fermentation

MDPI

Article

Batch Simultaneous Saccharification and Fermentation of Primary Sludge at Very High Solid Concentrations for Bioethanol Production

Cátia V. T. Mendes, Jorge M. S. Rocha and M. Graça V. S. Carvalho *

CIEPQPF, Department of Chemical Engineering, University of Coimbra, R. Sílvio Lima, Pólo II, 3030-790 Coimbra, Portugal; catvan@eq.uc.pt (C.V.T.M.); jrocha@eq.uc.pt (J.M.S.R.)
* Correspondence: mgc@eq.uc.pt

Abstract: A sustainable industrial future involves the exploitation of renewable resources to obtain a wide diversity of products and energy and the decrease of waste generation. Primary sludge (PS) from pulp and paper mills is a lignocellulosic residue mainly consisting of cellulose and hemicelluloses that can be converted to bioethanol. In the present work, bioethanol was produced from untreated PS by simultaneous saccharification and fermentation (SSF). Studies were carried out on initial solid concentration, yeast inoculum percentage, cellulolytic enzyme dosage, and co-application of two enzyme complexes (cellulolytic NS 22192 and xylanolytic Cellic® HTec2, Bagsværd, Denmark). Increasing solid content up to 22% improved ethanol concentration (59.1 g L^{-1}), productivity (1.97 g L^{-1} h^{-1}), and yield (86.3%); however, at the maximum solid concentration (28%), both yield and productivity decreased. At the highest solid concentration, a decrease of 33% in the cellulolytic enzyme dosage was observed (compared to reference enzyme loadings). The co-application of the two enzyme complexes had a positive effect on PS conversion efficiency. When a preliminary scale-up strategy was implemented from 50 mL to 2.5 L at 22% solids concentration, similar results were obtained despite the initial mixing difficulties of the heterogeneous system.

Keywords: batch; bioethanol; lignocellulosic residue; simultaneous saccharification fermentation

Citation: Mendes, C.V.T.; Rocha, J.M.S.; Carvalho, M.G.V.S. Batch Simultaneous Saccharification and Fermentation of Primary Sludge at Very High Solid Concentrations for Bioethanol Production. *Fermentation* **2023**, *9*, 888. https://doi.org/10.3390/fermentation9100888

Academic Editor: Alessia Tropea

Received: 20 August 2023
Revised: 27 September 2023
Accepted: 28 September 2023
Published: 30 September 2023

1. Introduction

Part of a sustainable industrial future involves the exploitation of renewable and biological resources to obtain a wide diversity of products and energy and the decrease of waste generation, for which biorefineries are destined to play an instrumental role [1,2]. The pulp and paper industries are at the forefront of the forest biorefinery concept. The main products of existing pulp and paper manufacturing facilities are pulp and paper, but it is possible to integrate other processes, enabling competitive advantages. Particularly, lignin and residual waste streams can be valorized into a broad range of marketable products (food, feed, chemicals, and materials) and energy (fuels, power, and/or heat) through chemical, thermal, or biological conversion technologies [2–5].

Pulp and paper manufacturing industries generate four main types of wastes: (i) primary sludge generated along the production line of virgin fibers; (ii) waste paper produced by removing inks from post-consumer paper (de-inking paper sludge); (iii) activated sludge from the secondary systems (secondary sludge); and (iv) combined sludge composed of waste paper and activated sludge [6,7]. Like most of the worldwide wastes, these residues are generally intended for landfilling, composting, or incineration. The disposal costs may represent around 60% of the total waste treatment operation costs, creating both economic and environmental concerns. Thus, sustainable waste management alternatives need to be developed to decrease waste [8–10]. Portuguese pulp and paper mills produce around 350,000 t of sludges per year, consisting of nearly 64% of the total solid waste. Landfilling (21%), energetic valorization (26%), agriculture and composting (12%), valorization by

other industry sectors (12%), and other not specified destinations are the current solutions of final waste management for those solid residues [11]. The fibers lost along the various stages of pulp and paper manufacturing are sent to a sedimentation tank to decrease the high content of suspended solids. This treatment produces the primary sludge (PS), which represents the highest waste generation output [7]. PS is usually mechanically dewatered to a solids content of up to 30%, making it undesirable for energetic valorization. Its chemical composition and physicochemical characteristics vary widely, depending on the raw material and the manufacturing and effluent treatment processes at the mills [6–8,12,13]. Generally, PS consists of short pulp fibers, clays, fillers, and other contaminants lost during the stages of pulp and paper production [12]. PS can contain solid contents of 20–50%, which includes, on a dry basis, 20–75% of carbohydrates (cellulose and hemicelluloses), 4.5–11% of lignin, and up to 50.8% of ash, among others [7–9,12–15]. Carbohydrates from PS are more amenable to enzymatic hydrolysis compared to raw lignocellulose since the crystalline structure of cellulose has been disrupted, and recalcitrant lignin has been removed during the previous extensive mechanical and chemical treatment during pulping and papermaking processes [8,10,12]. Therefore, PS has many positive features as a potential raw material for ethanol production: (i) renewable, non-edible and readily available; (ii) it carries zero or, in some cases, negative cost (elimination of disposal cost, reducing economic and environmental effects); (iii) the kraft pulping primary sludge has high carbohydrates and low lignin contents; (iv) pretreatment is not a mandatory step; (v) a processing unit of PS can be potentially integrated into an established paper mill [10,11]. A major challenge for PS bioconversion is the presence of fillers and impurities, such as calcium carbonate ($CaCO_3$), which increases the pH of sludge suspensions and obstructs the access of the enzymes to cellulose substrate, decreasing enzymatic hydrolysis efficiency. It also limits the solids/cellulose loading capacity [8,10,16]. Calcium carbonate contents of 24–44.7% can be found in different PS samples [6,7,12].

In separate processes of enzymatic hydrolysis and fermentation (SHF), a previous $CaCO_3$ removal step (de-ashing) is usually needed. Several methods, including mechanical fractionation or chemical pretreatment with different inorganic acids, have been tested [10,11,17]. Alternatively, the application of simultaneous saccharification and (co-)fermentation (SSF/SSCF) processes enables the utilization of untreated PS due to the partial neutralization of $CaCO_3$ by carbonic and other organic acids produced in the fermentation, acting afterward as a buffer to stabilize pH, promoting carbohydrates hydrolysis to fermentable sugars [14,18,19]. Another major challenge is that high solid loading must be applied in order to obtain high ethanol concentrations and develop an efficient and economically viable production process [8,13,20]. The high-water holding capacity of PS leads to high viscous mixtures, resulting in improper mixing and mass transfer when using high solids concentration. When solid contents higher than 15% (w/v) are used, specific conditions for agitation and mixing are required [8,10,16,20]. To overcome this issue, the fed-batch strategy is usually implemented with efficiency, resulting in high ethanol concentrations, and the feeding frequency is pointed as a major influence in ethanol yield and productivity [8,13,16,21,22]. Data information on ethanol production from high solids content at batch conditions is still scarce.

In our previous works, SSF of PS was carried out in fed-batch and batch at equal solids loading. Ethanol concentration, conversion yield, and productivity were higher when batch conditions were applied [23].

The present work aims to continue the studies on the production of ethanol by SSF of PS at very high solid concentrations in batch conditions. Studies on initial solid concentration (up to 28%), inoculum percentage, enzyme dosage decrease, and the co-application of two enzyme complexes were carried out. A preliminary scale-up strategy was also applied from 50 mL to 2.5 L working volume, in which different types of impellers were tested for mechanical agitation.

2. Materials and Methods

2.1. Raw Material, Enzymes, and Microorganisms

PS was provided by a Portuguese pulp and paper mill that produces kraft pulp mainly from *Eucalyptus globulus* chips. It was collected from the primary clarifier unit in the first stage of the wastewater treatment and stored at 4 °C until further use.

Two enzyme complexes were used for the enzymatic degradation of cellulose and hemicelluloses to fermentable sugars (glucose and xylose): (i) the cellulolytic enzyme complex NS 22,192 (a blend of cellulases, β-glucosidases, and hemicellulases) and the xylanolytic enzyme complex Cellic® HTec2 (endoxylanase with cellulase background). Both enzyme complexes were kindly provided by Novozymes® (Bagsværd, Denmark). Cellulolytic enzyme complex NS 22,192 was the main complex used to hydrolyze cellulose, whilst xylanolytic enzyme complex Cellic® HTec was primarily used as an auxiliary cellulase-boosting enzyme. Cellulase activity was determined by the paper filter assay at 38 °C [24]. Xylanase activity was measured by the amount of xylose liberated from oat spelt xylan as a result of enzyme action for 60 min at 38 °C [25]. NS 22,192 had cellulase and xylanase activities of 73 FPU mL^{-1} and 21 893 U mL^{-1}, respectively. Cellic® HTec2 had a cellulase and xylanase of 4.1 FPU mL^{-1} and 19 954 U mL^{-1}, respectively, at 38 °C. The protein content was also determined following the Bradford method [26]. NS 22,192 and Cellic® HTec2 had a protein content (expressed as bovine serum albumin equivalents) of 73 g L^{-1} and 108 g L^{-1}, respectively.

For ethanol production, *Saccharomyces cerevisiae* (ATCC® 26602™) (American Type Culture Collection, Manassas, VA, USA) was used. The yeast was kept in agar slants, with the Yeast Medium (YM) plus 15 g L^{-1} agar (HiMedia Laboratories) at 4 °C. The YM consisted of 3 g L^{-1} yeast extract, 3 g L^{-1} malt extract, 5 g L^{-1} peptone (all from Fluka), and 10 g L^{-1} glucose (Riedel de-Haën). For the SSF assays, a fresh inoculum was previously prepared with YM and kept overnight at 38 °C and 150 rpm.

2.2. Simultaneous Saccharification and Fermentation (SSF)

2.2.1. SSF in Shake Flasks

All SSF experiments were carried out in batch conditions. Small-scale SSF was performed in 250 mL Erlenmeyer flasks containing 50 mL working volume. The Erlenmeyer flasks (70 mm diameter) were incubated at 38 °C in an orbital shaker at a shaking frequency of 150 rpm. Studies were carried out on (i) initial solid concentration, from 8 to 28% (*w*/*w*); (ii) inoculum percentage, from 10.0 to 20% (*v*/*v*, working volume basis); (iii) cellulolytic enzyme complex NS 22,192 dosage (5, 10 or 15 filter paper units per gram of carbohydrates, FPU $g_{CH}{}^{-1}$) and (iv) the co-application of enzyme complexes NS 22,192 and Cellic® HTec2 (Table 1).

Table 1. Co-application of NS 22,192 (NS) and Cellic® HTec2 (HT) in the batch SSF of PS (28% total solids).

NS 22,192 Dosage		Cellic® HTec2 Dosage
FPU $g_{CH}{}^{-1}$	**Protein Conc. % (*w*/*w*)**	**Protein Conc. % (*w*/*w*)**
10.0	1.00	0.00
8.5	0.85	0.15
7.0	0.70	0.30
5.0	0.50	0.50

In the co-application of the two enzyme complexes, the NS 22,192 enzyme charge of 10 FPU g $_{CH}{}^{-1}$ was used as the control assay, which corresponds to a total protein concentration of 1% (*w*/*w*) based on carbohydrates; then 15% to 50% of the reference protein content was replaced by Cellic® HTec2, as presented in Table 1. Considering the mixture of different types of enzymes of both complexes, protein concentration was used instead of cellulase or xylanase activity to have a common calculation basis.

2.2.2. SSF in Stirred Tank Bioreactors

Batch SSF assays were also performed in different stirred tank bioreactors (STBR) at 38 °C and with an initial solid content of 22%: (i) 1.1 L vessel (STBR-1.1L), (ii) 3.4 L vessel (STBR-3.4L), and (iii) 5 L automatized bioreactor (Sartorius Biostat® B-plus, Goettingen, Germany) (STBR-5L). The schematic representation and respective nomenclature [27] of the STBR and the geometries of the available mechanical impellers used are shown in Figure 1. Table 2 shows the STBR dimensions.

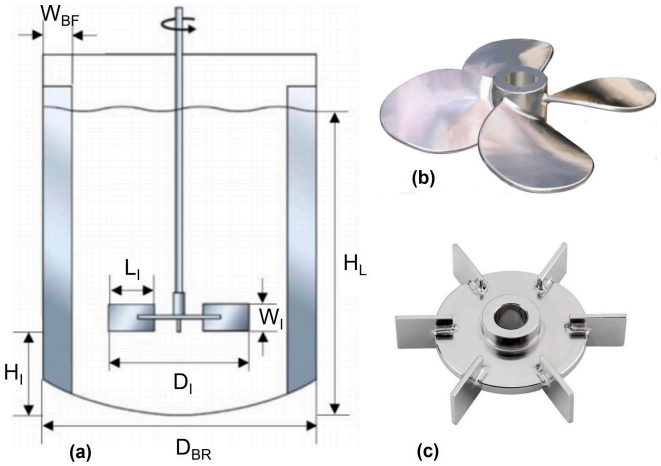

Figure 1. Schematic representation of (**a**) Stirred Tank Bioreactor (STBR); (**b**,**c**) available mechanical impellers used: (**b**) four-blade propeller (small and large); (**c**) Rushton disc turbine. Nomenclature for STBR: W_{BF}—Width of baffles; W_I—Width of the impeller; D_I—Diameter of the impeller; D_{BR}—Diameter of the bioreactor; H_L—Height of the mixture; H_I—Height between the impellers and the bottom of the bioreactor; L_I—Length of the impeller [27].

Table 2. Dimensions and configurations of available bioreactors.

Dimensions	STBR-1.1L	STBR-3.4L	STBR-5.0L
V_L, L	0.48	1.40	2.50
Impeller	4-blade small propeller	4-blade large propeller	Rushton
Number of impellers	1	1	2
Blades per impeller	4	4	6
D_{BR}, cm	10	17	16
D_I, cm	6.0	11	6.5
H_L, cm	6.1	6.2	31.8
H_I, cm	1.5	2.0	5.0
$H_{I\text{-}I}$, cm	n.a.	n.a.	10
L_I, cm	2.7	5.2	1.6
W_I, cm	1.9	3.3	1.5
D_{BR}/D_I	1.7	1.5	2.5
H_L/D_I	1.0	0.6	4.9

Legend: V_L, working volume; D_{BR}, diameter of the STBR; D_I, diameter of the impeller; H_L, height of the SSF broth; H_I, height between the impellers and the bottom of the STBR; $H_{I\text{-}I}$, distance between two impellers; L_I—Length of the impellers; W_I—Width of the impeller; n.a. not applicable.

Fresh inoculum of *S. cerevisiae* (ATCC® 26602™) (constant percentage of total working volume in all assays), nutrient solution, and an enzymatic dosage of 15 FPU $g_{CH}{}^{-1}$ were provided in all SSF experiments performed in the STBR.

2.3. Preliminary Scale-Up Strategy

The batch SSF of primary sludge for ethanol production was scaled up from 250 mL shake flasks to 5 L mechanically stirred vessels. In biological processes, the scale-up is usually carried out based on the constant impeller tip speed. However, when scale-up is directly performed from shake flasks to the stirred tank bioreactor, it is inadequate to

use the constant impeller tip speed since there is no geometrical similarity between vessel shapes and the agitation systems differ. Therefore, the scale-up was conducted from the shake flask to the 1.1 L bioreactor using the constant Reynolds number, whilst, for the mechanically stirred vessels (from 1.1 L to 5 L), the constant impeller tip speed was used as a scale-up criterion. Mathematical Equations (1)–(5) were applied in the shake flasks, and Equations (1) and (6)–(8) were used in the mechanically stirred vessels [28–30],

$$\eta = K.\left(\gamma_{eff}\right)^{m-1} \quad (1)$$

$$Re_{sf} = \frac{\rho.n.d_{sf}^2}{\eta} \quad (2)$$

$$N_e^{'} = 70.Re_{sf}^{-1} + 25.Re_{sf}^{-0.6} + 1.5.Re_{sf}^{-0.2} \quad (3)$$

$$\mathrm{P} = N_e^{'}.\rho.n^3.d_{sf}^4.V_L^{1/3} \quad (4)$$

$$\gamma_{eff,\,sf} = L^{\frac{1}{m}+1}.\left(\frac{\frac{\mathrm{P}}{V_L}}{K}\right)^{\frac{1}{m}+1}.\left(\frac{V_L^{1/3}}{d_f}\right)^{\frac{x}{m}+1}.\left(\frac{V_L^{1/3}}{d_0}\right)^{\frac{y}{m}+1} \quad (5)$$

$$Re_i = \frac{\rho.N_i.D_i^2}{\eta} \quad (6)$$

$$\vartheta_{tip} = \pi.N_i.D_i \quad (7)$$

$$\gamma_{eff,BSTR} = k_s.N_i \quad (8)$$

where: Re_i, impeller Reynolds number (dimensionless); N_i, impeller rotation speed (rpm); ρ, fluid density (kg m^{-3}); Di, impeller diameter (m); η, viscosity of the mixture (Pa.s); Re_{sf}, shake flasks Reynolds number (dimensionless); n, shaking frequency (rps); d_{sf}, largest inner diameter of shake flasks (m); ϑ_{tip}, impeller tip speed (m s^{-1}); P, power consumption for shake flasks (W); N'_e, modified power number for shake flasks (dimensionless); V_L, working volume (m^3); $\gamma_{eff,BSTR}$, shear rate for BSTR (s^{-1}); k_s, constant dependent on the impeller geometry (10–12, dimensionless); $\gamma_{eff,sf}$, effective shear rate correlation for shake flasks (s^{-1}); L, proportional factor (2.06, dimensionless); K, flow consistency index (128.1 mPa.sm); d_0, shaking diameter (m); m, flow behavior index (m = 0.54, dimensionless); x, exponent of the geometric number (x = −0.331, dimensionless); y, exponent (y = 0) [28–30].

2.4. Analytical Procedures

PS moisture and lignin, carbohydrates (cellulose and hemicelluloses), and ash contents were analyzed according to NREL standard protocols [31]. Water and solid contents of PS were analyzed by gravimetry after drying PS at 105 °C. Ash was also determined by gravimetry after PS samples were incinerated at 525 °C. To determine the lignin and carbohydrate contents, PS was previously submitted to sulfuric acid hydrolysis at 72% (*w*/*w*) for 60 min at 30 °C, followed by acid hydrolysis at 4% (*w*/*w*) for 60 min at 121 °C. The final hydrolysate was filtered with a glass fiber filter. The retained solids were weighed to estimate the acid-insoluble lignin by gravimetry after oven drying at 105 °C. Acid-soluble lignin was quantified in the supernatant by UV-Vis spectrophotometry at 205 nm (Jasco V-550). Total lignin corresponds to the sum of acid-insoluble and acid-soluble lignin. An aliquot of the supernatant was neutralized and analyzed by high-performance liquid chromatography (HPLC) to detect and quantify the monomeric sugars (glucose, xylose, mannose, arabinose, and galactose) liberated from the acid hydrolysis of cellulose and hemicelluloses. HPLC system consisted of a Knauer model K-301 connected to a refractive index detector (HPLC-RI). An Agilent Hi-Plex Ca (8 μm × 300 mm) column was used at 80 °C. The mobile phase was water pumped at 0.6 mL min^{-1} for 30 min. Neutralized supernatant samples were

previously centrifuged and filtrated with a 0.2 µm syringe filter membrane (Whatman). Cellulose and hemicelluloses were calculated based on the monomeric sugar content.

PS intrinsic viscosity was also evaluated following the T230 om-08 TAPPI standard [32]. Cellulose PS is dissolved in a cupriethylenediamine solution, and its time of efflux was measured through a capillary tube at a known pulp mass concentration of 25 °C. The degree of polymerization (DP) of cellulose was estimated through its correlation with intrinsic viscosity, as applied in a previous work [33].

The viscous flow behavior of the SSF broth was measured in a Haake™ Rheostress™ 1 rheometer (Thermo Scientific, Waltham, MA, USA), equipped with a measurement cup Z34 DIN53018 and a cylindrical rotor Z34 DIN53019. Samples were withdrawn at 17, 41, and 72 h from the SSF of primary sludge with 22% solid content and analyzed directly after sampling. The measurements were performed at the respective SSF temperature (38 °C). Values of shear stress (τ, Pa) and apparent viscosity (η_{app}, Pa.s) were obtained at a specific range of shear rates (γ, s^{-1}). The rheologic characteristics of the broth sample collected at 17 h were applied in the mathematical Equations (1-8).

Samples withdrawn from the SSF experiments were analyzed for ethanol and residual monomeric sugars by HPLC-RI, applying the method described above.

Process efficiency was evaluated by ethanol concentration, yield, and productivity. Ethanol yield ($Y_{EtOH/sug}$), based on the monomeric sugars existing in the PS, was calculated by Equation (9), where: $[EtOH]_{max}$ is the maximum ethanol concentration ($g_{EtOH}\ L^{-1}$) achieved; f is the fraction of monosaccharides in primary sludge (0.613 $g_{sug}\ g_{PS}{}^{-1}$, dry basis); [PS] is the initial concentration of dry weight primary sludge in batch operation ($g_{PS}\ L^{-1}$). The percentage of the theoretical yield ($Y'_{EtOH/sug}$) was determined by Equation (10), where 0.51 is the theoretical mass conversion factor of monomeric sugars to ethanol ($g_{EtOH}\ g_{sug}{}^{-1}$). Experimental ethanol yield, based on primary sludge used ($Y_{EtOH/PS}$, dry basis), was calculated by Equation (11). Ethanol productivity (P) at time t was determined for the maximum experimental ethanol concentration according to Equation (12) [34].

$$Y_{EtOH/sug}\ (g_{EtOH}\ g_{sug}^{-1}) = \frac{[EtOH]_{max}}{f \times [PS]} \quad (9)$$

$$Y'_{EtOH/sug}\ (\%) = \frac{Y_{EtOH/sug}}{0.51} \times 100 \quad (10)$$

$$Y_{EtOH/PS}\ (g_{EtOH}\ g_{PS}^{-1}) = \frac{[EtOH]_{max}}{[PS]} \quad (11)$$

$$P\left(gL^{-1}h^{-1}\right) = \frac{[EtOH]_{max}}{t} \quad (12)$$

3. Results and Discussion

3.1. PS Characterization

PS contained 50.0 ± 0.0% (*w*/*w*) of total solids. On a dry weight basis, PS consists of 2.9 ± 0.1% of acid-insoluble lignin, 0.6 ± 0.0% of acid-soluble lignin, 34.4 ± 1.8% of ash (29.4 ± 3.8% of $CaCO_3$), 9.3 ± 0.1% of xylose and 52.0 ± 1.1% of glucose. Considering that PS contains hardwood fibers, residual contents of mannose and galactose were obtained, as expected. PS cellulose had an intrinsic viscosity of 608 ± 1 L kg^{-1}, corresponding to a degree of polymerization of 2162. The referred values are lower than the usual reported for eucalypt kraft pulps [35], suggesting a higher level of degradation of polysaccharides in PS, favoring the following saccharification and fermentation processes. Based on the xylose and glucose contents, a maximum of 0.31 g of ethanol could theoretically be produced per gram of PS (dry weight basis).

3.2. Batch SSF in Shake Flasks

3.2.1. Effect of Initial Solid Concentration

Batch SSF of untreated PS was tested at different initial solids: 8.0, 16.0, 22.0 and 28.0%. Figure 2 shows ethanol concentration (1a) and monosaccharide content (1b) determined during the process.

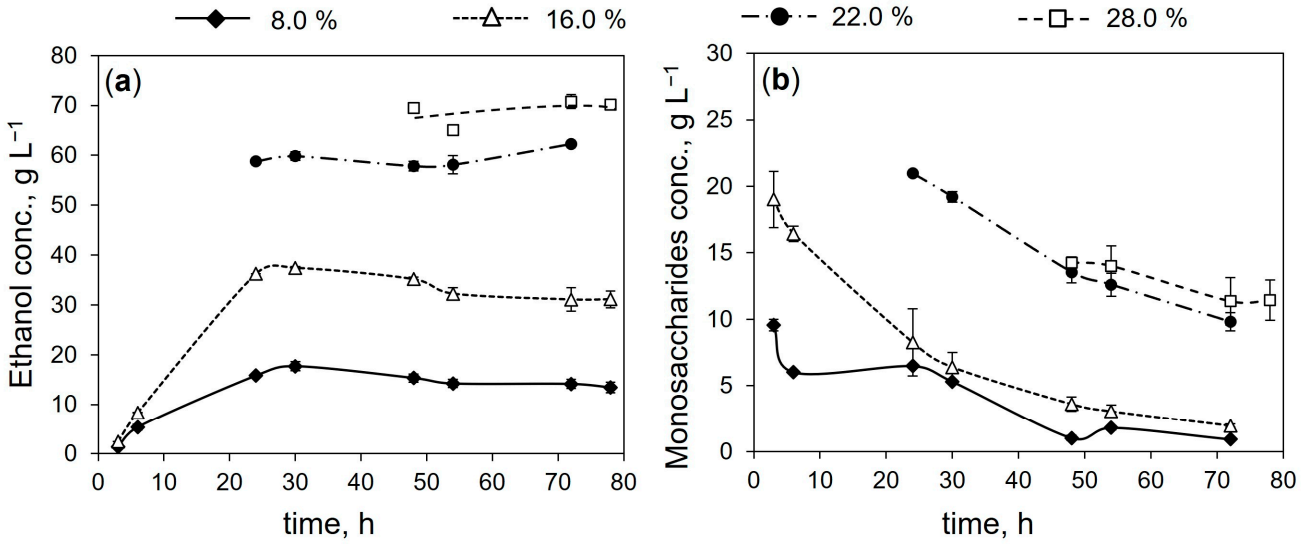

Figure 2. Batch SSF of untreated PS in shake flasks with different initial solid concentrations: (**a**) ethanol concentration; (**b**) monosaccharides concentration (glucose and xylose).

As expected, ethanol concentration increased as the initial solid content increased, as observed in Figure 1a. Higher solid loading means higher available carbohydrates for hydrolysis, and consequently, a higher amount of fermentable sugars can be converted to ethanol. Figure 1b shows that increasing the initial solid concentration also resulted in a higher residual sugar concentration in the SSF broth that is not converted; up to 13 g L^{-1} of monosaccharides were determined, mostly consisting of xylose a C5 sugar not fermented by *S. cerevisiae* (ATCC® 26602™). As the solid load increased, initial orbital mixing was more difficult to achieve since the SSF mixture was mostly a solid heterogeneous blend. It was observed that the SSF mixture remained mostly in the shake flask's bottom, and more time was needed for the reaction mixture to liquefy. Therefore, the first sample withdrawn from the shake flasks occurred after 3, 24, and 48 h of SSF of primary sludge at 8.0%, 16.0, 22.0, and 28.0% solid contents, respectively. Table 3 shows the main results in ethanol production. SSF efficiency was enhanced when initial solid content was increased from 8.0 to 22.0%. Ethanol concentration, yield, and productivity improved, as seen in Table 3. At 22% total solids, 59.1 g L^{-1} of ethanol was produced with a conversion yield of 86.3% and productivity of 1.97 g L^{-1} h^{-1}. Increasing solid content to 28.0% also led to higher ethanol concentration. However, it lowered yield and productivity. Even so, a high SSF efficiency was still attained: yield of 78.4% and productivity of 1.45 g L^{-1} h^{-1} for an ethanol concentration of 69.5 g L^{-1}. These results compare well to the ones achieved in the fermentation of glucose carried out in preliminary tests. The yeast was preliminary tested in the ethanolic fermentation of different initial glucose concentrations (50–240 g L^{-1}) in batch conditions. Ethanol concentration increased to 72.8 g L^{-1} when 200 g L^{-1} of glucose was used. An increase in ethanol productivity was registered (1.52 g L^{-1} h^{-1}), whilst a slight decrease was observed in the conversion yield (71%). All ethanolic fermentation parameters decreased when the initial glucose concentration was further increased to 240 g L^{-1}.

Table 3. Ethanol production parameters in the batch SSF of different initial solid loading of untreated PS.

Total Solids %	Initial Sugars [1] g L^{-1}	[Ethanol]$_{max}$ g L^{-1}	Time h	P_{EtOH} g L^{-1} h^{-1}	$Y_{EtOH/sug}$ g_{EtOH} $g_{sug}{}^{-1}$	$Y'_{EtOH/sug}$ %	$Y_{EtOH/PS}$ g_{EtOH} $g_{PS}{}^{-1}$
8	49	17.7 ± 0.8	30	0.59	0.36	70.6	0.22
16	98	37.4 ± 0.5	30	1.25	0.38	74.5	0.23
22	135	59.1 ± 1.0	30	1.97	0.44	86.3	0.27
28	172	69.5 ± 1.4	48	1.45	0.40	78.4	0.25

[1] maximum possibly obtained sugars via the hydrolysis of cellulose and hemicelluloses.

In the SSF of paper sludge reported in another work [16], an ethanol concentration of 22.5 g L^{-1} was produced with a solid loading of 13.5% and a conversion yield of 66.1% (based on glucan content) [16]. However, when solids loading was increased to 20.2%, the authors reported inefficient enzymatic hydrolysis, the sludge was not liquefied, and ethanol production data were not shown. Ethanol production was then slightly improved by increasing the agitation intensity, removing ashes from the paper sludge (de-ashed by CO_2 treatment), or changing the process configuration (SSCF—simultaneous saccharification and co-fermentation) [16]. A higher solid loading (27%) was used in the fed-batch SSF of corrugated recycle paper sludge, resulting in 45.5 g L^{-1} of ethanol with a conversion yield of 78.2% at a production rate of 0.45 g L^{-1} h^{-1} [8]. In the present study, higher ethanol concentrations were produced from PS, without pretreatment, using higher solids concentration (28%) in batch conditions with similar conversion yields.

3.2.2. Effect of Different Variables at the Highest Solids Concentration (28.0%)

Different variables were studied in the batch SSF of PS at the highest solids concentration (28.0%) since it enabled obtaining a higher ethanol concentration (an important parameter to achieve an economic separation after fermentation). The effect of (i) inoculum percentage, (ii) enzyme dosage decrease, and (iii) partial replacement of NS 22,192 by Cellic® HTec2 were assessed to improve the SSF yield.

Inoculum percentages of 10.0, 14.0, 17.5, and 20.0% (v/v), based on working volume, were studied in the batch SSF of primary sludge at 28.0% total solids concentration. Figure 3 shows the effect of inoculum dosage on ethanol production. Between 30 and 48 h of process, ethanol concentration was higher when a higher inoculum percentage was used, as supposed since higher inoculum is expected to accelerate the production process. For example, after 30 h, 65 g L^{-1} of ethanol was already produced, with 17.5 and 20.0% inoculum percentage; with 10.0 and 14.0% inoculum addition, 44.5 and 58.7 g L^{-1} of ethanol was achieved for the same period, respectively. However, no major differences were observed between the experiments after 53.5 h; similar ethanol concentration and yield were attained, as presented in Table 4. At 53.5 h, an ethanol concentration of 73.1–74.8 g L^{-1} was determined at 71.4–73.0% yield. In a different study [22], increasing the amount of inoculum used did improve ethanol production significantly. In the SSF of 16% (w/v) of total solids, ethanol concentration increased from 35.7 to 40.5 g L^{-1} at 66% yield after 80 h, when the inoculum was increased from 10 to 20% (v/v) [22].

Enzyme costs have a major contribution to the total process costs. Enzymes have been applied five to ten times the levels that can be economically feasible, and there are still efforts to reduce enzymatic doses at laboratory scale [10]. In the present study, 15 FPU $g_{CH}{}^{-1}$ of cellulase was mainly used, but lower enzymatic charges were also tested: 10 and 5 FPU $g_{CH}{}^{-1}$ at higher solids concentration (28.0%) and an inoculum percentage of 17.4%. As expected, reducing enzyme NS 22,192 dosage resulted in lower ethanol concentration, productivity, and yield, as shown in Figure 4 and Table 5. The differences registered in the SSF performances with 15 and 10 FPU $g_{CH}{}^{-1}$ were not so noteworthy, and ethanol concentrations higher than 70 g L^{-1} were produced at both enzyme dosages, with high productivity (1.35–1.55 g L^{-1} h^{-1}) and yield (71.4–72.5%). The decrease of enzyme dosage by 33.3% resulted in a decrease of only 1.6% in ethanol concentration. When an enzyme dosage of 5 FPU $g_{CH}{}^{-1}$ (66.7% reduction) was applied, a decrease of 11.3% was observed

in ethanol concentration. SSF of untreated PS at 5 FPU $g_{CH}{}^{-1}$ resulted in an ethanol concentration of 65.9 g L^{-1} at 64.3% conversion yield and 0.92 g L^{-1} h^{-1} productivity after 72 h of reaction.

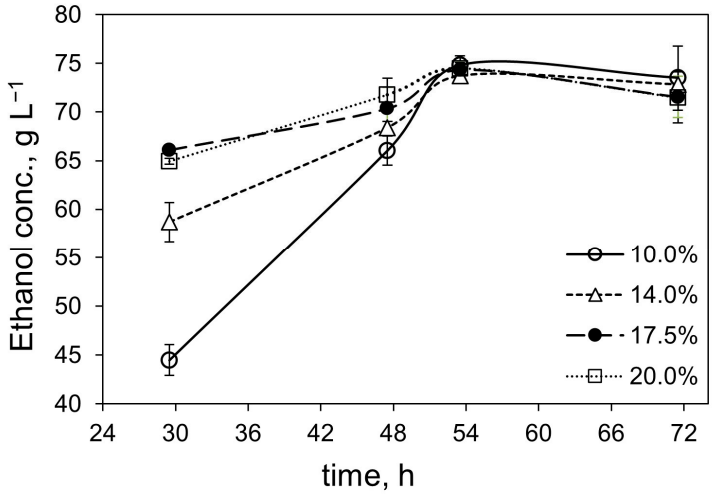

Figure 3. Effect of inoculum percentage in the batch SSF of PS at highest solids concentration (28.0%).

Table 4. Ethanol production parameters in the batch SSF of PS at highest solids concentration (28.0%), obtained at 53.5 h.

Inoculum Percentage %	$[Ethanol]_{max}$ g L^{-1}	P_{EtOH} g L^{-1} h^{-1}	$Y_{EtOH/sug}$ g_{EtOH} $g_{sug}{}^{-1}$	$Y'_{EtOH/sug}$ %	$Y_{EtOH/PS}$ g_{EtOH} $g_{PS}{}^{-1}$
10.0	74.8 ± 1.0	1.40	0.44	85.3	0.27
14.0	73.1 ± 0.7	1.37	0.43	83.3	0.26
17.5	74.3 ± 0.8	1.39	0.43	84.7	0.27
20.0	74.5 ± 0.6	1.39	0.43	84.9	0.27

Another attempt to improve the PS digestibility is by adding auxiliary enzymes, such as hemicellulases (xylanases, mannanases), to facilitate both cellulose and hemicellulose hydrolysis. Using these supplementary enzymes helps to expose the cellulose fibers, thus making them more accessible to cellulases. It is expected that the conjugated action of cellulases and hemicellulases can increase the rate of enzymatic hydrolysis and achieve higher sugar production. The synergy between NS 22,192 (cellulolytic enzyme complex) and Cellic® HTec2 (xylanolytic enzyme complex) was tested. The cellulase dosage of 10 FPU $g_{CH}{}^{-1}$ was used as a control, which also corresponds to a total enzyme protein dosage of 1% (*w*/*w*, gram of protein per gram of carbohydrates)—Table 1. The total enzyme protein (1% *w*/*w*) was then gradually replaced by Cellic® HTec2 up to 0.50% (*w*/*w*). The use of 8.5 FPU $g_{CH}{}^{-1}$ of NS 22,192 with 0.15% (*w*/*w*) Cellic® HTec2 replacement provided identical SSF efficiency parameters (with a slight improvement in productivity), when compared to the control experiments, as shown in Figure 4c (ethanol concentration curve) and Table 5. A negative effect was registered when NS 22,192 dosage was decreased to 7.0 FPU $g_{CH}{}^{-1}$ with 0.30% (*w*/*w*) of Cellic® HTec2 replacement and to 5.0 FPU $g_{CH}{}^{-1}$ with 0.50% (*w*/*w*) replacement. Nevertheless, for the same minimum dosage of enzyme consortium (5 FPU $g_{CH}{}^{-1}$), the addition of Cellic® HTec2 enhanced the SSF of untreated primary sludge (Figure 4 and Table 5).

In a fed-batch semi-simultaneous saccharification and fermentation (with pre-hydrolysis) of shredded copier substrate [21], 2.5% (*w*/*v*) total solids were initially applied with 16.0 FPU g^{-1} of cellulase and 30 U g^{-1} of β-glucosidase. Subsequent additions of substrate were carried out to increase the total solid content up to 65% (*w*/*v*) with no extra enzyme addition. Therefore, a final dosage of 3.7 FPU g^{-1} of cellulase and 6.9 U g^{-1} of β-glucosidase was effectively applied. This efficient strategy resulted in the production of 91.5 g L^{-1} of ethanol, although with low yield (54%) and productivity (0.22 g L^{-1} h^{-1}) [21]. Another

study reported the effects of cellulase dosage decrease with simultaneous β-glucosidase dosage increase in the SSF and SSCF of untreated and de-ashed primary sludge at low solids loading (6% (*w*/*v*)). A decrease in ethanol concentration, yield, and productivity was registered [16].

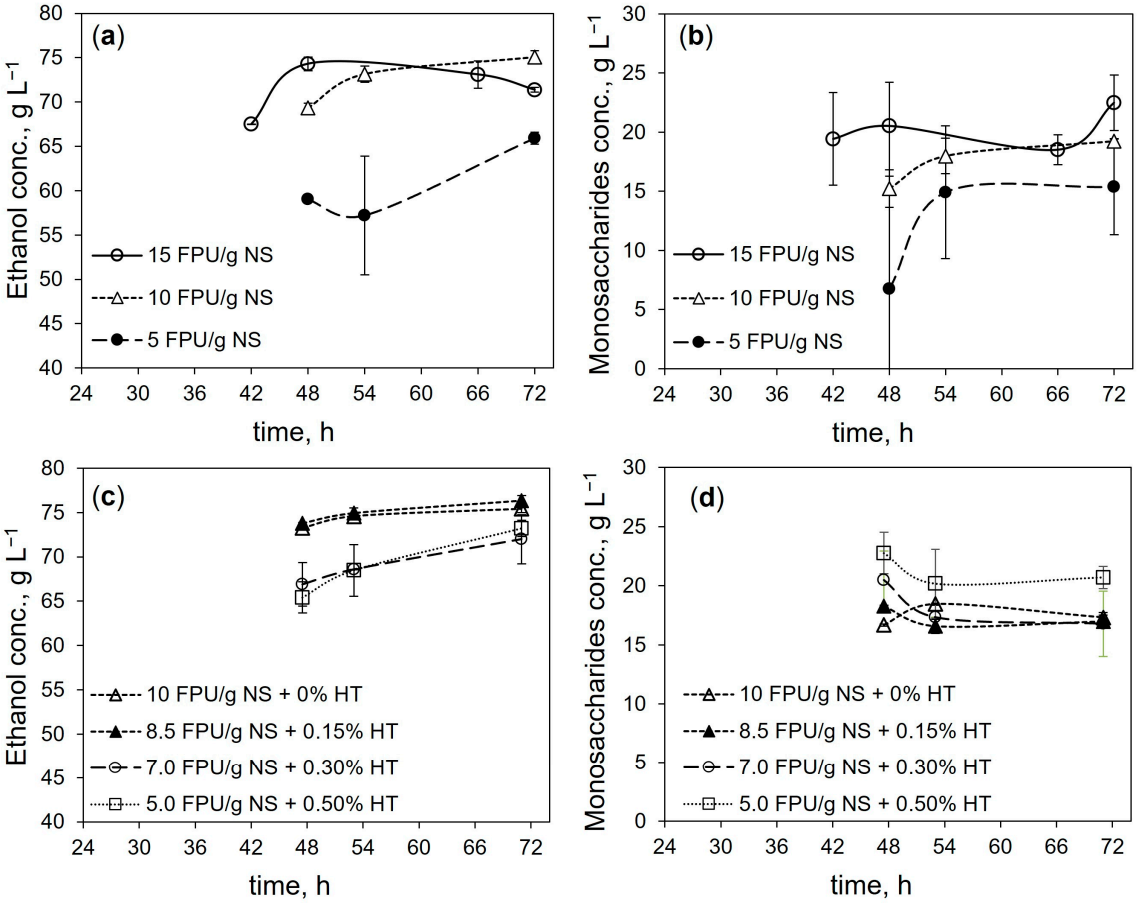

Figure 4. Effect of (**a**,**b**) NS 22,192 (NS) dosage decrease, and (**c**,**d**) Cellic® HTec2 (HT) partial replacement (Table 1) on ethanol production and monosaccharides concentration, in the batch SSF of PS at highest solids concentration (28.0%).

Table 5. Ethanol production parameters in the batch SSF of untreated PS at the highest solids concentration (28.0%), with enzyme dosage decrease and Cellic® HTec2 partial replacement.

NS 22,192 Dosage FPU $g_{CH}{}^{-1}$	Cellic® HTec2 Dosage Protein Conc., % (*w*/*w*)	$[Ethanol]_{max}$ g L^{-1}	Time h	P_{EtOH} g L^{-1} h^{-1}	$Y_{EtOH/sug}$ g_{EtOH} $g_{sug}{}^{-1}$	$Y'_{EtOH/sug}$ %	$Y_{EtOH/PS}$ g_{EtOH} $g_{PS}{}^{-1}$
15.0	0.00	74.3 ± 1.3	48	1.55	0.43	84.7	0.27
10.0		73.1 ± 1.5	54	1.35	0.43	84.3	0.26
5.0		65.9 ± 4.0	72	0.92	0.38	75.1	0.24
8.5	0.15	73.8 ± 2.1	48	1.54	0.43	84.3	0.26
7.0	0.30	72.0 ± 3.4	72	1.00	0.42	82.1	0.26
5.0	0.50	73.2 ± 1.3	72	1.02	0.43	84.3	0.26

Generally, the synergy between the cellulolytic enzyme complex NS 22,192 and the xylanolytic enzyme complex Cellic® HTec2 benefited the conversion of PS carbohydrates in ethanol in the present report. In further studies, it would be interesting to study the effect of lignin-degrading enzymes addition to the process. Additionally, studies on the costs of such synergy should be carried out since the expensive commercial enzymes have a major impact on the economic viability of bioprocesses.

3.3. Batch SSF in Stirred Tank Fermenters

In the stirred tank fermenters, the mixture hydrodynamics was studied. A compromise was established to choose an adequate solid concentration that would simultaneously lead to a high SSF efficiency and allow mixture liquefaction in a period as short as possible. The batch SSF was then carried out at 22% solid loading.

The scale-up criteria most used in the fermentation industry are constant specific power input (P/V), constant volumetric mass transfer coefficient (K_La), constant impeller tip speed of the agitator (ϑ_{tip}) and constant dissolved oxygen concentration (C_{O2}) [36]. The Reynolds number was kept constant in the scale-up performed from the shake flask to the 1.1 L bioreactor. In the scale-up from 1.1 L bioreactor to 5 L bioreactor, the impeller tip speed was kept constant. When the rheologic characteristics of the broth sample were applied in the mathematical equations (1-8), values of Reynolds number, impeller tip speed, shear rate, apparent viscosity, and rotation speed were determined (Table 6). Reynolds number was kept constant (785) from the shake flask to the 1.1 L bioreactor. Once calculated, the impeller tip speed remained constant (0.83 m s^{-1}) from the 1.1 L bioreactor to the 5 L bioreactor. The impeller tip speed value is lower than 3.2 m s^{-1}, which is known to be the maximum tip speed value to prevent microbial damage [36]. For the stirred-tank bioreactors, the impeller rotation speed was calculated to be 264, 144, and 243 rpm for 1.1, 3.4, and 5 L bioreactors, respectively.

Table 6. Scale-up of batch SSF of untreated PS (solid content of 22%) from 50 mL in shake flasks to 2.5 L in stirred-tank bioreactor.

Bioreactors	Shake Flask	BSTF-1.1L	BSTF-3.4L	BSTF-5L
Hydrodynamic parameters [1]				
Re	785	785	1032	735
ϑ_{tip} (m s^{-1})	n.a	0.83	0.83	0.83
γ (s^{-1})	84.6	48.4	26.4	46.7
η (Pa.s)	0.017	0.022	0.028	0.022
N (rpm)	150 (orbital)	264	144	243
SSF efficiency parameters				
$[Ethanol]_{max}$, g L^{-1}	59.1	48.5	59.9	54.5
P_{EtOH}, g L^{-1} h^{-1}	1.97	1.01	1.25	1.82
$Y_{EtOH/sug}$, g_{EtOH} $g_{sug}{}^{-1}$	0.38	0.31	0.38	0.35
$Y'_{EtOH/sug}$, %	73.8	60.6	74.9	68.0
$Y_{EtOH/PS}$, g_{EtOH} $g_{PS}{}^{-1}$	0.27	0.22	0.27	0.25

[1] Reference: viscous flow behavior of PS at 22% solid content and after 17 h of SSF.

Table 6 also shows the parameters obtained for the SSF of primary sludge at a solid content of 22% (at 38 °C) in the different stirred-tank reactors and compares with the ones obtained in the shake flasks.

According to Table 6, bioethanol production parameters differed significantly from the shake flask to the 1.1 L stirred-tank bioreactor. A decrease in ethanol concentration, yield, and productivity was registered: 48.5 g L^{-1}, 60.6%, and 1.01 g L^{-1} h^{-1}, respectively. Many factors may have contributed to this unfavorable scale-up study: the use of a single sample reference (viscous flow behavior of PS at 22% solid content, after 17 h of SSF) for the calculation of the hydrodynamic parameters; the complex and heterogeneous composition of primary sludge; the completely different geometry and configuration of bioreactors in this size process change.

In the scale-up from 1.1 L bioreactor to 3.4 L bioreactor, an improvement in the ethanol production parameters was attained. An ethanol concentration of 59.9 g L^{-1} was produced with a conversion yield of 74.9% and a productivity of 1.25 g L^{-1} h^{-1} in the 3.4 L bioreactor.

In the 5 L bioreactor, carbohydrates from primary sludge were converted to 54.5 g L^{-1} of ethanol with a yield of 68.0% and a productivity of 1.82 g L^{-1} h^{-1}. In this case, ethanol concentration and yield slightly decreased, but productivity was higher compared to the

production conditions reached with a 3.4 L bioreactor. Nevertheless, the comparison must be performed carefully since the bioreactors are not geometrically similar.

Figure 5 shows the different profiles obtained in the SSF carried out in the different reaction vessels. The first samples were withdrawn at 24 h of SSF, and Figure 5a shows that ethanol concentration remained practically constant after 48 h of reaction. A range of 10–20 g L^{-1} of monosaccharides was not metabolized by *S. cerevisiae* ATCC 26602, as observed in Figure 5b, corresponding mainly to xylose.

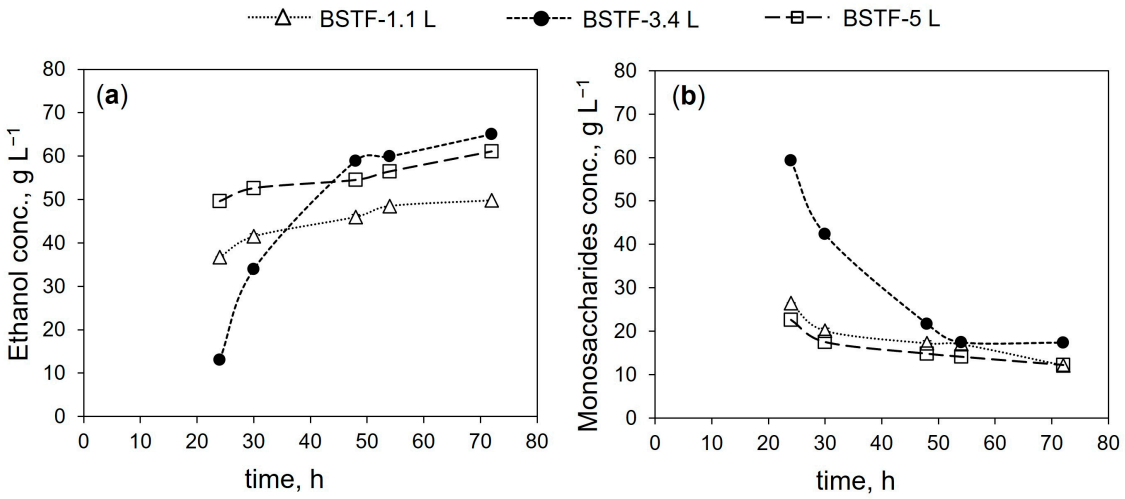

Figure 5. Batch SSF of untreated PS (22% solid concentration) in stirred-tank fermenters: (**a**) ethanol concentration; (**b**) monosaccharides concentration.

Stirred tank bioreactors with conventional impellers may not be the most suitable choice for high-solids loadings due to initial inefficient mixing, even at high stirring speeds. At the beginning of each experiment, unmixed areas were created inside the vessels, particularly at the bottom and top of the reactor. Often, cavern formation was observed: the mixture near the impeller had motion; contrarily, the suspension further from the impeller would move with negligible velocities. These problems can be attenuated by increasing the stirring speed, which will also increase energy requirements. Alternative bioreactors configuration with horizontal geometry and the use of anchor and helical impellers and multiple impellers in vertical bioreactors provide more desirable operating conditions compared to conventional bioreactors [37,38]. Additionally, experimental monitoring of the rheological behavior during the time-course of the bioprocess should be addressed to better describe the viscosity reduction and rheology changes of the PS slurries [20,37].

The rheological characteristics of PS slurries were obtained using samples withdrawn from the SSF of untreated PS with 22.0% solid content at 17, 41, and 72 h. Shear stress (τ, Pa) and apparent viscosity (η_{app}, Pa.s) were obtained at a specific range of shear rates (γ, s^{-1}), as shown in Figure 6. The viscosity of the mixture varied with reaction time: at the beginning, the broth was practically a heterogeneous solid mixture; during SSF, the mixture liquefied, and the viscosity decreased with time, particularly from the beginning until 41 h of reaction, remaining nearly constant until 72 h (Figure 6b). The mixture rheology changes mostly due to the depolymerization of cellulose and hemicelluloses into monomeric units during the enzymatic hydrolysis process [37]. Accurate measurement of the rheology parameters in high solids bioprocessing can be difficult since it depends on several factors, such as the concentration of the biomass, morphology, particle interaction, biomass nature, and pretreatment, and the gas bubbles produced during fermentation (that can disturb the measurements) [20,37,38]. Lignocellulosic biomass slurries exhibit non-Newtonian characteristics, with a non-linear relationship between the shear stress and shear rate. These slurries are shear-thinning, corresponding to a decrease in apparent viscosity with a shear rate increase (Figure 6b).

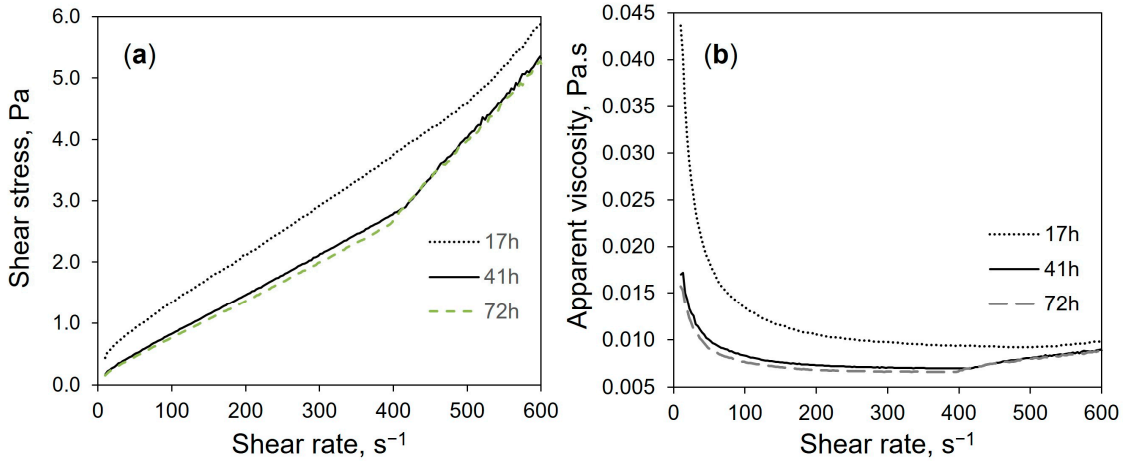

Figure 6. (**a**) Shear stress (τ, Pa) and (**b**) apparent viscosity (η_{app}, Pa.s) obtained at a specific range of shear rates (γ, s^{-1}) for samples withdrawn during the SSF of untreated PS at 17, 41 and 72 h.

One of the highest ethanol concentrations reported in the literature (91.5 g L^{-1}, Table 7) was achieved in a designed 10 L reaction vessel equipped with a high torque stirring capability [21]. Other studies using bioreactors geometrically similar to the one used in the present work reported lower ethanol concentrations [8]. Despite the initial problems with the inefficient mixture, high ethanol concentrations were achieved in the current work (batch SSF of untreated PS) compared to other findings regarding the conversion of paper wastes [8,13,15,16,21,22,39,40]. Further research will consist of accurate measurements of the rheological properties of PS slurries and the use of a tailored anchor impeller.

Table 7. Ethanol production processes from paper wastes with different operating conditions.

Substrate	Bioprocess Conditions	Main Results	Refs.
Primary sludge (untreated)	Batch SSF; 38 °C with 22–28% (*w*/*w*) solids; cellulase NS 22,192 at 5–15 FPU g^{-1}; *S. cerevisiae* ATCC 26602	54.5–74 g L^{-1} ethanol; 68–84% yield; 1.00–1.97 g L^{-1} h^{-1} productivity	This work
Primary paper sludge (pretreated by sequential steam explosion and NaOH)	Fed-batch S-SSCF; 48 h pre-hydrolysis; up to 18% (*w*/*w*) solids; crude cellulase at 158 FPU g^{-1}; *P. stipitis* NCIM 3499 and *S. cerevisiae*	42.3 g L^{-1} ethanol; 0.53 g g^{-1} yield; 0.71 g L^{-1} h^{-1} productivity	[13]
Corrugated recycle mill paper sludge	Fed-batch SSF; 37 °C; 27% (*w*/*w*) solids; cellulase Optiflow RC 2.0 at 11 FPU g^{-1}; *S. cerevisiae* MH1000	45.5 g L^{-1} ethanol; 78.2% yield; 0.45 g L^{-1} h^{-1} productivity	[8]
Shredded copier paper	Fed-batch SSSF; up to 65% (*w*/*v*) solids; final effective Accelerase 1500 dosage at 3.7 FPU g^{-1}; *S. cerevisiae* NCYC 2826	91.5 g L^{-1} ethanol; 54% yield; 0.22 g L^{-1} h^{-1} productivity	[21]
Pulp and paper sludge	Batch SSF; 40 °C; 6% (*w*/*w*) solids; cellulase at 40 U g^{-1}; *S. cerevisiae* CICC1001	42.5 g L^{-1} ethanol	[39]
Paper sludge	Fed-batch SSF; 16% (*w*/*v*) solids; non-commercial cellulase at 15 FPU g^{-1}; 20% inoculum *S. cerevisiae* TJ14	40 g L^{-1} ethanol; 64% yield; 0.52 g L^{-1} h^{-1} productivity	[22]
Paper sludge (de-ashed)	Fed-batch SSF; 23.1% (*w*/*v*) solids; cellulase Spezyme CP at 10 FPU g^{-1} + β-glucosidase Novozyme 188 at 20 CBU g^{-1}	60 g L^{-1} ethanol; 70% yield; 0.50 g L^{-1} h^{-1} productivity	[16]
Paper sludge (pretreated by sequential ball milling and phosphoric acid)	Batch SSF; 40 °C; 20% (*w*/*v*) solids; cellulase Meicelase at 20 FPU g^{-1}; *S. cerevisiae* AM12	30.5 g L^{-1} ethanol; 82% yield; 1.27 g L^{-1} h^{-1} productivity	[40]
Paper sludge	SSCF; 37 °C; 17% (*w*/*v*) solids; cellulase Spezyme CP at 10 FPU g^{-1} + β-glucosidase Novozyme 188 at 60 IU g^{-1}; *S. cerevisiae* RWB222	45 g L^{-1} ethanol; 76% yield; 0.33 g L^{-1} h^{-1} productivity	[15]

4. Conclusions

In shake flasks, increasing the solid content of primary sludge (PS) to 22% increased ethanol concentration, yield, and productivity; however, at the maximum solid content tested (28.0%), ethanol yield and productivity decreased despite the ethanol concentration increase. At the highest solid concentration (28%), a decrease of 33% in the cellulolytic enzyme complex (NS 22192) dosage was still feasible. A positive effect on PS conversion efficiency was observed when cellulolytic enzyme NS 22,192 and xylanolytic Cellic® HTec2 were co-applied in the process.

The scale-up of batch SSF of highly viscous PS slurries was challenging since there were several parameters influencing transport phenomena and fluid dynamics. The rheological nature and behavior of lignocellulosic suspensions (such as primary sludge), particularly at high solid loadings, is complex. The mixture rheology changes with reaction time; therefore, experimental monitoring of the rheological behavior during the time-course of the bioprocess should be further addressed to enhance the scale-up of ethanol production through SSF of primary sludge at high solids loading in batch conditions.

Nevertheless, the experiments carried out in this work represent a good start-up for further scale-up studies and process improvement in the SSF of PS for ethanol production from primary sludge of pulp and paper industries at batch conditions.

Author Contributions: Methodology and investigation, C.V.T.M.; writing—original draft preparation, C.V.T.M.; writing—review and editing, C.V.T.M., J.M.S.R. and M.G.V.S.C. All authors have read and agreed to the published version of the manuscript.

Funding: C.V.T.M. is grateful for the PhD grant (SFRH/BD/139908/2018), co-financed by the FCT and the European Social Fund (ESF) through the Regional Center Operational Programme (RCOP).

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Not applicable.

Acknowledgments: The authors are grateful to Ângelo Feliciano, who also performed the experimental work presented in this study. This work was partly developed within the scope of the CIEPQPF project (UIDB/00102/2020 and UIDP/00102/2020), financed by the FCT. The Portuguese Pulp and Paper Industries are acknowledged for providing primary sludge samples.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Hassan, S.S.; Williams, G.A.; Jaiswal, A.K. Moving towards the second generation of lignocellulosic biorefineries in the EU: Drivers, challenges, and opportunities. *Renew. Sustain. Energy Rev.* **2019**, *101*, 590–599. [CrossRef]
2. Liguori, R.; Amore, A.; Faraco, V. Waste valorization by biotechnological conversion into added value products. *Appl. Microbiol. Biotechnol.* **2013**, *97*, 6129–6147. [CrossRef] [PubMed]
3. Moreau, A.; Montplaisir, D.; Sparling, R.; Barnabé, S. Hydrogen, ethanol and cellulase production from pulp and paper primary sludge by fermentation with *Clostridium thermocellum*. *Biomass Bioenergy* **2015**, *72*, 256–262. [CrossRef]
4. Saha, N.; Saba, A.; Saha, P.; McGaughy, K.; Franqui-Villanueva, D.; Orts, W.J.; Hart-Cooper, W.M.; Reza, M.T. Hydrothermal Carbonization of Various Paper Mill Sludges: An Observation of Solid Fuel Properties. *Energies* **2019**, *12*, 858. [CrossRef]
5. Wang, L.; Wang, J.G.; Littlewood, J.; Cheng, H.B. Co-production of biorefinery products from kraft paper sludge and agricultural residues: Opportunities and challenges. *Green Chem.* **2014**, *16*, 1527–1533. [CrossRef]
6. Boni, M.R.; D'Aprile, L.; De Casa, G. Environmental quality of primary paper sludge. *J. Hazard. Mater.* **2004**, *B108*, 125–128. [CrossRef]
7. Azevedo, A.R.G.; Alexandre, J.; Pessanha, L.S.P.; Manhães, R.S.T.; Brito, J.; Marvila, M.T. Characterizing the paper industry sludge for environmentally-safe disposal. *Waste Manag.* **2019**, *95*, 43–52. [CrossRef]
8. Boshoff, S.; Gottumukkala, L.D.; van Rensburg, E.; Görgens, J. Paper sludge (PS) to bioethanol: Evaluation of virgin and recycle mill sludge for low enzyme, high-solids fermentation. *Bioresour. Technol.* **2016**, *203*, 103–111. [CrossRef]
9. Gottumukkala, L.D.; Haigh, K.; Collard, F.; van Rensburg, E.; Görgens, J. Opportunities and prospects of biorefinery-based valorisation of pulp and paper sludge. *Bioresour. Technol.* **2016**, *215*, 37–49. [CrossRef]
10. Gurram, R.N.; Al-Shannag, M.; Lecher, N.J.; Duncan, S.M.; Singsaas, E.L.; Alkasrawi, M. Bioconversion of paper mill sludge to bioethanol in the presence of accelerants or hydrogen peroxide pretreatment. *Bioresour. Technol.* **2015**, *193*, 529–539. [CrossRef]

11. Mendes, C.V.T.; Rocha, J.M.S.; Carvalho, M.G.V.S. Valorization of Residual Streams from Pulp and Paper Mills: Pretreatment and Bioconversion of Primary Sludge to Bioethanol. *Ind. Eng. Chem. Res.* **2014**, *53*, 19398–19404. [CrossRef]
12. Chen, H.; Venditti, R.; Gonzalez, R.; Phillips, R.; Jameel, H.; Park, S. Economic evaluation of the conversion of industrial paper sludge to ethanol. *Energy Econ.* **2014**, *44*, 281–290. [CrossRef]
13. Dey, P.; Rangarajan, V.; Nayak, J.; Das, D.B.; Wood, S.B. An improved enzymatic pre-hydrolysis strategy for efficient bioconversion of industrial pulp and paper sludge waste to bioethanol using a semi-simultaneous saccharification and fermentation process. *Fuel* **2021**, *294*, 120581–120593. [CrossRef]
14. Kang, L.; Wang, W.; Lee, Y.Y. Bioconversion of Kraft Paper Mill Sludges to Ethanol by SSF and SSCF. *Appl. Biochem. Biotechnol.* **2010**, *161*, 53–66. [CrossRef]
15. Zhang, J.; Lynd, L.R. Ethanol Production from Paper Sludge by Simultaneous Saccharification and Co-Fermentation Using Recombinant Xylose-Fermenting Microorganisms. *Biotechnol. Bioeng.* **2010**, *107*, 235–244. [CrossRef]
16. Kang, L.; Wang, W.; Pallapolu, V.R.; Lee, Y.Y. Enhanced ethanol production from de-ashed paper sludge by simultaneous saccharification and fermentation and simultaneous saccharification and co-fermentation. *Bioresources* **2011**, *6*, 3791–3808. [CrossRef]
17. Chen, H.; Han, Q.; Daniel, K.; Venditti, R.; Jameel, H. Conversion of Industrial Paper Sludge to Ethanol: Fractionation of Sludge and Its Impact. *Appl. Biochem. Biotechnol.* **2014**, *174*, 2096–2113. [CrossRef]
18. Dwiarti, L.; Boonchird, C.; Harashima, S.; Park, E.Y. Simultaneous saccharification and fermentation of paper sludge without pretreatment using cellulase from *Acremonium cellulolyticus* and thermotolerant *Saccharomyces cerevisiae*. *Biomass Bioenergy* **2012**, *42*, 114–122. [CrossRef]
19. Mendes, C.V.T.; Rocha, J.M.S.; Carvalho, M.G.V.S.C. Batch and fed-batch simultaneous saccharification and fermentation of primary sludge from pulp and paper mills. *Environ. Technol.* **2017**, *38*, 1498–1506. [CrossRef]
20. Hou, W.; Kan, J.; Bao, J. Rheology evolution of high solids content and highly viscous lignocellulose system in biorefinery fermentations for production of biofuels and biochemicals. *Fuel* **2019**, *253*, 1565–1569. [CrossRef]
21. Elliston, A.; Collins, S.R.A.; Wilson, D.R.; Roberts, I.N.; Waldron, K.W. High concentrations of cellulosic ethanol achieved by fed batch semi simultaneous saccharification and fermentation of waste-paper. *Bioresour. Technol.* **2013**, *134*, 117–126. [CrossRef] [PubMed]
22. Prasetyo, J.; Naruse, K.; Kato, T.; Boonchird, C.; Harashima, S.; Park, E.Y. Bioconversion of paper sludge to biofuel by simultaneous saccharification and fermentation using a cellulase of paper sludge origin and thermotolerant Saccharomyces cerevisiae TJ14. *Biotechnol. Biofuels* **2011**, *4*, 35–48. [CrossRef] [PubMed]
23. Mendes, C.V.T.; Cruz, C.H.G.; Reis, D.F.N.; Carvalho, M.G.V.S.; Rocha, J.M.S. Integrated bioconversion of pulp and paper primary sludge to second generation bioethanol using *Saccharomyces cerevisiae* ATCC 26602. *Bioresor. Technol.* **2016**, *220*, 161–167. [CrossRef] [PubMed]
24. Ghose, T.K. Measurement of cellulase activities. *Pure Appl. Chem.* **1987**, *59*, 257–268. [CrossRef]
25. Ghose, T.K.; Bisaria, V.S. Measurement of hemicellulase activities, Part 1: Xylanases. *Pure Appl. Chem.* **1987**, *59*, 1739–1752. [CrossRef]
26. Bradford, M.M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **1976**, *72*, 248–254. [CrossRef]
27. Pino, M.S.; Rodríguez-Jasso, R.M.; Michelin, M.; Flores-Gallegos, A.C.; Morales-Rodriguez, R.; Teixeira, J.A.; Ruiz, H.A. Bioreactor design for enzymatic hydrolysis of biomass under the biorefinery concept. *Chem. Eng. J.* **2018**, *347*, 119–136. [CrossRef]
28. Imamoglu, E.; Sukan, F.V. Scale-up and kinetic modeling for bioethanol production. *Bioresour. Technol.* **2013**, *144*, 311–320. [CrossRef]
29. Giese, H.; Klöckner, W.; Peña, C.; Galindo, E.; Lotter, S.; Wetzel, K.; Meissner, L.; Peter, C.P.; Büchs, J. Effective shear rates in shake flasks. *Chem. Eng. Sci.* **2014**, *118*, 102–113. [CrossRef]
30. Wilkens, R.J.; Henry, C.; Gates, L.E. How to Scale-up Mixing Processes in Non-Newtonian Fluids. *Chem. Eng. Prog.* **2003**, *99*, 44–52.
31. Sluiter, J.B.; Ruiz, R.O.; Scarlata, C.J.; Sluiter, A.D.; Templeton, D.W. Compositional Analysis of Lignocellulosic Feedstocks. 1. Review and Description of Methods. *J. Agric. Food Chem.* **2010**, *58*, 9043–9053. [CrossRef] [PubMed]
32. TAPPI T 230 om-08. Viscosity of Pulp (Capillary Viscometer Method). Available online: https://webstore.ansi.org/standards/tappi/230om08 (accessed on 15 January 2023).
33. Moreira, R.; Mendes, C.V.T.; Banaco, M.B.F.; Carvalho, M.G.V.S.; Portugal, A. New insights in the fractionation of *Pinus pinaster* wood: Sequential autohydrolysis, soda ethanol organosolv and acidic precipitation. *Ind. Crops Prod.* **2020**, *152*, 112499–112511. [CrossRef]
34. Chemical Analysis and Testing Laboratory. *Analytical Procedures (LAPs)*; National Renewable Energy Laboratory (NREL): Washington, DC, USA, 2008. Available online: https://www.nrel.gov/bioenergy/laboratory-analytical-procedures.html (accessed on 15 January 2023).
35. Neiva, D.M.; Gominho, J.; Pereira, H. Modeling and optimization of Eucalyptus globulus bark and wood delignification using response surface methodology. *BioRes* **2014**, *9*, 2907–2921. [CrossRef]
36. Deniz, I.; Imamoglu, E.; Sukan, F.V. Evaluation of scale-up parameters of bioethanol production from *Escherichia coli* KO11. *Turk. J. Biochem.* **2015**, *40*, 74–80. [CrossRef]

37. Barba, F.C.; Rodríguez-Jasso, R.M.; Sukumaran, R.K.; Ruiz, H.A.R. High-solids loading processing for an integrated lignocellulosic biorefinery: Effects of transport phenomena and rheology—A review. *Bioresour. Technol.* **2022**, *351*, 127044–127058. [CrossRef]
38. Afedzi, A.E.K.; Rattanaporn, K.; Parakulsuksatid, P. Impeller selection for mixing high-solids lignocellulosic biomass in stirred tank bioreactor for ethanol production. *Bioresour. Technol. Rep.* **2022**, *17*, 100935–100948. [CrossRef]
39. Lin, Y.; Wang, D.; Wang, T. Ethanol production from pulp & paper sludge and monosodium glutamate waste liquor by simultaneous saccharification and fermentation in batch condition. *Chem. Eng. J.* **2012**, *192*, 31–37. [CrossRef]
40. Yamashita, Y.; Sasaki, C.; Nakamura, Y. Development of efficient system for ethanol production from paper sludge pretreated by ball mill and phosphoric acid. *Carbohydr. Polym.* **2010**, *79*, 250–254. [CrossRef]