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# Insights on Monosaccharides and Bioethanol Production from Sweet Sorghum Stalks Using Dilute Acid Pretreatment

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**Abstract:** Sweet sorghum is a unique bioenergy crop that produces stalks with fermentable free sugars. The purpose of this study was to evaluate how the production of hemicellulosic saccharides and bioethanol from sweet sorghum stalks (SSS) can be influenced by a dilute sulfuric acid ($H_2SO_4$) pretreatment under different isothermal conditions. The bioethanol production from untreated SSS and pretreated solid phases was achieved through the Simultaneous Saccharification and Fermentation (SSF) process. A good SSS fractionation and an extensive hemicellulose hydrolysis into soluble saccharides were obtained, the most abundant hemicellulose-derived compounds present in the pretreated liquid phase being monosaccharides, with up to 17.22 g/L of glucose and 16.64 g/L of xylose in the pretreatments performed with 3% and 1% $H_2SO_4$ for 30 min at 134 °C, respectively. The SSF process of untreated SSS allowed a maximum bioethanol concentration of 9.78 g/L, corresponding to a maximum glucan conversion into ethanol of 49.8%. Bioethanol production from untreated SSS led to a higher bioethanol concentration and conversion than in the case of using acid pretreated solid phases obtained under the most severe conditions (with 3% $H_2SO_4$ for 30, 60 and 120 min at 134 °C), suggesting that, in the case of this biomass naturally rich in soluble sugars, the acidic pretreatment could negatively influence the fermentative process.

**Keywords:** sweet sorghum stalks; dilute acid pretreatment; monosaccharides; simultaneous saccharification and fermentation; bioethanol production

## 1. Introduction

The increasing demand for fossil fuels and the environmental concerns regarding global warming, greenhouse gas emissions and energy security have accelerated the uptake of biofuels from alternative feedstocks in the transport sectors. Biofuels such as bioethanol bring a number of important benefits: (i) they are obtained from renewable biomass resources: (ii) they are environmentally friendly; (iii) they can use a large part of the existing industrial infrastructure; and (iv) the carbon dioxide resulting from their combustion does not contribute to the intensification of the "greenhouse effect" [1,2].

In April 2009, the European Union adopted one of the most ambitious renewable energy policy to fight against climate change and reduce air pollution. The Renewable Energy Directive [3] established that at least 20% of the total energy needs of all European countries (including at least 10% of their

transport fuels) must be covered with renewables by 2020. In December 2018, a new binding target of at least 32% of energy from renewable sources by 2030 was set up by the revised Renewable Energy Directive [4].

Considering that, sweet sorghum is one of the most promising sources for bioethanol production that could limit food and feed crops-based biofuels from cereal and other starch-rich crops [5]. Compared to sugarcane or sugar beet, sweet sorghum requires less water, fertilizer and time, being tolerant to drought and flooding [6,7]. In addition, sorghum hybrids for silage production in semiarid conditions have a dry matter yield between 9.47 and 14.54 t/ha [8]. Sweet sorghum is a unique bioenergy crop that produces lignocellulosic stalks with high amounts of energy biomass and fermentable sugars [7,9] considered as a promising source for advanced biofuels production [10,11].

Dilute acid pretreatment is one of the most widespread processes used to enhance biomass digestibility, as a biomass hydrolysis stage [12,13]. The pretreatment step is able to solubilize hemicellulose, modify the lignin structure and reduce the crystallinity of cellulose as well as its degree of polymerization, leading to the recovery of hemicellulosic saccharides in the liquid phase while cellulose and lignin are retained in the solid phase [14]. Hemicellulose and cellulose are the polymeric carbohydrates of lignocellulosic material, which consist of pentose (xylose and arabinose) and hexose (glucose) sugars, respectively. Dilute acid pretreatment targets the hemicellulose fraction by releasing pentose sugars and breaks the crystalline structure of cellulose fibers [15]. Optimizing conditions for near-total hemicellulose solubilization comes with the risk of generating degradation products such as furaldehydes, furfural and 5-hydroxymethyl-2-furaldehyde (HMF), which are formed at severe acid pretreatments from hexoses and pentoses, respectively [16]. Monosaccharides represent one of the major units of non-structural carbohydrates with great importance as structural elements and energy sources [17]. Monosaccharides with functional value may facilitate the improvement of the human diet. Thus, hexoses (such as glucose) are manufactured at an industrial scale for food production [18]. Using dilute $H_2SO_4$ as a catalyst for acidic pretreatment at moderate temperatures (T <160 °C) and longer residence times, the hemicellulosic fraction is hydrolyzed into soluble sugars (mostly monomers) as the main reaction products [16].

Soluble sugars in SSS can be converted into bioethanol by fermentation technology using chopped stalks directly, thus avoiding the juice extraction step which has high energy consumption [19]. An economically profitable option for bioethanol production from SSS is to combine enzymatic hydrolysis and fermentation in a single process, the Simultaneous Saccharification and Fermentation (SSF) process, where enzymes and fermenting microorganisms are present in the same reaction vessel [20]. Cellulases represent an efficient enzymatic complex (including endo-glucanases, exo-glucanases and β-glucosidases) produced by filamentous fungi such as *Trichoderma* species, which are capable of hydrolyzing cellulose to fermentable sugars [21]. *Saccharomyces cerevisiae* is the most employed yeast for ethanol industry due to its ability to metabolize soluble sugars into bioethanol as the main fermentation product, having high tolerance to ethanol and other inhibitory compounds such as furfural and HMF [22].

This work aimed to investigate the effect of pretreatment with dilute sulfuric acid under various isothermal conditions on the production of hemicellulosic saccharides (mainly monosaccharides such as glucose and xylose) from Romanian sweet sorghum, as well as on the bioethanol production from untreated SSS and pretreated solid phases through SSF.

## 2. Materials and Methods

### 2.1. Raw Material

Sweet sorghum stalks (SSS) were collected from an experimental plantation located in Secuieni (Romania), air-dried and milled to a particle size of 10 mm using a Romer Analytical Sampling Mill (Romer Labs Division Holding GmbH, Getzersdorf, NÖ, Austria). To avoid compositional differences,

the raw material was homogenized in a single lot. The material was stored until use in a dark and dry place.

### 2.2. Dilute Acid Pretreatment of SSS

Milled SSS samples were mixed with $H_2SO_4$ at different concentrations (1%, 2% and 3% *v*/*v*) in 250 mL Erlenmeyer flasks, with a liquid to solid ratio (LSR) of 10 g dilute $H_2SO_4$/ g raw material. The reactions took place in a 63 L Astell autoclave model no. AMA440BT (Astell Scientific Ltd., Sidcup, LDN, UK), under different temperatures (121 °C (29 psi) and 134 °C (43.5 psi)) and reaction times (30, 60 and 120 min) according to Figure 1. The pretreatment conditions were optimized and adapted according to the method described by Vancov and McIntosh [23]. After dilute $H_2SO_4$ pretreatment, for further analysis, a separation by filtration of the solid and liquid phases was performed through filter paper using a Büchner Funnel. The solid phases recovered after dilute acid pretreatment were washed with distilled water until neutral pH and employed for solid yield determination.

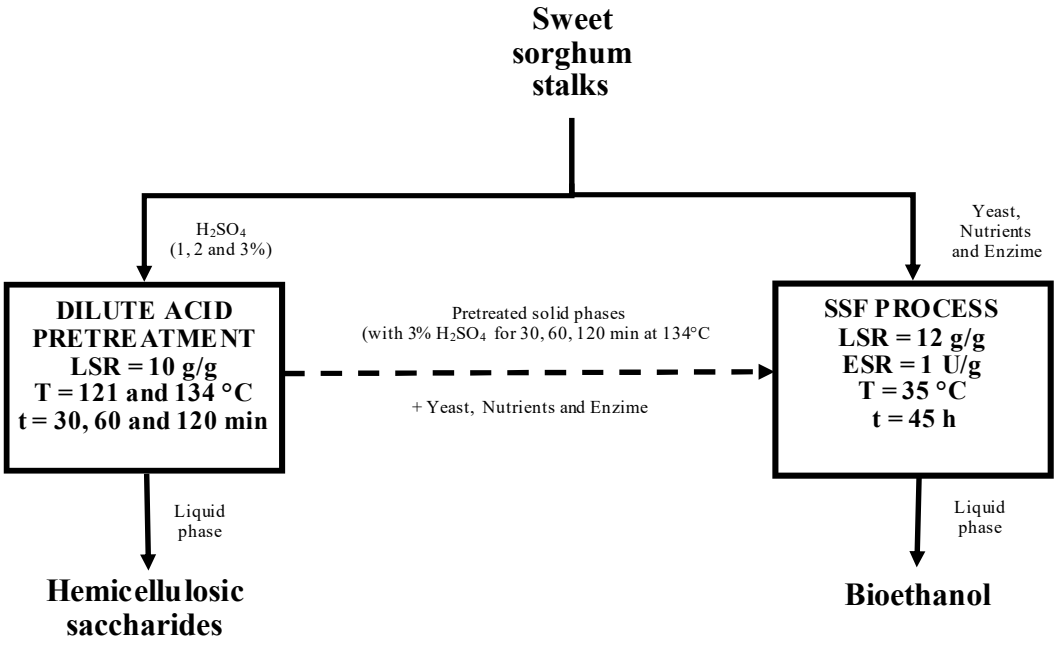


**Figure 1.** Scheme of the overall process considered in this work for the production of hemicellulosic saccharides from SSS and bioethanol from untreated SSS and selected pretreated solid phases (with 3% $H_2SO_4$ for 30, 60 and 120 min at 134 °C). SSF, Simultaneous Saccharification and Fermentation; LSR, liquid to solid ratio; ESR, enzyme to substrate ratio.

### 2.3. Yeast Cultivation and Inoculum Preparation

The microorganism selected for bioethanol production was Brewferm® Lager (Brouwland, Beverlo, Belgium), a sturdy lager brewing yeast. For inoculum preparation, cells were dissolved in physiological saline solution (0.9% *w*/*v* of sodium chloride NaCl) at a liquid to yeast ratio (LYR) of 10 g solution/g dry yeast and grown in shaken flasks (150 rpm) initially for 30 min at 22 ± 3 °C and then for 40 min at 20 °C. The temperature used to prepare the yeast inoculum (20–22 °C) was according to the temperature recommended by the manufacturer (21–26 °C) to make a fresh and ready-to-use yeast starter for fermentation (or for further multiplication). The number of viable cells in the yeast inoculum was determined by counting of colony forming units (CFU). Dry cell weight method was used for measuring the biomass concentration of the media.

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

SSF experiments on milled untreated SSS or selected solid phases of pretreated SSS were performed in 300 mL Erlenmeyer flasks containing 100 mL of medium at pH 4.8 (adjusted using 0.05 M citrate buffer), working with a liquid to solid ratio (LSR) of 12 g/g. The suspensions containing the desired amount of water and substrate were autoclaved at 121 °C for 15 min (separately from the nutrients). High temperature and short time sterilization of the substrates (at 121 °C for 15 min) was performed prior to their further utilization in SSF assays to eliminate all harmful microorganisms, thus preparing the substrates for yeast inoculation under sterile conditions [24]. Sterilized suspensions were supplemented with nutrients (5 mL, containing 5 g peptone/L, 3 g yeast extract/L and 3 g malt extract/L), yeast inoculum (10 mL) and enzyme, and then incubated without agitation in an Memmert incubator model INC108med (Memmert GmbH + Co. KG, Schwabach, Germany). A commercial enzyme, cellulase from *Trichoderma viride*, EC 3.2.1.4, obtained from Merck (Darmstadt, Germany), was used at a minimum enzyme loading (enzyme to substrate ratio (ESR) of 1 U/g oven-dry substrate). The specific activity of the cellulase lyophilized powder, as mentioned by the manufacturer, was 1.5 U/mg. One unit (U) will liberate 1.0 μmole of glucose from cellulose in 1 h at pH 4.5 and 37 °C (2 h of incubation time) on carboxymethyl cellulose (CMC) as substrate. To be used in the SSF process, the enzyme powder was dissolved at 1 mg/mL 0.05 M citrate buffer, pH 4.8. To achieve an appropriate degree of fermentation in the shortest possible time, a compromise between the optimum temperature for the enzyme and the yeast was needed since the yeast *Saccharomyces cerevisiae* acts at the optimum temperature around 30 °C and the cellulase enzyme around 48.5 °C. The fermentation temperature used in the SSF process was thus set at 35 °C. The kinetics of the SSF process was monitored by chromatographic determination of the ethanol concentration. The fermentation process was stopped after 45 h, when the ethanol concentration was already decreasing.

### 2.5. Analytical Methods

A high-performance liquid chromatography (HPLC) system with different modules was used to analyze the content of monosaccharides (glucose, xylose and arabinose), acetic acid and ethanol. The HPLC system consisted of Transgenomic$^{TM}$ ICSep COREGEL 87H3 column (Transgenomic, Inc., San Jose, CA, USA), Jasco PU-980 intelligent pump (Jasco Co. Ltd., Hachioji-shi, TYO, Japan) and ERC-7515A refractive index detector (ERC Inc., a part of IDEX Health & Science KK, Kawaguchi, Saitama, Japan). The chromatographic conditions for the separation of compounds were as follows: mobile phase of 0.008 N $H_2SO_4$, flow rate of 0.6 mL/min, column temperature of 35 °C and sample volume of 20 μL. The output signal was recorded and integrated using a Jasco Borwin Chromatography Software version 1.21.60 (Jasco Co. Ltd., Hachioji-shi, TYO, Japan).

Raw material was analyzed for moisture content (TAPPI T 264 cm-07), ash (TAPPI T 211 om-12), extractives (TAPPI T 264 cm-07) and subjected to two-step quantitative acid hydrolysis (QAH) (TAPPI T 249 cm-09), a first step with 72% *w/w* $H_2SO_4$ (for the conversion of polysaccharides into oligosaccharides) and a second step with 4% *w/w* $H_2SO_4$ (for the conversion of oligomers into monomers), and autoclaved at 121 °C for 60 min. The liquid phase resulting from QAH was filtered through 0.45 μm membranes and analyzed for glucose and xylose by HPLC. The obtained results measured the raw material content in glucan and xylan. The solid residue resulting from QAH was quantified and considered Klason lignin.

Samples of liquid phase recovered after dilute acid pretreatment were filtered through 0.45 μm membranes and used for direct HPLC determination of monosaccharides (glucose, xylose and arabinose) and acetic acid. The liquid phase was also analyzed for non-volatile compounds (NVC) content (by drying at 105 °C until constant weight). Other aliquots were subjected to quantitative posthydrolysis with 4% *w/w* $H_2SO_4$ and autoclaved at 121 °C for 40 min (to convert oligomers into monomers). The resulting samples from posthydrolysis step were filtered through 0.45 μm membranes and analyzed for monomers (glucose and arabinose) and acetic acid content by HPLC. The increases in monosaccharides and acetic acid concentrations respect to the pretreated liquor measured the content

of oligosaccharides and acetyl groups linked to oligomers. Samples of solids were air-dried, subjected to QAH and analyzed for glucan, xylan and Klason lignin.

Samples from SSF process (0.5 mL) were withdrawn from the media at preset reaction times (0, 3, 20, 25 and 45 h), centrifuged at 5000 rpm for 5 min, filtered through 0.20 μm membranes and analyzed for glucose, xylose and ethanol by HPLC.

Experiments were performed in triplicate, and the composition of solid and liquid phases after dilute acid pretreatment, as well as the ethanol concentration and conversion in the SSF process, was presented as average values with standard deviations below 1%.

## 3. Results and Discussion

### *3.1. SSS Fractionation and Saccharides Production*

Tables 1 and 2 presents data concerning SSS composition (expressed in g/100 g raw material, oven-dry basis), the operational conditions employed in SSS pretreatment at temperature of 121 and 134 °C, respectively, total material balance (solid yield and non-volatile compounds) and solid and liquid phase composition.

Depending on temperature and reaction time used in the experiments, the increase in $H_2SO_4$ concentration caused a decrease in the solid yield, from 53.49 g solid phase/100 g oven dry raw material (odrm) (with 1% $H_2SO_4$ for 30 min at 121 °C) to 41.85 g solid phase/100 g odrm (with 3% $H_2SO_4$ for 120 min at 134 °C). The NVC content in liquid phase increased with the increasing of $H_2SO_4$ concentration for each temperature tested up to 57.26 g of NVC/100 g odrm (with 3% $H_2SO_4$ for 120 min at 121 °C) and up to 57.05 g of NVC/100 g odrm (with 3% $H_2SO_4$ for 60 min at 134 °C). The glucan content in the hydrolyzed solid phase increased with $H_2SO_4$ concentration for each temperature and reaction time tested, from 55.92 g/100 g PSS, oven-dry basis (in the experiment carried out with 1% $H_2SO_4$ for 30 min at 121 °C) to a maximum content of 67.23 g/100 g PSS, oven-dry basis (in the experiment carried out with 3% $H_2SO_4$ for 30 min at 134 °C). Klason lignin followed the same variation pattern as glucan, reaching values in the range of 24.21–38.80 g/100 g PSS, oven-dry basis, for pretreatments at the lowest and highest isothermal conditions, respectively. The hemicellulosic content of pretreated solids (in the form of xylan) decreased with the increasing of the pretreatment conditions. Under the severest operational conditions assayed (with 3% $H_2SO_4$ for 30, 60 and 120 min at 134 °C), the pretreated solids showed minimum contents of xylan (1.88, 0.96 and 0.5 g/100 g PSS, oven-dry basis, respectively), while high amounts of glucan and lignin were retained in the solid phase, confirming the extensive solubilization of hemicellulosic fraction.

**Table 1.** Operational conditions employed in SSS dilute acid pretreatment at temperature of 121 °C and experimental results concerning material balances and composition of solid and liquid phase.

| **$H_2SO_4$ conc. (%)** | **Untreated** | **1** | **2** | **3** | **1** | **2** | **3** | **1** | **2** | **3** |
|---|---|---|---|---|---|---|---|---|---|---|
| Reaction Time (min.) | SSS | 30 | 30 | 30 | 60 | 60 | 60 | 120 | 120 | 120 |
| Material balance data (g PSS/100 g raw material, oven-dry basis) | | | | | | | | | | |
| Solid recovered | - | 53.49 | 49.04 | 46.31 | 51.69 | 46.20 | 45.82 | 50.73 | 47.99 | 42.21 |
| Non-volatile compounds | - | 38.11 | 44.29 | 53.57 | 36.05 | 44.51 | 53.22 | 41.39 | 46.64 | 57.26 |
| Solid phase composition (g/100 g PSS, oven-dry basis) | | | | | | | | | | |
| Glucan | 44.07 ± 0.56 | 55.92 ± 0.48 | 58.79 ± 0.62 | 62.92 ± 0.35 | 58.25 ± 0.45 | 60.80 ± 0.32 | 64.42 ± 0.32 | 61.06 ± 0.68 | 64.81 ± 0.12 | 66.00 ± 0.50 |
| Xylan | 14.31 ± 0.15 | 14.94 ± 0.11 | 8.39 ± 0.12 | 6.64 ± 0.13 | 12.41 ± 0.02 | 7.10 ± 0.08 | 5.05 ± 0.12 | 9.35 ± 0.12 | 5.21 ± 0.16 | 3.38 ± 0.10 |
| Klason lignin | 19.46 ± 0.19 | 24.21 ± 0.23 | 27.99 ± 0.13 | 30.77 ± 0.15 | 28.18 ± 0.35 | 32.34 ± 0.12 | 33.27 ± 0.12 | 29.45 ± 0.21 | 32.58 ± 0.25 | 34.39 ± 0.32 |
| Extractives | 14.67 ± 0.03 | | | | | | | | | |
| Ash | 4.00 ± 0.02 | | | | | | | | | |
| Proteins | 1.33 ± 0.02 | | | | | | | | | |
| Liquid phase composition (g/L or g monomer equivalent/L) | | | | | | | | | | |
| Glucose | - | 14.62 ± 0.06 | 13.84 ± 0.06 | 14.68 ± 0.12 | 13.97 ± 0.02 | 14.06 ± 0.09 | 13.89 ± 0.13 | 14.11 ± 0.14 | 13.81 ± 0.21 | 14.89 ± 0.05 |
| Xylose | - | 12.34 ± 0.05 | 15.81 ± 0.09 | 16.27 ± 0.14 | 13.31 ± 0.17 | 16.22 ± 0.05 | 15.27 ± 0.17 | 15.73 ± 0.09 | 15.39 ± 0.15 | 14.45 ± 0.02 |
| Arabinose | - | 2.26 ± 0.01 | 2.20 ± 0.05 | 2.50 ± 0.04 | 2.13 ± 0.04 | 2.27 ± 0.08 | 2.42 ± 0.07 | 2.07 ± 0.05 | 2.31 ± 0.06 | 2.58 ± 0.01 |
| Acetic acid | - | 1.45 ± 0.01 | 2.69 ± 0.08 | 3.02 ± 0.06 | 2.08 ± 0.02 | 3.13 ± 0.04 | 3.14 ± 0.08 | 2.73 ± 0.05 | 2.94 ± 0.02 | 3.61 ± 0.01 |
| Gluco-oligomers | - | 1.79 ± 0.02 | 1.84 ± 0.09 | 1.13 ± 0.03 | 2.33 ± 0.03 | 2.06 ± 0.04 | 1.27 ± 0.08 | 1.39 ± 0.05 | 1.16 ± 0.01 | 0.48 ± 0.01 |
| Arabino-oligomers | - | 0.28 ± 0.01 | 0.42 ± 0.08 | 0.16 ± 0.08 | 0.41 ± 0.07 | 0.44 ± 0.01 | 0.28 ± 0.05 | 0.37 ± 0.08 | 0.21 ± 0.06 | 0.02 ± 0.08 |
| Acetyl groups-oligomers | - | 0.16 ± 0.01 | 0.18 ± 0.01 | 0.02 ± 0.00 | 0.27 ± 0.02 | 0.05 ± 0.00 | 0.34 ± 0.02 | 0.02 ± 0.00 | 0.51 ± 0.01 | 0.00 ± 0.00 |

PSS, pretreated sorghum stalks. Values are expressed as average ± standard deviation of three replicates.

**Table 2.** Operational conditions employed in SSS dilute acid pretreatment at temperature of 134 °C and experimental results concerning material balances and composition of solid and liquid phase.

| **$H_2SO_4$ conc. (%)** | **1** | **2** | **3** | **1** | **2** | **3** | **1** | **2** | **3** |
|---|---|---|---|---|---|---|---|---|---|
| Reaction Time (min.) | 30 | 30 | 30 | 60 | 60 | 60 | 120 | 120 | 120 |
| Material balance data (g PSS/100 g raw material, oven-dry basis) | | | | | | | | | |
| Solid recovered | 46.26 | 45.63 | 42.40 | 45.59 | 42.40 | 42.16 | 44.54 | 42.53 | 41.85 |
| Non-volatile compounds | 46.05 | 47.15 | 54.66 | 45.27 | 49.08 | 57.05 | 45.84 | 47.43 | 55.63 |
| Solid phase composition (g/100 g PSS, oven-dry basis) | | | | | | | | | |
| Glucan | 60.56 ± 0.23 | 66.29 ± 0.56 | 67.23 ± 0.52 | 65.60 ± 0.41 | 66.75 ± 0.63 | 66.85 ± 0.29 | 66.21 ± 0.35 | 66.61 ± 0.31 | 66.69 ± 0.29 |
| Xylan | 7.50 ± 0.09 | 3.40 ± 0.12 | 1.88 ± 0.23 | 6.33 ± 0.14 | 2.16 ± 0.05 | 0.96 ± 0.01 | 3.78 ± 0.06 | 1.10 ± 0.01 | 0.50 ± 0.01 |
| Klason lignin | 30.54 ± 0.14 | 34.25 ± 0.03 | 36.01 ± 0.08 | 30.59 ± 0.18 | 35.08 ± 0.12 | 36.79 ± 0.05 | 32.69 ± 0.06 | 38.06 ± 0.06 | 38.80 ± 0.11 |
| Liquid phase composition (g/L or g monomer equivalent/L) | | | | | | | | | |
| Glucose | 15.97 ± 0.13 | 14.88 ± 0.08 | 17.22 ± 0.09 | 14.82 ± 0.18 | 15.73 ± 0.17 | 15.31 ± 0.07 | 16.24 ± 0.18 | 16.01 ± 0.04 | 15.27 ± 0.05 |
| Xylose | 16.64 ± 0.04 | 14.10 ± 0.19 | 12.44 ± 0.02 | 15.37 ± 0.14 | 14.24 ± 0.01 | 11.74 ± 0.09 | 15.23 ± 0.09 | 11.76 ± 0.04 | 9.97 ± 0.12 |
| Arabinose | 2.06 ± 0.02 | 2.29 ± 0.01 | 2.59 ± 0.08 | 1.99 ± 0.06 | 2.34 ± 0.04 | 2.47 ± 0.07 | 1.92 ± 0.09 | 2.24 ± 0.02 | 2.22 ± 0.02 |
| Acetic acid | 2.81 ± 0.05 | 3.28 ± 0.06 | 3.37 ± 0.07 | 2.90 ± 0.01 | 3.66 ± 0.06 | 3.44 ± 0.09 | 3.43 ± 0.09 | 2.70 ± 0.07 | 3.30 ± 0.08 |
| Gluco-oligomers | 2.10 ± 0.05 | 1.30 ± 0.05 | 0.00 ± 0.00 | 1.44 ± 0.04 | 1.05 ± 0.01 | 1.47 ± 0.01 | 1.06 ± 0.02 | 1.08 ± 0.01 | 0.85 ± 0.01 |
| Arabino-oligomers | 0.51 ± 0.01 | 0.27 ± 0.01 | 0.16 ± 0.01 | 0.35 ± 0.02 | 0.23 ± 0.01 | 0.21 ± 0.01 | 0.19 ± 0.01 | 0.10 ± 0.01 | 0.13 ± 0.01 |
| Acetyl groups-oligomers | 0.20 ± 0.01 | 0.16 ± 0.01 | 0.09 ± 0.01 | 0.13 ± 0.01 | 0.00 ± 0.00 | 0.39 ± 0.02 | 0.15 ± 0.01 | 0.75 ± 0.03 | 0.22 ± 0.02 |

PSS, pretreated sorghum stalks. Values are expressed as average ± standard deviation of three replicates.

The most abundant hemicellulose-derived compounds present in the liquid phase were monosaccharides, with important fractions of them corresponding to glucose and xylose. The maximum concentration of glucose (17.22 g/L) was reached in the experiment carried out with 3% $H_2SO_4$ for 30 min at 134 °C, whereas the maximum content of xylose (16.64 g/L) was obtained in the experiment performed with 1% $H_2SO_4$ for 30 min at 134 °C, with a clear dependence on pretreatment conditions. The obtained results suggest that high hemicellulose solubilization and degradation of the solubilized carbohydrates occurs at high pretreatment temperature (134 °C) combined with high $H_2SO_4$ concentration (3%), favoring the monosaccharides (glucose and xylose) production. The use of higher $H_2SO_4$ concentrations promotes the sugars hydrolysis from SSS but leads to the formation of degradation products such as furfural and HMF. The release of inhibitory products leads to the degradation of xylose into furfural, thus obtaining lower concentrations in prehydrolysates [25]; therefore, a lower $H_2SO_4$ concentration is required to obtain a higher xylose concentration. Choudhary et al. [26] evaluated the effect of $H_2SO_4$ on the pretreatment of sorghum stalks from *Sorghum bicolor* (YSS-10R variety) using different parameters (acid concentration, residence time and temperature) at a solid loading of 12% (*w*/*v*) and reported that maximum glucose yield (7.66 g/L) was obtained in the pretreatment with 0.5% $H_2SO_4$ for 10 min at 100 °C, whereas maximum xylose yield (7.62 g/L) was reached with 0.5% $H_2SO_4$ for 20 min at 130 °C. Vancov and McIntosh [23] used $H_2SO_4$ with different strengths to pretreat milled sorghum straw samples at a solid loading of 10% (*w*/*v*) and reported xylose yields of 150 mg/g (approximately 55% of hemicellulose solubilization) in the presence of 2% $H_2SO_4$ for 60 min at 121 °C. Deshavath et al. [15] reported that the dilute acid pretreatment of sorghum brown midrib IS11861 biomass in the presence of 0.2 M $H_2SO_4$ for 120 min at 121 °C significantly hydrolyzed the hemicellulose (with 97.6% conversion efficiency) mainly into xylose (225.2 mg/g). Deshavath et al. [27] also reported 89% of xylan conversion (which yields 150.2 mg of xylose) using dilute acid pretreatment of sorghum stalks with 0.2 M $H_2SO_4$ for 120 min at 121 °C. Another monomeric sugar in liquid phase was arabinose, with values up to 2.59 g/L with 3% $H_2SO_4$ for 30 min at 134 °C. Acetic acid generated by cleavage of acetyl groups linked to oligomers increased up to a concentration of 3.66 g/L with 2% $H_2SO_4$ for 60 min at 134 °C. Among oligomers, the gluco-oligosaccharides concentration reached a maximum of 2.33 g/L with 1% $H_2SO_4$ for 60 min at 121 °C. Other oligosaccharide-substituents with minor amounts in liquid phase were arabino-oligosaccharides (at concentrations below 0.5 g/L) and acetyl groups linked to oligomers (at concentrations below 0.8 g/L), with no defined dependence on pretreatment. Based on the material balance and chemical composition, the dilute acid pretreatments carried out under the severest conditions (with 3% $H_2SO_4$ for 30, 60 and 120 min at 134 °C) were considered favorable in terms of a biorefinery approach and selected for further SSF tests, because they represent a compromise between: (i) good fractionation of the feedstock; and (ii) extensive hemicellulose solubilization.

### *3.2. SSF of Untreated and Pretreated SSS*

To assess the production of ethanol, untreated SSS samples (Experiment SSF1 in Table 3) were used as substrate for SSF process in batch mode, operating at low solid loading. Substrate, nutrients, inoculum and enzyme were added in the same reaction vessel from the beginning of the process, respecting an overall LSR of 12 g/g and a minimum cellulase charge of 1 U/g. The SSF assays were performed at an inoculum level of $2.1 \times 10^7$ CFU/mL (leading to an initial yeast cell concentration of 13 g/L). For comparative purposes, similar experiments were performed using the solid phases pretreated under the selected operational conditions (Experiments SSF2–SSF4 in Table 3).

**Table 3.** Experimental results obtained in the SSF of untreated SSS and pretreated solid phases from selected pretreatments.

| Experiment | Maximum Ethanol Concentration (g/L) | Maximum Ethanol Conversion (%) |
|---|---|---|
| SSF1 | 9.78 ± 0.19 | 49.80 ± 0.31 |
| SSF2 | 1.23 ± 0.12 | 4.06 ± 0.08 |
| SSF3 | 1.39 ± 0.13 | 4.62 ± 0.06 |
| SSF4 | 1.24 ± 0.12 | 4.10 ± 0.05 |

SSF1, SSF of raw material; SSF2, SSF of solid phase from pretreatment with 3% $H_2SO_4$ for 30 min at 134 °C; SSF3, SSF of solid phase from pretreatment with 3% $H_2SO_4$ for 60 min at 134 °C; SSF4, SSF of solid phase from pretreatment with 3% $H_2SO_4$ for 120 min at 134 °C. Values are expressed as average ± standard deviation of three replicates.

Further insight on the substrate utilization was obtained by maximum ethanol conversion ($C_{EMAX}$) (g maximum ethanol concentration/100 g potential ethanol concentration), calculated using the following equation [28]:

$$C_{EMAX} = 100 \times \frac{E_{MAX}}{E_{POT}} \quad (1)$$

where $E_{MAX}$ is the maximum ethanol concentration achieved in the experiment (g/L) and $E_{POT}$ is the potential ethanol concentration calculated assuming a stoichiometric conversion of the glucan present in the substrate into ethanol (g/L).

Table 3 presents the experimental conditions employed in the SSF process and the values of the maximum ethanol concentration and ethanol conversion, whereas Figure 2 presents the time course of ethanol concentration in the SSF assays performed with selected SSS samples.

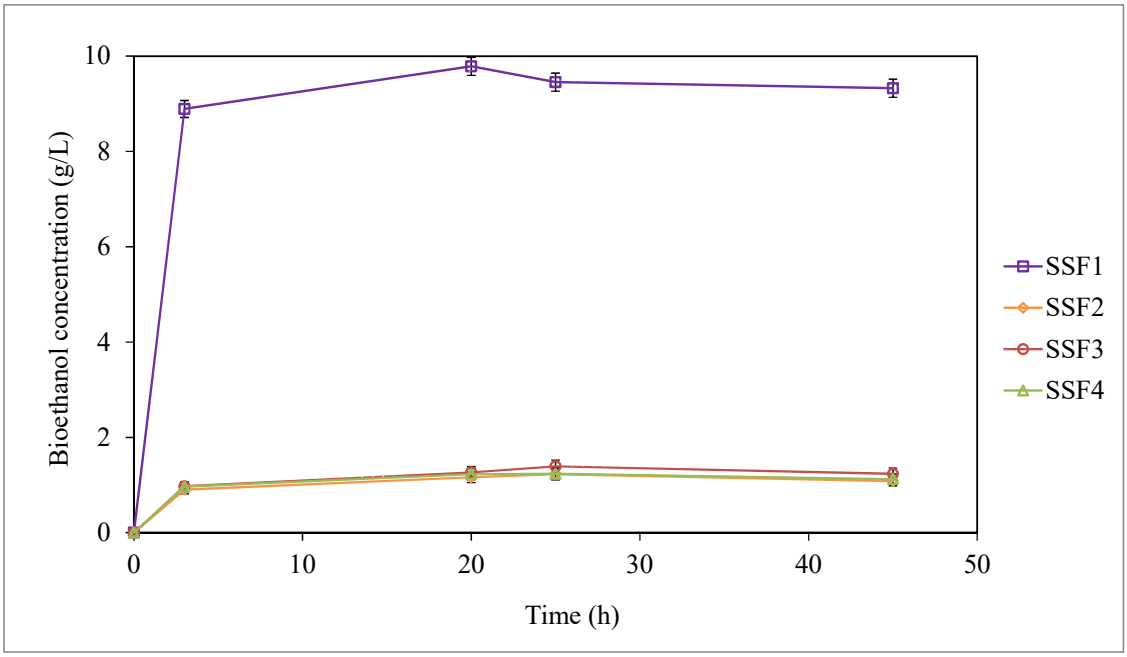


**Figure 2.** Time course of bioethanol concentration in the SSF experiments (carried out at LSR of 12 g/g and ESR of 1 U/g, with 5 mL of nutrients and 10 mL of yeast inoculum) using SSS samples and solid phases from selected pretreatments (see Table 3 for nomenclature).

The experimental data show that the untreated raw material behaved as satisfactory substrate for ethanol production, leading to a maximum ethanol concentration after only 20 h. The SSF assay performed with the untreated SSS (Experiment SSF1 carried out at LSR of 12 g/g, ESR of 1 U/g, nutrients of 5 mL and yeast inoculum of 10 mL) led to $E_{MAX}$ of 9.78 g ethanol/L (corresponding to $C_{EMAX}$ of 49.80%). When operating the SSF process at low solid loading (LSR of 12 g/g), a fast increase in $E_{MAX}$

along the first 3 h (up to 8.89 g/L) with raw material as substrate was observed, while incubation times longer than 20 h resulted in moderate $E_{MAX}$. (9.45 g/L after 25 h and 9.33 g/L after 45 h). In comparison, the SSF of the solid samples obtained after selected dilute acid pretreatments (Experiments SSF2 to SSF4 carried out at the same operational conditions as Experiment SSF1) showed slow kinetics and poor substrate conversions after 25 h of incubation, leading to $E_{MAX}$ up to 1.39 g ethanol/L (corresponding to $C_{EMAX}$ up to 4.62%, in Experiment SSF3). Bioethanol efficiency with respect to the raw material (expressed in g ethanol/100 g raw material) for SSF processes was as follows: 21.95, 1.79, 2.04 and 1.81 for SSF1, SSF2, SSF3 and SSF4, respectively. After SSF process of the selected substrates, it was found that only a small concentration of residual sugars (especially glucose and xylose) remained, even if we worked in stationary condition, which indicates that the lack of agitation did not decisively influence the transformation of sugars into bioethanol. The maximum concentrations of residual sugars after SSF were as follows: 0.30, 0.03, 0.01 and 0.01 g glucose/L and 0.00, 0.06, 0.09 and 0.10 g xylose/L in SSF1, SSF2, SSF3 and SSF4, respectively.

Nozari et al. [29] investigated the bioethanol production from untreated SSS by SSF under anaerobic conditions, at 37 °C and 120 rpm for 72 h, using a flocculent strain of *Saccharomyces cerevisiae* (CCUG 53310) and enzymes mixture. Their results show an ethanol production of 31.10% of the theoretical yield, corresponding to an ethanol concentration of 3.10 g/L.

The lower bioethanol concentrations and conversion yields obtained in the SSF assays with selected pretreated solid phases, compared with those obtained with untreated SSS, could have multiple causes. On the one hand, it may be due to a lower free sugars content in the pretreated solid phases, due to the release of free soluble sugars in the liquid phase after pretreatment and also to a too severe acidic pretreatment conditions (with 3% $H_2SO_4$ for 30, 60 and 120 min at 134 °C), which could have caused the degradation of free sugars from the SSS structure to various by-products (acetic acid, furfural, HMF, etc.). On the other hand, the low enzyme loading (ESR of 1 U/g) used and the fact that the enzyme used in the present study was an endo-glucanase, and no beta-glucosidase or exo-cellulases were added compared to other studies [30], could suggest the lack of a proper cellulolytic activity. The absence of full cellulase activities might have limited the saccharification process and only a small amount of glucose was released from the polysaccharidic matrix. In addition, it should be also noted that SSF was done at a lower temperature than optimal for the used enzyme, thus influencing hydrolysis yields, and implicitly the low ethanolic conversion yields.

From this level, the addition of enzymes in the SSF process can be increased, improving the bioethanol concentrations obtained. The enzyme charge is clearly important for the process economy, but the economic sensitivity towards the enzyme loading in SSF is difficult to predict due to the cost of enzymes [30].

## 4. Conclusions

The obtained results show that the Romanian sweet sorghum can be used as an important source of monosaccharides and as biomass for bioethanol production. Important concentrations of monomers such as glucose (17.22 g/L) and xylose (16.64 g/L) were obtained from SSS using dilute acid pretreatment under various isothermal conditions. Using a sturdy lager yeast that delivers a consistent neutral fermentation, a maximum ethanol concentration was recorded at a short fermentation time (after only 20 h). Higher ethanol concentration (9.78 g/L) was obtained from raw SSS as compared to pretreated solid phases (up to 1.39 g/L), probably due to the release of the soluble sugars in the liquid phase after dilute acidic pretreatment, a too harsh acidic pretreatment, a too lower enzyme dose used and the absence of full cellulase activity. Since the results suggest that the acidic pretreatment could have negative impact on overall yield of ethanol production from SSS, other pretreatments could be used in the case of this biomass, as suggested by Ostovareh et al. [24]. Our future research on the SSF process with sweet sorghum stalks intends also to use higher enzyme concentrations and a mix of enzymes, combined with different pretreatments, in order to obtain higher bioethanol concentrations.

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