the bleached kraft pulp used in this study was supplied as dried sheets with a solids content near 90%. The drying of cellulosic materials causes a phenomenon known as hornification, characterized by the formation of irreversible bonds between cellulose microfibrils and fibrils during water removal. As a consequence, the swelling capacity, porosity, and accessible surface area of the fibers decrease [32,33]. These structural modifications can reduce the enzyme's accessibility to hydrolysis sites, limiting the penetration of the enzyme complex into the cell wall and decreasing saccharification efficiency. Therefore, the hornification generated during the pre-drying of the pulp could have contributed to the observed hydrolysis yields [34], even though the material had a low lignin content and a high proportion of glucans.

Furthermore, the pulp needs to be resuspended, with water accounting for 60% of the resuspended pulp mass even after centrifugation.

## 4. Discussion of Technological Alternatives

Due to the significance of process configuration, kinetic behavior, and integration strategy, various authors have proposed alternative solutions to address the key challenges of biomass hydrolysis for bioethanol production. Table 4 summarizes the proposed technologies, along with their advantages and limitations.

**Table 4.** Comparative analysis of process configurations for lignocellulosic biomass hydrolysis.

| Configuration | Typical Solids Loading | Key Advantages | Disadvantages and Limitations | Kinetic and Inhibitor Impact | References |
|---|---|---|---|---|---|
| SHF (separate hydrolysis and fermentation) | Low to Moderate (<15%) | Independent optimization of pH and temperature (50 °C for enzymes/ 30–37 °C for microbes). Allows detoxification before fermentation. | High capital expenditures (CAPEX) are required because separate reaction vessels are needed. Prolonged overall process times (72–96 h). Potential sugar losses during intermediate solid–liquid separation. | Severe feedback inhibition; the accumulation of glucose and cellobiose drastically blocks cellulase and β-glucosidase active sites. | [35–38] |
| SSF (simultaneous saccharification and fermentation) | Moderate to High (≥15%) | Lowers CAPEX and contamination risks by integrating both steps into a single vessel. Shorter overall processing times and high volumetric productivity. | Requires a compromised temperature (≥37 °C) that severely penalizes enzymatic hydrolysis rates. Incompatible with oxidative enzymes (LPMOs) requiring specific cofactors. | Mitigates end-product inhibition; fermenting microorganisms continuously consume sugars as they are released, sustaining the catalytic driving force. | [35,37–39] |
| pSSF (pre-saccharification followed by SSF) | High (≥15% to 30%) | Rapidly liquefies biomass at optimal enzymatic temperature (50 °C) during the first 6–24 h before inoculation. Combines the benefits of SHF and SSF. | Requires an intermediate cooling step before introducing the yeast inoculum to prevent thermal cell death. | Drastically reduces initial slurry viscosity and mass/heat transfer limitations, overcoming the “solids effect” and protecting enzymes from shear stress. | [35–39] |
| CBP (consolidated bioprocessing) | Moderate to High | Maximum integration: a single engineered microbe produces enzymes, deconstructs biomass, and ferments sugars. Eliminates costs associated with commercial enzymes. | Currently has a very low biological conversion efficiency. Candidate strains (e.g., *Clostridium thermocellum*) exhibit low ethanol tolerance (<2.5 g $L^{-1}$). | Prone to carbon catabolite repression and carbon diversion toward undesirable organic acid by-products. | [36–39] |
| CBS (consolidated bio-saccharification) | Moderate to High | Integrates on-site enzyme production and saccharification without chemical addition, isolating fermentation to avoid thermal compromises. | The resulting hydrolysate is a complex fermentation broth containing bacterial cell remnants and metabolic intermediates. | Native cellulosomes suffer steric hindrance and inhibition from dissolved xylan. Requires supplemental glucose-tolerant β-glucosidases. | [36] |

**Table 4.** *Cont.*

| Configuration | Typical Solids Loading | Key Advantages | Disadvantages and Limitations | Kinetic and Inhibitor Impact | References |
|---|---|---|---|---|---|
| CEH (continuous enzymatic hydrolysis) | Moderate (10%) | Achieves equivalent conversions with a 50% reduction in enzyme loading. Increases glucose (~15.6%) and xylose yields via in situ diafiltration. | Technical challenges related to membrane fouling and gasket failure under low-flow and low-pressure conditions. Dilutes the product stream if the flow rate is unoptimized. | Eliminates sugar end-product feedback inhibition in real time by continuously passing monomers through 10 kDa membranes, keeping cellobiose <0.5%. | [40] |
| Mechanocatalysis (chemo-mechanical route) | Very High (Up to 100% Solids) | Enzyme-free process driven by ball milling and mineral acid impregnation. Ultrashort reaction times (<6 h). Reduces the minimum sugar selling price by up to 30%. | High electricity and energy consumption at an industrial scale due to mechanical milling. Requires post-hydrolysis acid neutralization and carbon detoxification. | Independent of biomass lignin content. Mechanical shear ruptures β-1,4-glucosidic bonds, yielding 100% water-soluble oligosaccharides (WSO). | [37] |

The comparative analysis in Table 4 shows that the transition from laboratory to industrial scale involves more than increasing reactor volume; it also changes the dominant limitations. At the low solids loading, reaction kinetics and enzyme accessibility are the major constraints. Conversely, at the industrially relevant solids loading (>15%), mass transfer, slurry rheology, enzyme deactivation, and inhibitor management become increasingly important. The various process configurations represent successive attempts to enhance productivity while minimizing enzyme usage and simplifying the process.

SHF offers the greatest operational flexibility because hydrolysis and fermentation can be optimized independently, but it suffers from strong product inhibition and longer overall processing times. SSF can partially alleviate glucose and cellobiose inhibition by coupling sugar release with sugar consumption in a single reactor, thereby generally improving volumetric productivity. However, the compromise temperature required for simultaneous hydrolysis and fermentation can reduce enzymatic performance. Among the integrated configurations, the pSSF option offers the best balance, as the pre-hydrolysis step facilitates biomass liquefaction, reduces viscosity, and enhances enzyme accessibility before yeast inoculation.

CBP and CBS aim to reduce or eliminate the need for externally produced enzymes by integrating enzyme production with biomass deconstruction. Although these approaches are attractive from a process integration perspective, current microbial systems still show lower conversion efficiency and limited tolerance to inhibitors and ethanol. Consequently, they should presently be regarded as promising long-term biotechnological strategies rather than immediate industrial solutions.

More recent intensified approaches focus on overcoming the kinetic limitations of enzymatic hydrolysis. Continuous enzymatic hydrolysis with in situ sugar removal has been reported to increase sugar release and reduce enzyme requirements by alleviating product inhibition. Meanwhile, mechanocatalytic methods aim to shorten processing times and decrease reliance on enzyme cocktails. Despite their potential, these technologies still face significant engineering and economic challenges. Overall, the literature suggests that future industrial biorefineries will likely rely on hybrid solutions that combine improved pretreatment, high-solids pSSF, intensified hydrolysis strategies, and enhanced reactor operation rather than on any single configuration alone.

Based on the hydrolysis profiles obtained in the stirred reactor, the lower ethanol yields were primarily associated with incomplete enzymatic hydrolysis and the resulting limitation in the availability of fermentable sugars, which may have been influenced by high-solids phenomena such as mass-transfer limitations and product inhibition. Accordingly, the comparison between flask and reactor experiments reflects the combined influence of increased solids loading, mixing configuration, hydrodynamics, and scale, rather than scale-up alone. However, the present experimental design does not allow the relative contribution of scale-dependent hydrodynamic and mixing effects to be separated from those arising from the increase in solids loading.

Since the study did not include experiments specifically designed to quantify the contribution of product inhibition, mass-transfer limitations, reduced water availability, hornification, or mixing constraints, these mechanisms are interpreted as literature-supported hypotheses rather than as phenomena directly demonstrated by the experimental data. Future studies should focus on mitigating these constraints using approaches such as post-hydrolysis solids separation and recirculation, mechanical pretreatment of the pulp, and other process-intensification strategies.

## 5. Conclusions

Bleached *Pinus taeda* kraft pulp was evaluated as a model substrate for second-generation bioethanol production using SHF, SSF, and pSSF configurations. In all cases, enzymatic hydrolysis was identified as the main process bottleneck. The maximum hydrolysis yields were 57.6% for SHF, 58.1% for SSF, and 64.6% for pSSF, whereas *Saccharomyces cerevisiae* efficiently fermented virtually all available glucose.

Hydrolysis conducted under optimal enzymatic conditions accelerated glucose release, as evidenced by the similar hydrolysis yield of 54.8% achieved after 24 h in SHF and at 48 h in SSF. Under the conditions investigated in this work, pSSF showed the highest ethanol yield (61.8%) and volumetric productivity (0.61 g $L^{-1}$ $h^{-1}$) among the evaluated configurations (although no techno-economic or energy assessment was performed).

Although complete conversion was achieved in SSF at the laboratory scale (2% solids), ethanol yield decreased to approximately 58% at the bench scale (12.5% solids), highlighting the challenges associated with high-solids processing and scale-dependent mixing and hydrodynamic effects.

The current experimental design did not permit a separation of the effects of increased solids loading from those related to scale-up. Additionally, there was no evaluation of enzyme costs, energy requirements for agitation, heating, and cooling needs, or the overall economics of the process. Therefore, future work should focus on improving enzymatic hydrolysis through strategies such as fed-batch operation, enhanced reactor designs, post-hydrolysis solids recirculation, mechanical pretreatment of the pulp, and reprocessing of the residual unhydrolyzed glucan fraction.

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