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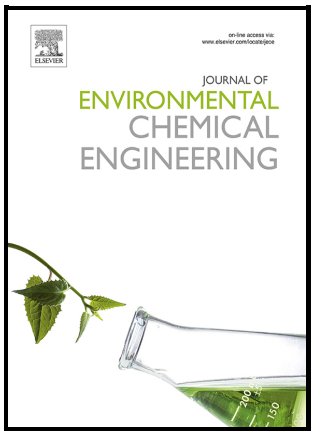

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Improving simultaneous saccharification and fermentation by pre-saccharification and high solids operation for bioethanol production from *Eucalyptus globulus* bark

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Abstract

The European Green Deal emerged as a package of policy initiatives for a green transition, aiming to reach climate neutrality by 2050. Accordingly, the pulp and paper industry has been focused on upgrading residues into value-added products within the forest-based circular economy business model. In this context, this study considered the possibility of using bark, a residue available in high volume on factory floors, for cellulosic ethanol production instead of typical burning for energy generation. Bioconversion of lignocellulosic polysaccharides of bark is a challenge and simultaneous saccharification and fermentation (SSF) setup was chosen to boost cellulosic ethanol production. The introduction of a short pre-saccharification (PS) stage (0, 1 and 4 h) in bioethanol production from *Eucalyptus globulus* bark, previously submitted to a kraft pretreatment, following an integrated configuration, PS-SSF, at the bioreactor scale improved bioethanol concentration. It was observed that the longer pre-saccharification, the higher productivity. Shifting from this batch PS-SSF (4 h) to a fed-batch PS-SSF (4 h) configuration allowed to increase the solids loading from 8 to 20% (w/v), raising the final bioethanol concentration from 27.4 to 75.9 g L^{-1}, and improving by itself the overall productivity more than 25%. These results show that in the pulp and paper mills, an integrated biorefinery should be considered to foster the total resources use within the circular economy model.

Keywords: Bioethanol; *Eucalyptus globulus*; simultaneous saccharification and fermentation (SSF); pre-saccharification (PS); fed-batch; high solids loading;

1. Introduction

The European Green Deal has developed recently as a response to progressive climate change. This strategy aimed to reach carbon neutrality by 2050, promoting a

resource-efficient and competitive economy. Therefore, the industrial sector has a key role as an accelerator of change, innovation and economic growth. The pulp and paper (P&P) industry has been committed to turning the low-carbon bioeconomy into reality. This sector has joined efforts toward decarbonization and process efficiency improvement, becoming more energy self-sufficient (Lipiäinen et al. 2022; Mäki et al. 2021). The share of energy derived from biomass in this sector has grown over time, accounting for about 60% in 2020 (Confederation of European Paper Industries - CEPI 2021) These actions align with recent government policies emerging worldwide, promoting a deep energy transition from fossil to renewable resources (Amândio et al. 2022b). Furthermore, a strong bet in research and development for innovative technologies and bioproducts has been observed, with a commitment to meet regulatory requirements and new consumption patterns (Haile et al. 2021; Mäki et al. 2021). There is a growing interest in upgrading the wastes, sub-products and side-streams generated during all the process stages for producing biofuels, biocomposites and bioplastics (Amândio et al. 2022a; Haile et al. 2021).

Eucalyptus globulus, a fast-growing species, is the most widely used wood source in the Portuguese pulp and paper sector. Due to its negative impact on pulp quality, the bark is an abundant industrial residue derived from wood handling. Currently, most of the bark is burned for energy and steam generation due to its low economic value and chemical complexity (Domingues et al. 2010; Neiva et al. 2018). About 200 kg of bark are generated per ton of pulp produced (Haile et al. 2021). Therefore, about 0.5 Mton of bark are generated considering the total production of 2.66 Mton of virgin fiber pulp from eucalyptus in Portugal in 2021 (Biond - Forest fibers from Portugal 2022). However, the bark is still an unrecognized valuable lignocellulosic feedstock. Its conversion into cellulosic sugars is one promising valorization pathway, recognized as a platform for a

wide range of biochemicals and biofuels, namely bioethanol (Isikgor and Becer 2015; Neiva et al. 2018; Rodrigues et al. 2018). Bioethanol is a renewable transportation fuel typically blended with gasoline in different proportions (Deshavath et al. 2021; Hossain et al. 2021).

The cellulosic ethanol from *E. globulus* bark entails three main steps: 1) pretreatment, to promote lignin separation and facilitate enzymes access to the polysaccharides; 2) hydrolysis of cellulose and hemicelluloses for its conversion into monosaccharides; and 3) fermentation to convert these sugars into bioethanol through the metabolic activity of the microorganisms (Jönsson and Martín 2016). Already in the late 80's of last century, Wright, Wyman, and Grohmann were pioneers in presenting a complete technical and economic evaluation of wood biotransformation to bioethanol (Wright et al. 1988). This work brought a deep contribution to the scientific community, promoting several studies of integrated processes, concerning other raw materials, namely solid lignocellulosic wastes, with innovative variants as shown below.

Pre-saccharification (PS) and simultaneous saccharification and fermentation (SSF) are the main integrated configurations investigated for second-generation bioethanol production (Pratto et al. 2020). There is a growing interest in these integrated approaches since enzymatic hydrolysis and fermentation are performed in the same reaction vessel, reducing the number of unit operations, time, and costs (Mendes et al. 2017). No further processing of the hydrolysate is required before fermentation, minimizing the loss of sugars and preventing the contamination risk (Ask et al. 2012; Hans et al. 2019).

SSF approach has been widely applied for alleviating issues regarding enzyme inhibition caused by end-product (in particular, cellobiose and glucose). The pretreated lignocellulosic biomass, enzymatic consortium and inoculum are added simultaneously at the beginning of the process. Therefore, the sugars released from enzymatic hydrolysis

are converted into ethanol by microorganisms almost immediately, reducing the processing time. By maintaining low sugar concentrations along the process, this setup minimizes the inhibition of enzymatic activity, reducing the risk of contamination (Hans et al. 2019; Pratto et al. 2020; Saini et al. 2018; Wyman et al. 1992). The main disadvantage is related to the operation temperature, which is a trade-off between the optimum value required by the enzymatic consortium catalyzing hydrolysis (50 °C) and the microbial culture promoting ethanolic fermentation (typically between 28 and 30 °C) (Mendes et al. 2016). Consequently, a slowdown in the hydrolysis rate may occur, resulting in lower sugar yield compared to the separated hydrolysis and fermentation (SHF) configuration (Paulova et al. 2015; Pratto et al. 2020). The low ethanol productivity in the early stages of the process due to limited sugar availability is another limitation of this configuration (Paulova et al. 2015).

Following a PS-SSF configuration, some drawbacks of the SSF methodology could be overcome. Several designations of this setup were used in the literature, including SSF with delayed inoculation, non-isothermal SSF, and semi-SSF. This approach starts with a pre-saccharification period, conducted at the optimal conditions for enzymatic hydrolysis (between 45 and 50 °C, depending on the enzymatic consortium). Then, the reaction system is cooled down until the trade-off temperature, commonly around 38 °C (Paulova et al. 2015). Selecting a suitable pre-saccharification period is crucial to ensure high productivity (Pratto et al. 2020). However, this parameter highly depends on the substrate, namely concentration, pretreatment, and microbial strain. The main aim of the pre-saccharification stage is to boost the hydrolysis rate and promote the conversion of cellulose into glucose in the early stages, reducing the initial consistency of the slurry considerably before inoculation. Therefore, an increase in ethanol production is expectable by overcoming the glucose limitation at the beginning of SSF (Paulova et al.

2015). This approach could be particularly advantageous for high solids loading (above 15% w/v), another strategy to boost bioethanol production (Pino et al. 2018; Pratto et al. 2020).

The operation using high substrate loading could offer significant economic benefits, including reduced capital investment, lower energy requirements and lower disposal and wastewater treatment costs (He et al. 2018; Pino et al. 2018; Zhang et al. 2010). However, some technical challenges still hinder its implementation, namely mass and heat transfer limitations due to the high consistency of these complex mixtures (Chen and Liu 2017; He et al. 2018). Consequently, the mixing energy requirements increase and a decline in hydrolysis yield could be observed (He et al. 2018; Zhang et al. 2012). Moreover, the higher the initial consistency, the longer the liquefaction process takes, imposing decreased productivity (Geng et al. 2015; He et al. 2018; Zhang et al. 2009). Another barrier could be related to sugar feedback enzyme inhibition (Chen and Liu 2017). Therefore, the fed-batch operation mode could be a promising alternative to overcome some of these limitations (Chen and Liu 2017; Mendes et al. 2016). This configuration promotes the homogenization of the reactional mixture and efficient mass transfer since the new solids loading is added to the system only after the liquefaction of almost the feedstock initially added (Gomes et al. 2018).

Based on the above considerations, the present work aimed to assess bioethanol production from *E. globulus* bark (previously pretreated by kraft pulping) from SSF and PS-SSF configurations in a nominal 5L-bioreactor scale. Moreover, a fed-batch PS-SSF strategy was evaluated to increase the solids loading gradually and boost bioethanol production. Despite all the research conducted regarding bioethanol production, to our knowledge, this is the first time that pretreated *E. globulus* bark is converted into cellulosic ethanol through fed-batch PS-SSF configuration at the bioreactor scale.

2. Materials and Methods

2.1. Raw material and pretreatment

Pretreated *Eucalyptus globulus* bark was kindly provided by RAIZ - Instituto de Investigação da Floresta e do Papel (Eixo, Portugal). Bark was pretreated by kraft pulping in a laboratory rotary digester (Apineq, Leça do Balio, Portugal) using a solid-to-liquid ratio of 1:8. The pretreatment was carried out at 170 °C for 75 min using an active alkali of 24 wt %. Active alkali means that the pretreatment solution contains 24% of alkali content (measured as Na_2O equivalents) in relation to the biomass' dry weight. The pretreatment solution contained NaOH, Na_2S and Na_2CO_3 at a ratio of 65:25:10.

The chemical composition of the raw material, namely its carbohydrates and lignin contents, was determined according to NREL standard protocols (Sluiter et al. 2012). The kraft pulp is mainly composed of cellulose (79.8 ± 3.8 wt %), hemicelluloses (15.5 ± 0.6 wt %) and lignin (2.6 ± 0.3 wt %) (Amândio et al. 2021).

2.2. Enzymatic consortium

Cellic® CTec2, a commercial cellulases consortium from Novozymes, was acquired from Sigma-Aldrich with an enzymatic activity of 168.7 FPU mL^{-1}. The activity was checked before each round of experiments to ensure an enzyme dosage of 25 FPU g_{CH}^{-1}.

2.3. Microorganism

Ethanol Red® was kindly provided by Leaf by Lesaffre Advanced Fermentations. The strain was grown at 28 °C and maintained at 4 °C on Petri dishes with solid yeast medium (YM) composed of 10 g L^{-1} glucose, 5 g L^{-1} peptone, 3 g L^{-1} malt extract, 3 g L^{-1} yeast extract and 20 g L^{-1} agar.

2.4. Pre-inocula and inocula

Pre-inocula were prepared in duplicate by transferring 2-3 colonies from a maintenance YM Petri dish to a 100 mL Erlenmeyer flask containing 40 mL of liquid YM (similar to solid YM, except agar) and then incubated at 28 °C and 180 rpm for 24 h (Stuart, SI500).

Inocula were prepared in duplicate by transferring the pre-inoculum to 200 mL fresh liquid YM. The inocula was incubated at 28 °C and 180 rpm for 14 h.

2.5. SSF Integrated assays

Assays were carried out in a 5 L BIOSTAT A plus bioreactor (Sartorius Stedim Biotech, Göttingen, Germany) with a working volume of 2 L and automatic control of stirring, temperature, pH by micro-DCU software and data acquisition by MFCS/DA 3.0 system (Sartorius Stedim Systems). The pH was monitored using a 405-DPAS-SC-K8S probe (Mettler Toledo™) and controlled to 5.50 ± 0.05 by adding KOH 5 M and H_2SO_4 1 M. The stirring was set at 250 rpm by two six-bladed Rushton turbines.

Each experiment started with 8% (w/v) substrate loading and the enzymatic consortium dosage of 25 FPU $g_{carbohydrates}^{-1}$, which was added once at the beginning. The inoculum represented 10% (v/v) of the total working volume. Supplementation of 2.0 g L^{-1} $(NH_4)_2HPO_4$, 1.0 g L^{-1} $(NH_4)_2SO_4$, 0.5 g L^{-1} $MgSO_4.7H_2O$, and 2.5 g L^{-1} yeast extract, was added simultaneously with the inoculum.

2.5.1. Batch simultaneous saccharification and fermentation (SSF)

In the SSF assay, the enzymatic consortium and the yeast strain were added simultaneously at the beginning of the process. The process was carried out at 38 °C with 8% (w/v) solids loading. These operational conditions are summarized in Table 1.

2.5.2. Batch pre-saccharification and simultaneous saccharification and fermentation (PS-SSF)

In the PS-SSF assays, a pre-liquefaction period of 1 h for PS-SSF (1 h) or 4 h for PS-SSF (4 h) was carried out under optimal conditions for saccharification (50 °C). After this first period, the temperature was quickly reduced to 38 °C (taking advantage of the automatic bioreactor system for temperature control), and then the mixture was inoculated and supplemented. Batch PS-SSF assays were carried out using 8% (w/v) total solids loading and an enzyme dosage of 25 FPU $g_{carbohydrates}^{-1}$ according to Table 1.

2.5.3. Fed-batch PS-SSF

Similar to the PS-SSF (4 h) assay, the fed-batch PS-SSF (4 h) experiment was initiated with 8% (w/v) solids loading. Two more loads were added during the process until 20% (w/v) total solids loading. The main goal was to gradually increase the amount of substrate added, to avoid mixing issues due to the high consistency of the mixture, and also, the inhibition of the hydrolytic enzymes by high glucose concentration. The schedule of the two additions was related to the knowledge of the consistency from previous experiments, and also to the night period for logistic reasons. The first feed of 6% (w/v) was added after 12.8 h, whereas the second one with the same solids loading was fed after 23.5 h. The inoculation and supplementation were added 4 h after the beginning. Before that, the temperature was quickly reduced from 50 to 38 °C (Table 1). The experimental assays described above are represented in Figure 1.

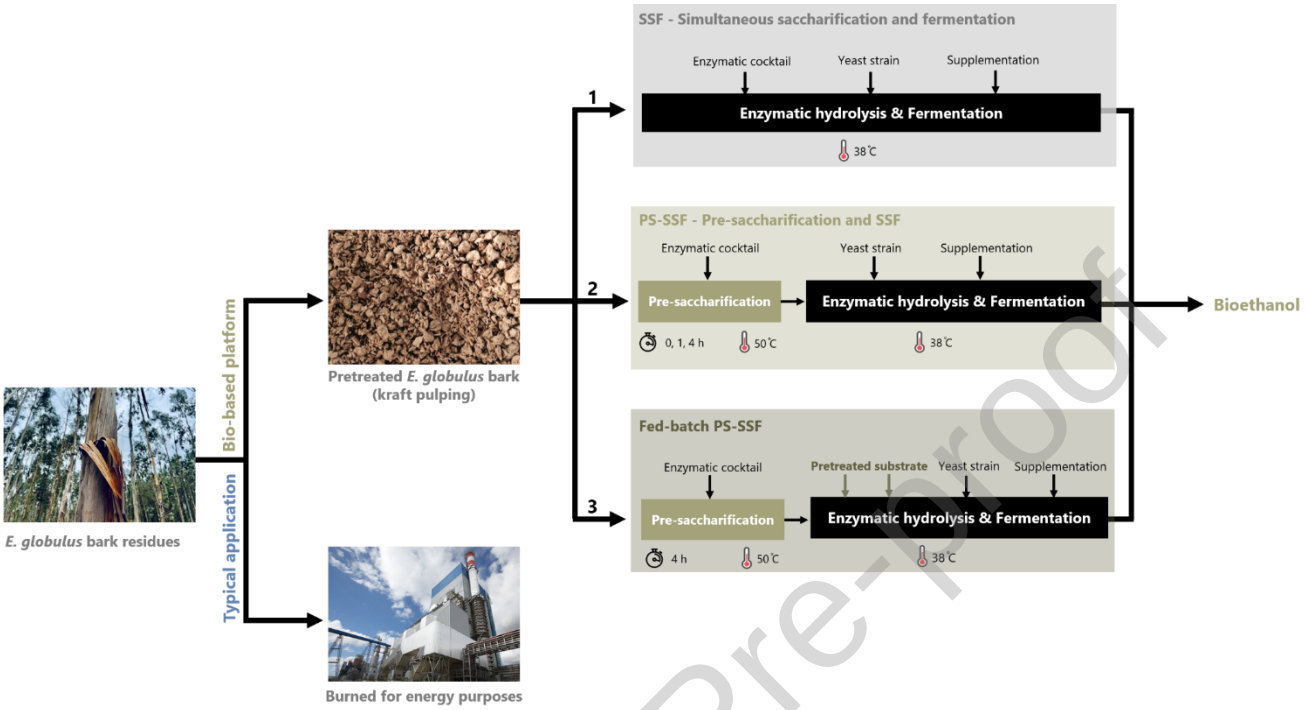

Figure 1. Schematic representation of the experimental assays carried out following three different SSF configurations.

Table 1. Operational conditions of experimental assays at bioreactor scale using an enzyme dosage of 25 FPU g_{CH}^{-1} and agitation rate of 250 rpm.

Assay	Operational conditions		
	Solids loading	**Temperature**	**Working volume**
SSF	8% (w/v)	38 °C	2 L
PS-SSF (1 h)	8% (w/v)	50 °C: 0 h – 1 h 38 °C: 1 h – end	2 L*
PS-SSF (4 h)	8% (w/v)	50 °C: 0 h – 4 h 38 °C: 4 h – end	2 L*

Fed-batch PS-SSF (4 h)	8% (w/v) (0 h) + 6% (w/v) (12.8 h) + 6% (w/v) (23.5 h) Total: 20% (w/v)	50 °C: 0 h – 4 h 38 °C: 4 h – end	2 L*

* Total working volume of 2 L was achieved only after inoculation. Before that, the working volume was 1.7 L.

2.6. Analytical Methods

HPLC was used to quantify glucose, xylose, ethanol, glycerol and acetic acid. After properly diluted, a total of 500 µL of each sample was filtered using centrifugal filters (VWR) with a pore size membrane of 0.2 µm (VWR) at 8000 rpm (Eppendorf, MiniSpin) for 8 min before HPLC injection. Samples were injected on a Rezex ROA-Organic Acid H^+ (8%) 300 x 7.8 mm ion-exchange column (Phenomenex) at 65 °C (oven Gecko 2000) connected to a refraction index detector L-2490 (Hitachi). The injection volume was 10 µL and the eluent was H_2SO_4 0.005 N, with a flow rate of 0.500 mL min^{-1} (Hitachi, pump L-2130). Standard calibration curves were obtained frequently using freshly prepared standards in the range of 0-5 g L^{-1} for sugars, ethanol, glycerol and acetic acid to ensure method linearity.

2.7. Calculations

The overall productivity, $Prod_{vol,\ overall}$ (g L^{-1} h^{-1}), was calculated according to Equation (1), considering the ratio of the maximum ethanol concentration reached and the corresponding time since the beginning of the experiment (pre-saccharification + simultaneous saccharification and fermentation).

$$Prod_{vol,\ overall}\ (g\ L^{-1}\ h^{-1}) = \frac{\Delta\ [Ethanol]}{\Delta t} \quad (1)$$

The maximum glucose concentration in the pretreated bark estimated from kraft pulp composition, $[Glucose]_{pretreated\ bark}$ (g L^{-1}), was calculated based on Equation (2). This

corresponds to the glucose concentration if the cellulose present in the kraft pulp would be fully hydrolyzed in glucose.

$$[\text{Glucose}]_{\text{pretreated bark}}\ (\text{g L}^{-1}) = 1.11 \times \text{Cellulose}_f \times \frac{m_{\text{dry pulp}}}{V} \quad (2)$$

where 1.11 is the mass conversion factor of cellulose to glucose ($g_{glucose}\ g_{cellulose}^{-1}$); Cellulose_f is the cellulose fraction in the dry weight kraft pulp (0.798 $g_{cellulose}\ g_{dry\ weight\ kraft\ pulp}^{-1}$); $m_{\text{dry pulp}}$ is the dry weight kraft pulp ($g_{dry\ weight\ kraft\ pulp}$) and V is the working volume (2 L).

The maximum xylose concentration in the pretreated bark estimated from kraft pulp composition, $[\text{Xylose}]_{\text{pretreated bark}}$ (g L^{-1}), was calculated based on Equation (3). This corresponds to the xylose concentration if the hemicelluloses present in the kraft pulp would be fully hydrolyzed in xylose.

$$[\text{Xylose}]_{\text{pretreated bark}}\ (\text{g L}^{-1}) = 1.14 \times \text{Hemicelluloses}_f \times \frac{m_{\text{dry pulp}}}{V} \quad (3)$$

where 1.14 is the mass conversion factor of hemicelluloses to xylose ($g_{xylose}\ g_{hemicelluloses}^{-1}$) and Hemicelluloses_f is the hemicelluloses fraction in the dry weight kraft pulp (0.155 $g_{hemicelluloses}\ g_{dry\ weight\ kraft\ pulp}^{-1}$).

The maximum theoretical ethanol concentration, $[\text{Ethanol}]_{\text{max, theoretical}}$ (g L^{-1}), was calculated considering the maximum glucose concentration present in the pretreated bark and the theoretical yield for ethanol fermentation (0.511 $g_{ethanol}\ g_{glucose}^{-1}$), according to Equation (4):

$$[\text{Ethanol}]_{\text{max, theoretical}}\ (\text{g L}^{-1}) = [\text{Glucose}]_{\text{pretreated bark}} \times 0.511 \quad (4)$$

Estimated glucose consumption, [Glucose consumption for ethanol] (g L^{-1}), was calculated from the produced ethanol concentration and considering the theoretical yield for ethanol, according to Equation (5):

$$[\text{Glucose consumption for ethanol}]\ (\text{g L}^{-1}) = \frac{[\text{Ethanol}]}{0.511} \quad (5)$$

The volumetric glucose consumption rate, $r_{glucose}$ (g L^{-1} h^{-1}) was estimated by the ratio of glucose consumption for ethanol production, and the corresponding time period, following Equation (6). Similar calculations were followed for volumetric ethanol production rate determination.

$$r_{glucose}\ (g\ L^{-1}\ h^{-1}) = \frac{\Delta[\text{Glucose consumption for ethanol}]}{\Delta t} \quad (6)$$

The overall conversion efficiency (%) was calculated by the ratio between the maximum ethanol concentration reached and the maximum glucose concentration in the pretreated bark based on kraft pulp composition, based on Equation (7):

$$\text{Overall conversion efficiency (\%)} = \frac{[\text{Ethanol}]_{max}}{0.511 \times [\text{Glucose}]_{\text{pretreated bark}}} \times 100 \quad (7)$$

where $[\text{Ethanol}]_{max}$ is the maximum ethanol concentration accomplished ($g_{ethanol}$ L^{-1}), 0.511 is the theoretical yield and $[\text{Glucose}]_{\text{pretreated bark}}$ is the maximum glucose concentration in the pretreated bark estimated from kraft pulp composition.

3. Results and discussion

Several different assays were carried out following an integrated approach to boost ethanol production and overall productivity. In this type of configuration, it was not possible to take samples at the initial time (t = 0 h) since there was no homogeneity in the reaction medium due to the solid nature of the substrate. In the graphs presented, the point depicted at time 0 h is not an experimental result but a result of an estimative. Typically, these lignocellulosic materials have relatively low density and are highly hygroscopic. Consequently, the substrate rapidly absorbed most of the initial free liquid

3.1. SSF

In the SSF assay, the *E. globulus* bark kraft pulp, the enzymatic consortium and the Ethanol Red® yeast were added simultaneously at the beginning of the process. The SSF was conducted at 38 °C, which is a trade-off between the optimum temperature required

by the enzymatic consortium (50 °C) and the fermentative yeast (28-30 °C). (Mendes et al. 2017). Figure 2 represents the concentration profile of metabolites determined experimentally, namely glucose, xylose, ethanol and glycerol (full points). Moreover,

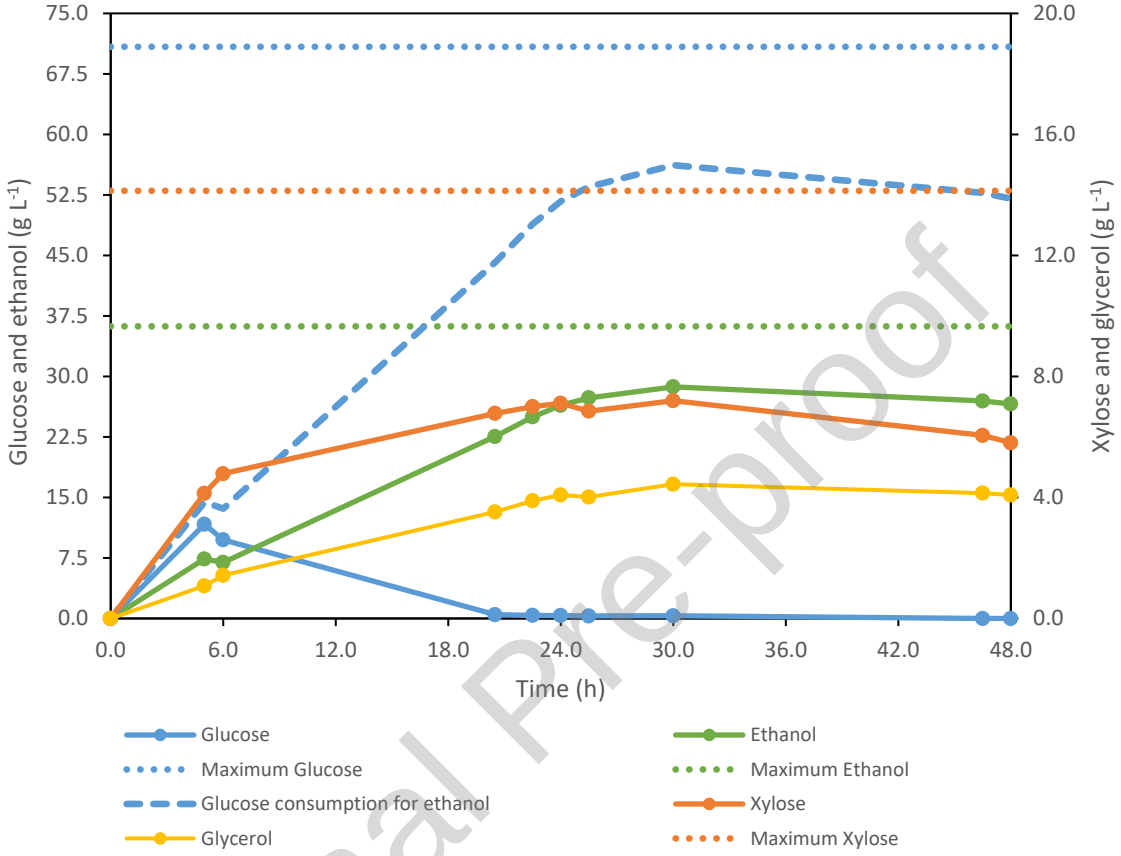

Figure 2 depicts the estimated glucose consumption based on ethanol production (dashed line), the calculated maximum glucose and xylose concentrations based on kraft pulp composition and the solids loading employed, and still the maximum ethanol concentration based on the calculated maximum glucose concentration (dotted lines). The same pattern was followed for all subsequent Figures.

Figure 2. Profile of glucose, ethanol, xylose and glycerol concentrations for SSF assay (2000 mL, 250 rpm, 8% (w/v) solids loading, 25 FPU g_{CH}^{-1}). The dotted lines represented the maximum glucose and xylose concentrations (based on kraft pulp composition) and the maximum theoretical ethanol concentration (coming from these sugars). The estimated glucose consumption from the produced ethanol is represented by the dashed line.

In this assay, it was impossible to take samples before 5 h of operation since there was no homogeneity in the bioreaction medium. At this time, it was detected about 11.7 g L^{-1} of glucose. This glucose was released by enzymatic hydrolysis, but some other was already consumed by yeast fermentation since, at this time, the ethanol concentration had already reached 7.4 g L^{-1}. At least 14.5 g L^{-1} of glucose was required to produce this ethanol amount. Furthermore, some of the consumed glucose was certainly directed toward the metabolic growth of the microbial culture, the biocatalysis agent for ethanol production. At this time, the xylose concentration was 4.1 g L^{-1} and increased slowly until reaching its maximum of about 7.2 g L^{-1} at 30 h. Reduced production of other by-products occurred, namely glycerol, which has attained its maximum concentration of 4.4 g L^{-1}.

Between 6.0 h and 20.5 h, there was the most significant ethanol production. It is estimated that about 30.5 g L^{-1} of glucose were consumed for ethanol during this period (assuming only glucose was fermented and based on the theoretical yield of fermentation), corresponding to an estimated glucose consumption rate of about 2.11 g L^{-1} h^{-1}. This glucose consumption rate decreased considerably to 1.27 g L^{-1} h^{-1} from 20.5 h to 30 h. Despite the glucose concentration being almost null from 20.5 h, there was still a gradual increase in ethanol concentration until 30 h. This means simultaneous hydrolysis and fermentation were occurring, being the limiting step the glucose release from the enzymatic hydrolysis. As soon as glucose was available, it was fermented by the yeast for ethanol production. Fernandes et al. (2018) observed a similar trend in bioethanol production. The maximum ethanol concentration of 28.7 g L^{-1} was achieved after 30 h, corresponding to the volumetric productivity of 0.957 g L^{-1} h^{-1}. The estimated overall conversion efficiency was about 79.3%, based on the ratio between the ethanol produced and the glucose concentration in the kraft pulp. It can be observed that extending the process beyond 30 h was not advantageous.

3.2. Batch PS-SSF (1 h)

To overcome the previous assay limitations, a pre-saccharification and simultaneous saccharification and fermentation (PS-SSF) configuration was investigated. Adopting a pre-saccharification stage could minimize issues related to mass and heat transfer phenomena, limiting the SSF operation mode (Gomes et al. 2021). The pre-saccharification should accelerate the liquefaction process since it operates at the optimal conditions for the hydrolytic enzymatic consortium, namely the temperature of 50 °C, for a certain period in the initial stage of the process (Silva et al. 2020).

In the PS-SSF (1h) assay, the Ethanol Red® yeast inoculation occurred at 38 °C, 1 h after the beginning of the hydrolysis, marked with the black vertical line in Figure 3.

After 1.5 h of the beginning of the process, the glucose and xylose reached a concentration of 25.2 g L^{-1} and 5.0 g L^{-1}, respectively. These values corresponded to about one-third and half of the total theoretical concentration of glucose and xylose, respectively. At that moment, 2.1 g L^{-1} of ethanol was already produced, only half an hour after yeast addition. This means that the fermentation process started immediately, with practically no lag phase. From 3.0 h to 8.0 h, glucose consumption for ethanol production was evident, with a consumption rate of about 4.99 g L^{-1} h^{-1}, more than double of the previous assay, 2.11 g L^{-1} h^{-1}.

Similar to the previous assay, no glucose was detected in the medium from about 22 h. However, ethanol concentration increased progressively until 28.5 h, attaining its maximum of 29.2 g L^{-1}. This means that a small fraction of glucose was simultaneously released and instantaneously consumed, as confirmed by the glucose consumption for the ethanol production. The overall productivity of 1.023 g L^{-1} h^{-1} and the conversion efficiency of 80.6% was reached. After this moment, the process shows a plateau,

indicating the end of enzymatic hydrolysis and, consequently, the cessation of fermentation due to lack of substrate.

The xylose concentration did not change significantly throughout the process, reaching its maximum of 6.8 g L^{-1} after 22.3 h. The glycerol production was also detected with a concentration of about 3.7 g L^{-1} after 30 h. The acetic acid concentration was almost negligible (0.3 g L^{-1}). Therefore, its profile was not represented.

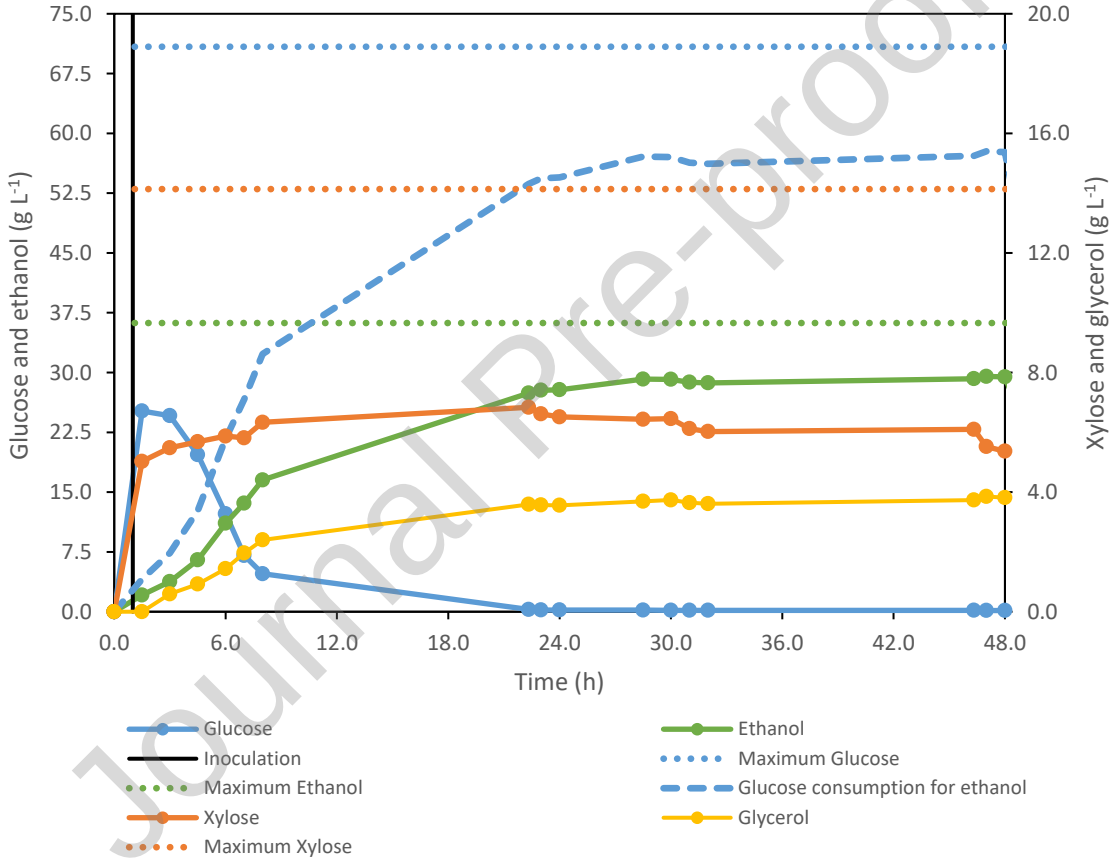

Figure 3. Profile of glucose, ethanol, xylose and glycerol concentrations for PS-SSF (1h) assay (2000 mL, 250 rpm, 8% (w/v) solids loading, 25 FPU g_{CH}^{-1}). The dotted lines represented the maximum glucose and xylose concentrations (based on kraft pulp composition) and the maximum theoretical ethanol concentration (coming from these sugars). The estimated glucose consumption from the produced ethanol is represented by the dashed line.

Overall, a short pre-saccharification (1 h only) before inoculation leads to a slight improvement in overall conversion efficiency and productivity over SSF. However, there

is still no agreement in the literature concerning the effect of extending the pre-liquefaction period. Barros et al. (2017) concluded that a pre-saccharification stage of 12 h did not enhance the overall conversion efficiency or productivity over SSF in bioethanol production from cashew apple bagasse. On the other hand, Chilari et al. (2017) already stated that the short time to achieve the liquefaction of the mixture is one of the main advantages of the pre-saccharification stage. These authors evaluated the effect of the pre-saccharification period (6, 14, 24 and 36 h) on bioethanol production from alkali-treated cotton stalks using Cellic® CTec2 with 80 FPU $g_{cellulose}^{-1}$ of enzymatic dosage (Chilari et al. 2017). Overall, no significant differences in ethanol titer regarding the pre-saccharification time were found. Still, it was found that the optimal pre-saccharification time was 14 h for both substrate loadings evaluated (15 and 20% w/v) Likewise, Chen and Fu (2016) found that a prolonged pre-saccharification period (up to 24 h) may have an undesirable effect on productivity, despite increasing sugar yield during the pre-saccharification period. Moreover, a more prolonged pre-liquefaction stage may result in feedback sugar inhibition (Chen and Fu 2016; Chen and Liu 2017). In contrast, Gomes et al. (2021) stated that extending the pre-saccharification period from 24 h to 48 h improved ethanol yield from *E. globulus* bark, and this effect was most evident in the assays supplemented with yeast extract and peptone. Based on these remarks, another experiment was carried out with an extended pre-liquefaction period. Even so, the pre-saccharification stage was not too extended (only 4 h) to not compromise the overall productivity from the outset.

3.3. Batch PS-SSF (4 h)

In the PS-SSF (4 h) assay, the inoculation of the Ethanol Red® yeast occurred 4 h after the beginning of the hydrolysis (Figure 4). Just before it, glucose and xylose concentrations were about 34.4 g L^{-1} and 6.4 g L^{-1}, respectively. Three additional hours

of pre-saccharification increased the glucose and xylose concentrations by about 36 and 27%, compared to the previous experiment. The xylose concentration was almost constant until the end. In contrast, glucose was quickly consumed after inoculation, and ethanol production started to be detected thereupon. At 5.2 h, only 1.2 h after inoculation, an ethanol concentration of about 3.2 g L^{-1} was already attained, showing that the lag phase was almost inexistent. Between 5.2 h and 8.2 h, the glucose decreased to more than half, corresponding to a consumption rate of about 4.41 g L^{-1} h^{-1}. After 4 h of the beginning of the fermentation (at 8.2 h), 10 g L^{-1} of ethanol was already produced, corresponding to a production rate of 2.40 g L^{-1} h^{-1}. Between 8.2 h and 21.2 h, the glucose consumption rate decreased to about half, and consequently, the ethanol production rate dropped to a similar extent (1.12 g L^{-1} h^{-1}) compared to the first hours afterwards inoculation.

After 24 h of operation, the maximum ethanol concentration of 27.4 g L^{-1} h was attained, corresponding to the productivity of 1.142 g L^{-1} h^{-1}. Regarding the overall conversion efficiency, this assay of PS-SSF (4 h) reached about 75.7% and attained the highest productivity. The pre-saccharification of 4 h resulted in a productivity improvement of about 12% and 19% compared to the previous PS-SSF (1h) and SSF, respectively. The extension of pre-saccharification from 1 h to 4 h allowed the maximum ethanol concentration to be reached around 4.5 h before the previous assay, considering the overall time process. Concerning the time of fermentation (counting only from inoculation), PS-SSF (4 h) required only 20 h to achieve its maximum, while PS-SSF (1

h) required 7.5 h more. Both assays with the pre-saccharification period were very similar regarding the by-products formation, namely glycerol and acetic acid.

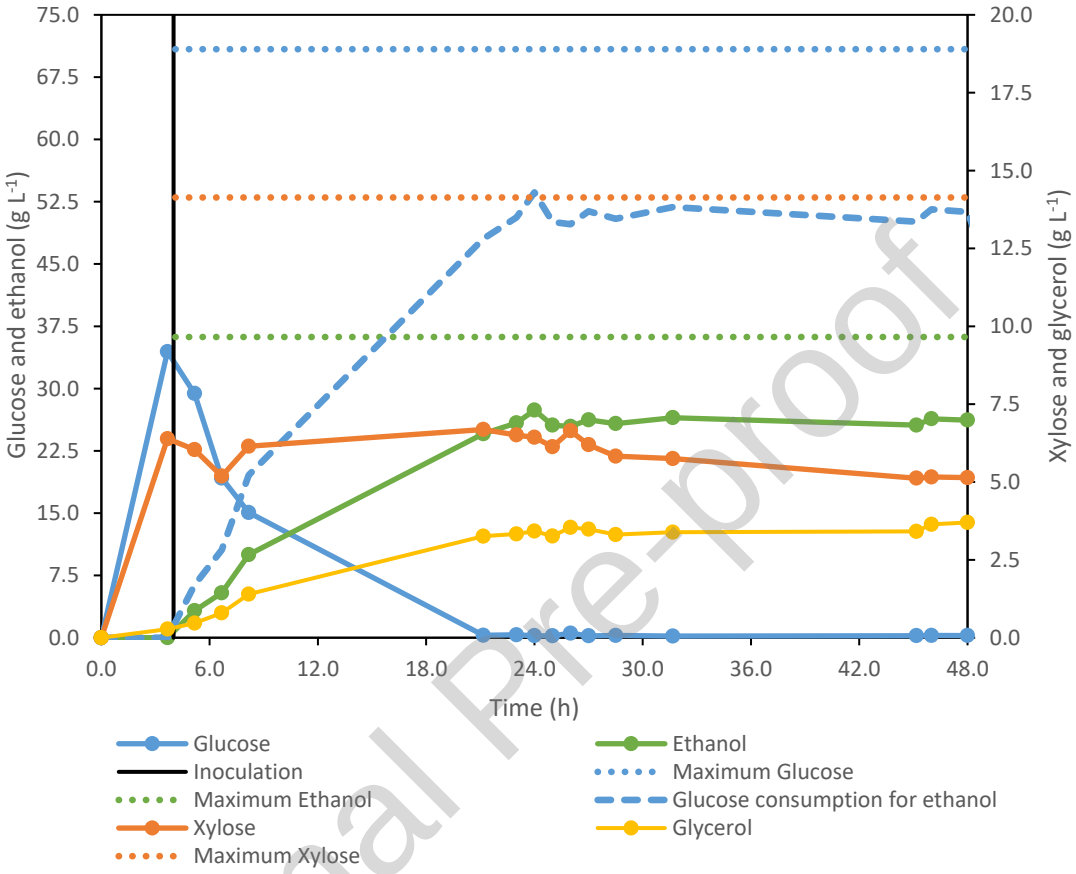

Figure 4. Profile of glucose, ethanol, xylose and glycerol concentrations for PS-SSF (4 h) assay (2000 mL, 250 rpm, 8% (w/v) solids loading, 25 FPU g_{CH}^{-1}). The dotted lines represented the maximum glucose and xylose concentrations (based on kraft pulp composition) and the maximum theoretical ethanol concentration (coming from these sugars). The estimated glucose consumption from the produced ethanol is represented by the dashed line.

Although the effect of the pre-saccharification period is still not consensual in the literature, the present work showed that the PS-SSF strategy seems to have slightly benefited the overall productivity over the SSF configuration for the conditions evaluated. Probably, this is due to the difference between the operating temperature and the optimum value for the hydrolytic enzymatic consortium. The whole SSF process was conducted at a temperature of 38 °C, a value lower than the optimal for the hydrolytic enzymatic

consortium, 50 °C, which slowed down the enzymatic hydrolysis rate (Paulova et al. 2015; Pratto et al. 2020). Moreover, when the SSF experiment was inoculated (time zero), the reaction mixture presented a significantly reduced liquid phase, whose availability of fermentable sugars was quite limited. The hindering of the cellulases activity in suspensions of solids with a high consistency has already been stated by Gomes et al. (2021). All these factors negatively affected the overall productivity of SSF. On the other hand, the PS-SSF favoured homogeneity throughout the fermentation period. The significant mixing constraints occurred at the beginning and were limited to the enzymatic hydrolysis step.

In general, ethanol production ranged from 26 to 29 g L^{-1}, attaining overall conversion efficiencies varying from 71 to 80% and productivities from 0.957 to 1.124 g L^{-1} h^{-1}. The maximum ethanol concentration did not differ significantly within these three experiments. However, the last assay, with a pre-saccharification period of 4 h, stood out by the considerable improvement in productivity (about 19% and 12% compared to SSF and PS-SSF (1h), respectively). These results compares favourably with the range found in the literature, as shown in Table 2. For other raw materials, productivities ranged from 0.20 to 1.83 g L^{-1} h^{-1}, with overall conversion efficiencies varying from 55 to 92% for solids loading ranging from 13 to 20% (w/v), following SSF and PS-SSF configurations. Although, there is still an opportunity for enhancement since the operation using 8% (w/v) solids loading limited the maximum ethanol concentration below 30 g L^{-1}, in the present work. Therefore, aiming to boost ethanol production, it is essential to increase solids loading considerably. Nonetheless, the high solids loading operation is known for higher mixing energy requirements and sugars feedback inhibition effect (Chen and Fu 2016; Chen and Liu 2017; He et al. 2018; Zhang et al. 2012), besides the hindrance mass and heat transfer phenomena (Chen and Liu 2017). Adopting a fed-

batch configuration should be a promising alternative to overcome these limitations and simultaneously boost ethanol production.

Table 2. Experimental and literature results from SSF and PS-SSF experiments for bioethanol production.

Feedstock	**Pretreatment**	**Enzymatic consortium**	**Yeast strain**	**Operation mode**	**Solids loading (w/v %)**	**[Ethanol$_{max}$ (g L^{-1})**	**Prod$_{vol,\ overall}$ (g L^{-1} h^{-1})**	**Overall conversion efficiency (%)**	**Reference**
Eucalyptus globulus bark	Kraft pulping	Cellic® CTec2 (25 FPU g_{CH}^{-1})	Ethanol Red®	SSF	8	28.7 (30.0 h)	0.957	79.3	This work
				PS-SSF (1 h)	8	29.2 (28.5 h)	1.023	80.6	
				PS-SSF (4 h)	8	27.4 (24.0 h)	1.142	75.7	
				Fed-batch PS-SSF (4 h)	20 (8 + 6 +6)	75.9 (52.3 h)	1.453	83.9	
Cashew apple bagasse	Two-stage acidic-alkaline	Celluclast 1.5 L (30 FPU g_{glucan}^{-1}) + β-glucosidase (60 CBU g_{glucan}^{-1})	*Kluyveromyces marxianus* ATCC 36907	SSF	15	58.67 ± 0.74 (32 h)	1.83 ± 0.02	92.68 ± 1.16	(Barros et al. 2017)
				PS-SSF (12 h)	15	50.11 ± 1.53 (36 h)	1.39 ± 0.04	79.51 ± 2.51	
Cotton Stalks	Alkaline	Cellic® CTec2 (80 FPU $g_{cellulose}^{-1}$)	Baker's yeast	PS-SSF (14 h)	20	34.80 ± 0.42	N.R.	55.40 ± 0.68	(Chilari et al. 2017)
Eucalyptus globulus bark	Hydrothermal	Cellic® CTec2 (20 FPU g_{solids}^{-1})	Ethanol Red®	SSF	17.5	33.43 ± 1.82 (72 h)	0.46	64.28	(Gomes et al. 2021)
Triticale straw	Steam explosion	Spezyme® CP (15 FPU $g_{cellulose}^{-1}$)	Ethanol Red®	SSF	15	29.31 (144 h)	0.20	84.7	(Kossatz et al. 2017)

Eucalyptus grandis sawdust	Autohydrolysis + Soda pulping	Cellic® CTec2 (25 FPU g_{glucan}^{-1})	*S. cerevisiae* PE-2	PS-SSF (24 h) – Erlenmeyer flask	13	58 (48 h)	1.2	85	(Guigou et al. 2019)
				PS-SSF (24 h) – lab reactor	13	52 (65 h)	0.8	64	

N.R. - Not reported.

3.4. Fed-batch PS-SSF (4 h)

According to the abovementioned considerations, a fed-batch PS-SSF strategy was carried out to increase the solids loading gradually from 8 to 20% (w/v), aiming to boost the production of sugars and ethanol, without compromising mixing and mass transfer phenomena. Therefore, this last experiment started with 8% (w/v) solids loading (the same as the previous assays) and two additional feeds of kraft pulp (6% (w/v) solids loading, each) were added at 12.8 h and 23.5 h, achieving a final solids loading of 20% (w/v). These additions are signalled in Figure 5. It was selected for a pre-saccharification period of 4 h due to higher productivity since maximum ethanol production was not significantly different between the three last experiments evaluated (Table 2).

Glucose already achieved a high concentration of about 64.6 g L^{-1} after 4 h of operation, almost double that of the PS-SSF (4 h) assay for the same period. The reason is the increased availability of enzymatic consortium in the early stage. Although the enzyme dosage is the same for both experiments (25 FPU g_{CH}^{-1}), all the enzymatic consortium was fed at once at the beginning of each assay. This means that in the fed-batch experiment, the same initial solids loading (8% (w/v)) had available the enzyme dosage required for the 20% (w/v) total solids loading. This higher enzymatic consortium availability until the last feed promoted a higher reaction rate between the enzymatic

consortium and the polysaccharides, accelerating the enzymatic hydrolysis process and, consequently, the release of sugars to be consumed for ethanol production. Moreover, the viscosity of the reaction mixture decreased faster, facilitating the mixing and, therefore, mass and energy transfer phenomena. This could justify the significant increase in productivity (above 27%) compared to the batch PS-SSF (4 h) experiment. These observations are in accordance with Gao et al. (2018), which concluded that the release of sugars enhanced considerably in the first 12 h when all the enzymes were added at the beginning. Some authors also reported a similar effect, finding that splitting the addition of the enzymes may lead to lower glucose yield (Cardona et al. 2015; Jung et al. 2017; Tareen et al. 2021).

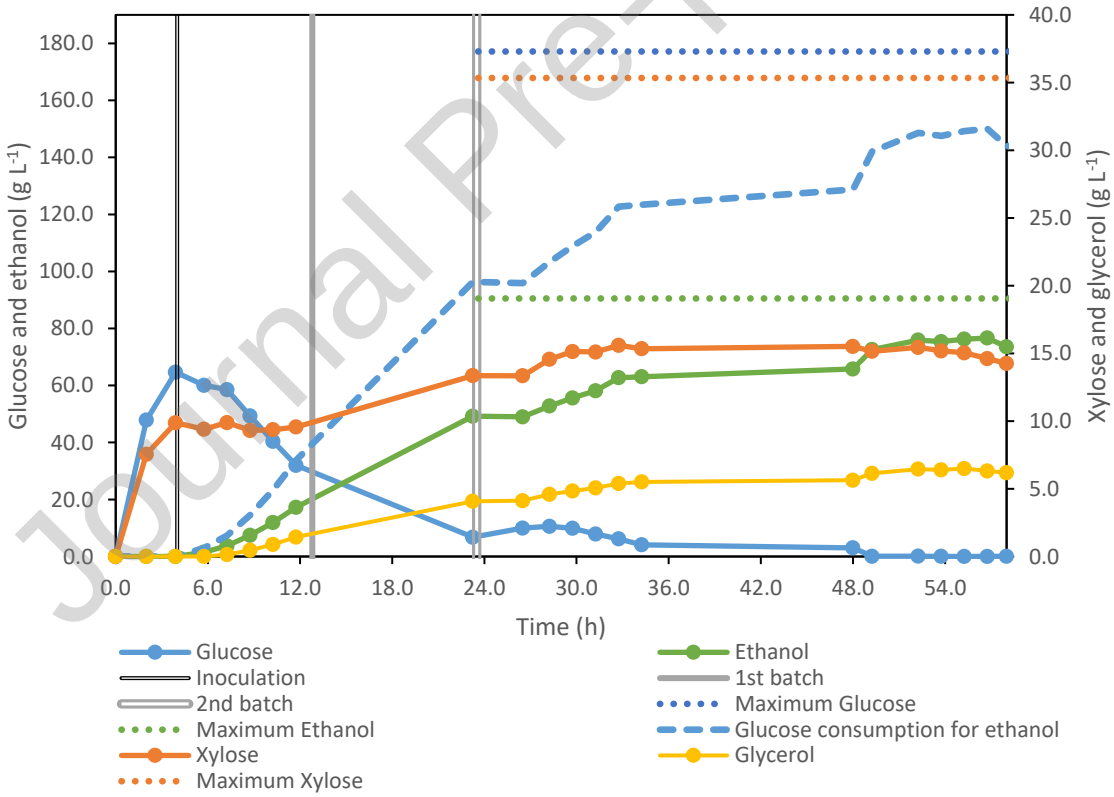

Figure 5. Profile of glucose, ethanol, xylose and glycerol concentrations for Fed-batch PS-SSF (4 h) assay (2000 mL, 250 rpm, 20% (w/v) solids loading, 25 FPU g_{CH}^{-1}). The dotted lines represented the maximum glucose and xylose concentrations (based on kraft pulp composition) and the maximum theoretical ethanol concentration (coming from these sugars). The estimated glucose consumption from the produced ethanol is represented by the dashed line.

Glucose and ethanol concentration profiles show that the lag phase was very short. A slight amount of ethanol, around 1.5 g L^{-1}, was already detected 1.7 h after inoculation. Between 5.7 h and 11.7 h, a pronounced drop in glucose concentration was observed, corresponding to a consumption rate of 5.14 g L^{-1} h^{-1}. Consequently, bioethanol increased from 1.5 g L^{-1} to 17.3 g L^{-1} during this 6 h, attaining a production rate of about 2.62 g L^{-1} h^{-1}. Due to logistic restrictions, it was not possible to monitor the progress of the process after adding the first pulse at 12.8 h. Nevertheless, about 32 g L^{-1} of ethanol were produced between 11.7 h and 23.3 h, corresponding to a volumetric production rate of 2.78 g L^{-1} h^{-1}. This ethanol titer required at least 62.5 g L^{-1} of glucose.

An adaptation period seems to have occurred after adding the second feed at 23.5 h since ethanol concentration was kept constant until 26.5 h. After that, a slight increase in glucose concentration was observed between 26.5 h and 32.7 h, meaning that the fermentation process was the limiting step in this period, i.e. glucose release by hydrolysis was faster than its fermentation to ethanol. Still, the ethanol production rate remained high (about 2.20 g L^{-1} h^{-1}). A similar trend was observed for the glucose consumption profile, corresponding to a consumption rate of 4.30 g L^{-1} h^{-1}.

From 48 h, the glucose detected was almost zero. From this moment, the ethanol concentration slowly increased until it reached its maximum of 75.9 g L^{-1} at 52.2 h, the highest value attained so far. The extension of the process beyond 80 h (data not shown) allowed the ethanol concentration to be increased until 80 g L^{-1}. Still, this increment does not justify the extra time required, resulting in a productivity decline. At least 148 g L^{-1} of glucose was consumed to reach this level of ethanol concentration.

A slight deceleration of glucose consumption and consequent ethanol production was found after adding the second feed of kraft pulp (at 23.5 h). This slowdown was evident

from 52 h when ethanol concentration reached a plateau and remained almost constant. The main reasons were probably the yeast inhibition effect due to the high ethanol concentration and eventually the lack of micronutrients. However, the overall conversion efficiency was already relatively high (83.9%).

Overall, it was observed that the fed-batch configuration favoured overall process performance for the conditions evaluated. With increasing solids loading from 8 to 20% (w/v), ethanol production increased about 2.8-fold (from 27.4 to 75.9 g L^{-1}). Moreover, further substantial improvements were also noticed in overall conversion efficiency and productivity, probably due to the faster liquefaction stage. A similar trend was stated by Chilari et al. (2017), reporting an enhancement of almost 60% in ethanol production from alkali-treated cotton stalks by increasing substrate loading from 15 to 20% (w/v), considering the pre-saccharification time of 14 h. In terms of productivity, this was equivalent to an improvement of 9% (Chilari et al. 2017). For 20% (w/v) substrate loading, Chilari and co-workers (2017) achieved a maximum ethanol concentration of 34.80 g L^{-1} after around 65 h of fermentation and the highest overall conversion efficiency, 55.4 %. In the present work, ethanol production more than doubled by using the same solids loading, with an unprecedented increase in productivity and overall conversion efficiency. This great performance could be related to the fed-batch strategy.

Nevertheless, it was found that the higher solids loading led to a considerable increase in the processing time required to reach the maximum ethanol concentration (24 to 52.3 h), but the additional ethanol production still compensated. Furthermore, it was found that higher solids loading operation led to higher by-products formation, namely glycerol (6.5 g L^{-1}) and acetic acid (1.4 g L^{-1}). This observation agrees with Kossatz et al. (2017), who showed that higher glycerol concentration is obtained for

higher substrate loading. Moreover, Mendes et al. (2017) also noticed an increment in by-products concentration after following a fed-batch SSF approach.

Compared to the studies already published in the literature regarding bioethanol production from integrated configuration, the results from the present work can be considered promising, particularly from the fed-batch configuration. Recently, Gomes et al. (2021) compared the SSF and PS-SSF performance using the *E. globulus* bark previously subjected to hydrothermal pretreatment as a raw material. The assays were carried out at the Erlenmeyer scale with a working volume of 50 mL using Cellic® CTec2 with an enzyme dosage of 20 FPU g_{solids}^{-1}. The maximum ethanol concentration of 38.03 ± 0.33 g L^{-1} was achieved for 17.5% (w/v) solids loading using nutrient supplementation (20 g L^{-1} of peptone and 10 g L^{-1} of yeast extract) following a PS-SSF strategy with a pre-saccharification period of 48 h. This approach increased conversion efficiency from 64.28 to 73.14% and productivity from 0.46 to 0.52 g L^{-1} h^{-1} over SSF. However, the increase in solids loading from 15 to 17.5% (w/v) led to a reduction of conversion efficiency of about 5%, probably due to increased mechanical constraints. The shorter pre-saccharification period (4 h instead of 48 h) adopted in the present work seems advantageous, reaching more than two times higher productivity than Gomes et al. (2021). The conversion efficiency was similar, but these authors used a 4-fold yeast extract concentration coupled with a significant amount of peptone, representing considerable extra costs. Furthermore, the small working volume (50 mL) compared to the present work (2000 mL) makes the results presented here more reliable. Finally, the bioreactor configuration is similar to the one to be adopted on a larger scale. Nevertheless, Gomes et al. (2021) have the advantage of co-production of ethanol (from autohydrolysed solid fraction) and xylooligosaccharides (from hydrothermal liquor

fraction) from two fractions obtained after hydrothermal pretreatment. The co-production could be crucial to ensure economic feasibility at a commercial scale.

There are still few works regarding the scale-up of both SSF and PS-SSF configuration to bioreactor scale. Kossatz et al. (2017) successfully carried out a scale-up for a 1 L bioreactor following a simultaneous configuration for bioethanol production from steam-exploded triticale straw. An enzyme dosage of 15 FPU $g_{cellulose}^{-1}$ of Spezyme® CP and 15% (w/v) substrate loading were used. An ethanol concentration of 29.31 g L^{-1} was attained after 144 h, with productivity and conversion efficiency of 0.20 g L^{-1} h^{-1} and 84.7%, respectively (Kossatz et al. 2017). Guigou et al. (2019) subjected sawdust from *Eucalyptus grandis* (previously submitted to a combined autohydrolysis and soda pulping pretreatments) to PS-SSF strategy at both Erlenmeyer and bioreactor scales. A substrate loading of 13% (w/v) and an enzyme dosage of 25 FPU g_{glucan}^{-1} of Cellic® CTec2 were used. After a pre-saccharification period of 24 h, the mixture was supplemented (3 g L^{-1} yeast extract, 3 g L^{-1} malt extract, and 5 g L^{-1} peptone) and inoculated using the *S. cerevisiae* PE-2 strain. At the Erlenmeyer scale, a maximum ethanol concentration of 58 ± 3 g L^{-1} was reached after about 48 h, corresponding to 1.2 ± 0.3 g L^{-1} h^{-1} of productivity and 85 ± 1% of conversion efficiency The scale-up to bioreactor using 2.5 L of working volume considerably reduced the overall performance, achieving a maximum ethanol production of 52 g L^{-1}, corresponding to 64% conversion efficiency and 0.8 g L^{-1} h^{-1} of productivity (Guigou et al. 2019). Although a higher solids loading was used in the present work, higher productivity and conversion efficiency were accomplished. This was most likely due to the short pre-saccharification period combined with the fed-batch operation mode.

4. Conclusions

The present work demonstrated that SSF setup, particularly with appropriate operation conditions, can be successfully applied for bioethanol production from eucalyptus bark, a waste material. Batch experiments with 8% (w/v) solids loading provided ethanol production ranging from 26 to 29 g L^{-1}, attaining overall conversion efficiencies varying from 71 to 80% and productivities from 0.957 to 1.124 g L^{-1} h^{-1}. The best performance was accomplished by a fed-batch PS-SSF strategy with a pre-saccharification of 4 h, and 20% (w/v) solids loading. Ethanol concentration of 75.9 g L^{-1}, corresponding to the productivity of 1.453 g L^{-1} h^{-1} and conversion efficiency of 80% was obtained. This setup boosted maximum ethanol concentration 2.7-fold and overall productivity by around 27% over the batch SSF configuration with 8% (w/v) solids. These results have a great potential to be integrated into a pulp and paper mill, as an integrated biorefinery, contributing to the total resources use within the circular economy model.

Patent

Mariana S. T. Amândio, Jorge M. S. Rocha and Ana M. R. B. Xavier. Integrated bioconversion as a strategy to boost cellulosic ethanol production from industrial *Eucalyptus globulus* bark. PT Provisional Patent Application No. PT118408.

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References

Amândio, M. S. T., Pereira, J. M., Rocha, J. M. S., Serafim, L. S., and Xavier, A. M. R. B. (2022a). "Getting Value from Pulp and Paper Industry Wastes : On the Way to Sustainability and Circular Economy," *Energies*, 15(4105), 1–31.doi:10.3390/en15114105

Amândio, M. S. T., Rocha, J. M. S., Serafim, L. S., and Xavier, A. M. R. B. (2021). "Cellulosic Bioethanol from Industrial *Eucalyptus globulus* Bark Residues Using Kraft Pulping as a Pretreatment," *Energies*, 14(2185), 1–18.doi:10.3390/en14082185

Amândio, M. S. T., Rocha, J. M. S., Serafim, L. S., and Xavier, A. M. R. B. (2022b). "Bioethanol from wastes for mobility: Europe on the road to sustainability," in: *Clean Fuels for Mobility*, G. Di Blasio, A. K. Agarwal, G. Belgiorno, and P. C. Shukla, eds., Springer, Singapore, 97–123.doi:10.1007/978-981-16-8747-1_6

Ask, M., Olofsson, K., Di Felice, T., Ruohonen, L., Penttilä, M., Lidén, G., and Olsson, L. (2012). "Challenges in enzymatic hydrolysis and fermentation of pretreated *Arundo donax* revealed by a comparison between SHF and SSF," *Process Biochemistry*, Elsevier Ltd, 47(10), 1452–1459.doi:10.1016/j.procbio.2012.05.016

Barros, E. M., Carvalho, V. M., Rodrigues, T. H. S., Rocha, M. V. P., and Gonçalves, L. R. B. (2017). "Comparison of strategies for the simultaneous saccharification and fermentation of cashew apple bagasse using a thermotolerant *Kluyveromyces marxianus* to enhance cellulosic ethanol production," *Chemical Engineering Journal*, Elsevier B.V., 307, 939–947.doi:10.1016/j.cej.2016.09.006

Biond - Forest fibers from Portugal. (2022). "Boletim Estatístico Biond 2021," Lisboa, <https://www.biond.pt/publicacoes/boletim-estatistico-2021/> (Nov. 28, 2022).

Cardona, M. J., Tozzi, E. J., Karuna, N., Jeoh, T., Powell, R. L., and McCarthy, M. J. (2015). "A process for energy-efficient high-solids fed-batch enzymatic liquefaction of cellulosic biomass," *Bioresource Technology*, Elsevier Ltd, 198, 488–496.doi:10.1016/j.biortech.2015.09.042

Chen, H., and Fu, X. (2016). "Industrial technologies for bioethanol production from lignocellulosic biomass," *Renewable and Sustainable Energy Reviews*, Elsevier, 57, 468–478.doi:10.1016/j.rser.2015.12.069

Chen, H. Z., and Liu, Z. H. (2017). "Enzymatic hydrolysis of lignocellulosic biomass from low to high solids loading," *Engineering in Life Sciences*, 17(5), 489–499.doi:10.1002/elsc.201600102

Chilari, D., Dimos, K., Georgoula, G., Paschos, T., Mamma, D., Louloudi, A., Papayannakos, N., and Kekos, D. (2017). "Bioethanol Production from Alkali-Treated Cotton Stalks at High Solids Loading Applying Non-isothermal Simultaneous Saccharification and Fermentation," *Waste and Biomass Valorization*, Springer Netherlands, 8(6), 1919–1929.doi:10.1007/s12649-016-9818-4

Confederation of European Paper Industries - CEPI. (2021). "European Pulp & Paper Industry - Key Statictics 2020," Brussels, <https://www.cepi.org/key-statistics-2020/> (Oct. 20, 2022).

Deshavath, N. N., Goud, V. V., and Veeranki, V. D. (2021). "Curtailing citrate buffer inhibition effect on S. cerevisiae to enhance the fermentability of cellulosic hydrolysate," *Journal of Environmental Chemical Engineering*, Elsevier Ltd, 9(4), 105696.doi:10.1016/j.jece.2021.105696

Domingues, R. M. A., Sousa, G. D. A., Freire, C. S. R., Silvestre, A. J. D., and Neto, C. P. (2010). "*Eucalyptus globulus* biomass residues from pulping industry as a source of high value triterpenic compounds," *Industrial Crops and Products*, 31(1), 65–70.doi:10.1016/j.indcrop.2009.09.002

Fernandes, M. C., Ferro, M. D., Paulino, A. F. C., Chaves, H. T., Evtuguin, D. V., and Xavier, A. M. R. B. (2018). "Comparative study on hydrolysis and bioethanol production from cardoon and rockrose pretreated by dilute acid hydrolysis,"

Industrial Crops and Products, Elsevier, 111, 633–641.doi:10.1016/j.indcrop.2017.11.037

Gao, Y., Xu, J., Yuan, Z., Jiang, J., Zhang, Z., and Li, C. (2018). "Ethanol production from sugarcane bagasse by fed-batch simultaneous saccharification and fermentation at high solids loading," *Energy Science and Engineering*, 6(6), 810–818.doi:10.1002/ese3.257

Geng, W., Jin, Y., Jameel, H., and Park, S. (2015). "Strategies to achieve high-solids enzymatic hydrolysis of dilute-acid pretreated corn stover," *Bioresource Technology*, Elsevier Ltd, 187, 43–48.doi:10.1016/j.biortech.2015.03.067

Gomes, A. C., Moysés, D. N., Santa Anna, L. M. M., and Castro, A. M. (2018). "Fed-batch strategies for saccharification of pilot-scale mild-acid and alkali pretreated sugarcane bagasse: Effects of solid loading and surfactant addition," *Industrial Crops and Products*, Elsevier, 119, 283–289.doi:10.1016/j.indcrop.2018.04.026

Gomes, D. G., Michelin, M., Romaní, A., Domingues, L., and Teixeira, J. A. (2021). "Co-production of biofuels and value-added compounds from industrial *Eucalyptus globulus* bark residues using hydrothermal treatment," *Fuel*, 285(119265), 119265.doi:10.1016/j.fuel.2020.119265

Guigou, M., Cabrera, M. N., Vique, M., Bariani, M., Guarino, J., Ferrari, M. D., and Lareo, C. (2019). "Combined pretreatments of eucalyptus sawdust for ethanol production within a biorefinery approach," *Biomass Conversion and Biorefinery*, Biomass Conversion and Biorefinery, 9(2), 293–304.doi:10.1007/s13399-018-0353-3

Haile, A., Gelebo, G. G., Tesfaye, T., Mengie, W., Mebrate, M. A., Abuhay, A., and Limeneh, D. Y. (2021). "Pulp and paper mill wastes: utilizations and prospects for high value-added biomaterials," *Bioresources and Bioprocessing*, Springer Singapore, 8(35), 1–22.doi:10.1186/s40643-021-00385-3

Hans, M., Kumar, S., Chandel, A. K., and Polikarpov, I. (2019). "A review on bioprocessing of paddy straw to ethanol using simultaneous saccharification and fermentation," *Process Biochemistry*, Elsevier, 85, 125–134.doi:10.1016/j.procbio.2019.06.019

He, L., Han, Q., Jameel, H., Chang, H. min, Phillips, R., and Wang, Z. (2018). "Comparison of One-Stage Batch and Fed-Batch Enzymatic Hydrolysis of Pretreated Hardwood for the Production of Biosugar," *Applied Biochemistry and Biotechnology*, Applied Biochemistry and Biotechnology, 184(4), 1441–1452.doi:10.1007/s12010-017-2633-y

Hossain, N., Mahlia, T. M. I., Miskat, M., Chowdhury, T., Barua, P., Chowdhury, H., Nizamuddin, S., Ahmad, N. B., Zaharin, N. A. B., Mazari, S. A., and Soudagar, M. E. M. (2021). "Bioethanol production from forest residues and life cycle cost analysis of bioethanol-gasoline blend on transportation sector," *Journal of Environmental Chemical Engineering*, Elsevier Ltd, 9(4), 105542.doi:10.1016/j.jece.2021.105542

Isikgor, F. H., and Becer, C. R. (2015). "Lignocellulosic biomass: a sustainable platform for the production of bio-based chemicals and polymers," *Polymer Chemistry*, 6, 4497–4559.doi:10.1039/c5py00263j

Jönsson, L. J., and Martín, C. (2016). "Pretreatment of lignocellulose: Formation of inhibitory by-products and strategies for minimizing their effects," *Bioresource Technology*, 199, 103–112.doi:10.1016/j.biortech.2015.10.009

Jung, Y. H., Park, H. M., Kim, D. H., Yang, J., and Kim, K. H. (2017). "Fed-Batch Enzymatic Saccharification of High Solids Pretreated Lignocellulose for Obtaining High Titers and High Yields of Glucose," *Applied Biochemistry and Biotechnology*, Applied Biochemistry and Biotechnology, 182(3), 1108–1120.doi:10.1007/s12010-016-2385-0

Kossatz, H. L., Rose, S. H., Viljoen-Bloom, M., and van Zyl, W. H. (2017). "Production of ethanol from steam exploded triticale straw in a simultaneous saccharification and fermentation process," *Process Biochemistry*, Elsevier Ltd, 53, 10–16.doi:10.1016/j.procbio.2016.11.023

Lipiäinen, S., Kuparinen, K., Sermyagina, E., and Vakkilainen, E. (2022). "Pulp and paper industry in energy transition: Towards energy-efficient and low carbon operation in Finland and Sweden," *Sustainable Production and Consumption*, 29, 421–431.doi:10.1016/j.spc.2021.10.029

Mäki, E., Saastamoinen, H., Melin, K., Matschegg, D., and Pihkola, H. (2021). "Drivers and barriers in retrofitting pulp and paper industry with bioenergy for more efficient production of liquid, solid and gaseous biofuels: A review," *Biomass and Bioenergy*, 148(106036), 1–19.doi:10.1016/j.biombioe.2021.106036

Mendes, C. V. T., Cruz, C. H. G., Reis, D. F. N., Carvalho, M. G. V. S., and Rocha, J. M. S. (2016). "Integrated bioconversion of pulp and paper primary sludge to second generation bioethanol using *Saccharomyces cerevisiae* ATCC 26602," *Bioresource Technology*, 220, 161–167.doi:10.1016/j.biortech.2016.07.140

Mendes, C. V. T., Rocha, J. M. S., Menezes, F. F., and Carvalho, M. G. V. S. (2017). "Batch and fed-batch simultaneous saccharification and fermentation of primary sludge from pulp and paper mills," *Environmental Technology*, 38(12), 1498–1506.doi:10.1080/09593330.2016.1235230

Neiva, D. M., Araújo, S., Gominho, J., Carneiro, A. C., and Pereira, H. (2018). "Potential of *Eucalyptus globulus* industrial bark as a biorefinery feedstock: Chemical and fuel characterization," *Industrial Crops & Products*, 123, 262–270.doi:10.1016/j.indcrop.2018.06.070

Paulova, L., Patakova, P., Branska, B., Rychtera, M., and Melzoch, K. (2015). "Lignocellulosic ethanol: Technology design and its impact on process efficiency," *Biotechnology Advances*, Elsevier Inc., 33(6), 1091–1107.doi:10.1016/j.biotechadv.2014.12.002

Pino, M. S., Rodríguez-Jasso, R. M., Michelin, M., Flores-Gallegos, A. C., Morales-Rodriguez, R., Teixeira, J. A., and Ruiz, H. A. (2018). "Bioreactor design for enzymatic hydrolysis of biomass under the biorefinery concept," *Chemical Engineering Journal*, Elsevier, 347, 119–136.doi:10.1016/j.cej.2018.04.057

Pratto, B., dos Santos-Rocha, M. S. R., Longati, A. A., de Sousa Júnior, R., and Cruz, A. J. G. (2020). "Experimental optimization and techno-economic analysis of bioethanol production by simultaneous saccharification and fermentation process using sugarcane straw," *Bioresource Technology*, Elsevier, 297(122494), 1–11.doi:10.1016/j.biortech.2019.122494

Rodrigues, A. E., Pinto, P. C. de O. R., Barreiro, M. F., Costa, C. A. E. da, Mota, M. I. F. da, and Fernandes, I. (2018). *An Integrated Approach for Added-Value Products from Lignocellulosic Biorefineries: Vanillin, Syringaldehyde, Polyphenols and Polyurethane*, Springer Cham, Cham, Switzerland.doi:10.1007/978-3-319-99313-3

Saini, P., Beniwal, A., Kokkiligadda, A., and Vij, S. (2018). "Response and tolerance of yeast to changing environmental stress during ethanol fermentation," *Process Biochemistry*, Elsevier, 72, 1–12.doi:10.1016/j.procbio.2018.07.001

Silva, A. S. da, Espinheira, R. P., Sposina, R., Teixeira, S., Souza, M. F. De, Leitão, V. F., and Bon, E. P. S. (2020). "Biotechnology for Biofuels Constraints and advances in high - solids enzymatic hydrolysis of lignocellulosic biomass : a critical review," *Biotechnology for Biofuels*, BioMed Central, 13(58), 1–28.doi:10.1186/s13068-020-01697-w

Sluiter, A., Hames, B., Ruiz, R., Scarlata, C., Sluiter, J., Templeton, D., and Crocker, D. (2012). "Laboratory Analytical Procedure (LAP): Determination of Structural Carbohydrates and Lignin in Biomass," *Technical Report NREL/TP-510-42618*, National Renewable Energy Laboratory, Colorado, <https://www.nrel.gov/docs/gen/fy13/42618.pdf> (Oct. 27, 2021).

Tareen, A. K., Punsuvon, V., Sultan, I. N., Khan, M. W., and Parakulsuksatid, P. (2021). "Cellulase Addition and Pre-hydrolysis Effect of High Solid Fed-Batch Simultaneous Saccharification and Ethanol Fermentation from a Combined Pretreated Oil Palm Trunk," *ACS Omega*, 6(40), 26119–26129.doi:10.1021/acsomega.1c03111

Wright, J. D., Wyman, C. E., and Grohmann, K. (1988). "Simultaneous Saccharification and Fermentation of Lignocellulose: Process Evaluation," *Applied Biochemistry and Biotechnology*, 18, 75–90.

Wyman, C. E., Spindler, D. D., and Grohmann, K. (1992). "Simultaneous saccharification and fermentation of several lignocellulosic feedstocks to fuel ethanol," *Biomass and Bioenergy*, 3(5), 301–307.

Zhang, J., Chu, D., Huang, J., Yu, Z., Dai, G., and Bao, J. (2010). "Simultaneous saccharification and ethanol fermentation at high corn stover solids loading in a helical stirring bioreactor," *Biotechnology and Bioengineering*, 105(4), 718–728.doi:10.1002/bit.22593

Zhang, X., Qin, W., Paice, M. G., and Saddler, J. N. (2009). "High consistency enzymatic hydrolysis of hardwood substrates," *Bioresource Technology*, Elsevier Ltd, 100(23), 5890–5897.doi:10.1016/j.biortech.2009.06.082

Zhang, Y., Liu, Y.-Y., Xu, J.-L., Yuan, Z.-H., Qi, W., Zhuang, X.-S., and He, M.-C. (2012). "High solid and low enzyme loading based saccharification of agricultural biomass," *BioResources*, 7(1), 345–353.doi:10.15376/biores.7.1.0345-0353

CRediT authorship contribution statement

Mariana S. T. Amândio: Investigation, Methodology, Data curation, Writing – original draft. **Jorge M. S. Rocha**: Conceptualization, Writing – review, Funding acquisition, Validation. **Ana M. R. B. Xavier**: Conceptualization, Supervision, Validation, Writing – review & editing.

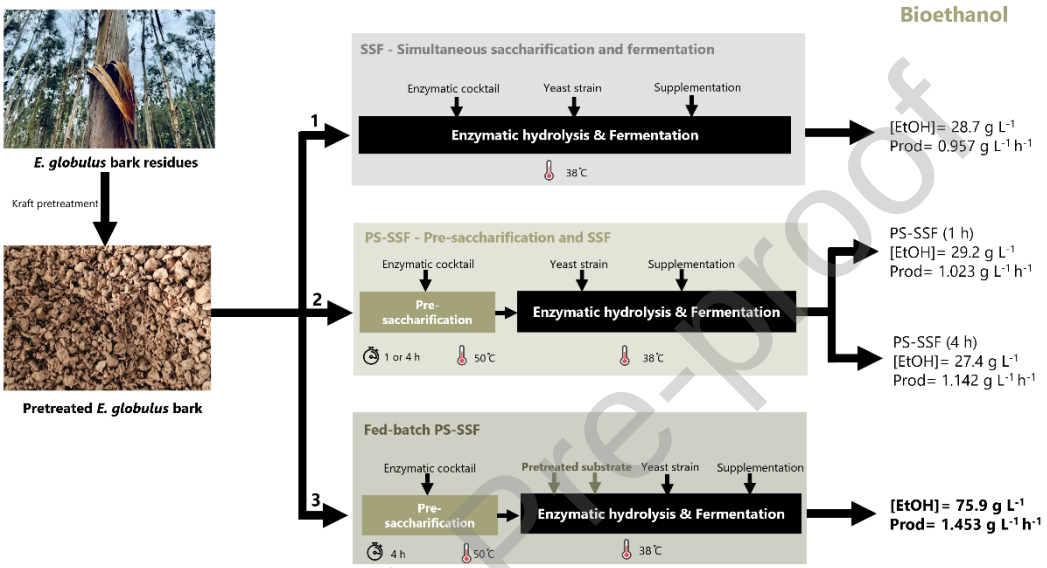
Declaration of interests

☐ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☒ The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Ana M. R. B. Xavier reports was provided by University of Aveiro CICECO. Ana M R B Xavier has patent pending to PT Provisional Patent Application No. PT118408.

Graphical abstract



Highlights

- Bioethanol from eucalyptus bark by Simultaneous Saccharification & Fermentation (SSF)
- A short pre-saccharification (PS) step improved bioethanol productivity
- High solids loading by Fed-batch PS-SSF boosted bioethanol productivity more than 25%
- Increasing solids loading from 8 to 20% raised bioethanol from 27.4 to 75.9 g L^{-1}