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Bioethanol Production from Steam-Exploded Barley Straw by Co-Fermentation with *Escherichia coli* SL100

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Abstract: Second-generation bioethanol is considered a suitable option for replacing fossil fuels. Agricultural residues are being studied as feedstocks for sugar generation, which are in turn converted into ethanol. Among them, barley straw (BS) is a promising raw material, due to its high abundance, lignocellulosic composition and lack of other practical applications. Under these assumptions, the central aim of this study is to suggest an efficient bioethanol production scheme from BS at different levels of integration in co-fermentation with *Escherichia coli* SL100, including separate hydrolysis and co-fermentation (SHCF), simultaneous saccharification and co-fermentation (SSCF), and presaccharification and simultaneous saccharification and co-fermentation (PSSCF), using the water-insoluble solid (WIS) and slurry fractions obtained after steam explosion (SE) pretreatment. The best results in terms of ethanol yield were achieved following the SHCF process, using the WIS and the slurry as substrates, with yields of 89.1% and 78.8% of the theoretical maximum, respectively. Considering all of the above points, the following scheme is proposed for the conversion of BS into ethanol: SE pretreatment (160 °C, 30 min) of BS previously soaked overnight in 2.88% *w*/*v* phosphoric acid solution, filtration of the slurry, followed by enzymatic hydrolysis and co-fermentation of the two fractions obtained separately, with previous detoxification of the prehydrolysate with ammonium hydroxide (5 N). Under these conditions, 19.43 g of bioethanol was produced from 100 g of BS.

Keywords: barley straw; steam explosion; *Escherichia coli* SL100; bioethanol; SHCF; SSCF; PSSCF

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1. Introduction

Today, the world is highly dependent on fossil fuels, with biofuels being an alternative to gasoline, diesel and aviation fuels. Owing to the depletion of fossil fuels and the aims of achieving more environmentally-friendly processes, strategic changes in the global economy will be required. In addition, it is estimated that biofuels will represent between 10% and 40% of the market place in the long term, depending on global energy system models, implying a significant increase in their use [1]. The production of biofuels at a competitive price is being considered in Spain, which will contribute to the nation's economic and energy security [2]. Biomass, and in particular lignocellulosic biomass, appears to be the most viable renewable source of carbon for providing biofuels and chemicals following a biorefinery scheme. In this context, the European Union estimates an increase of up to 30% in power and heat generated from biomass by 2030 [3].

Among the biofuels available, a suitable option is second-generation bioethanol, where the raw material is biomass from agricultural and agro-industrial residues. This kind of biomass has several advantages: It is low cost, there is a lack of dependence on the food chain and it is highly available—there are between 3.7 and 5 billion metric tons of lignocellulosic waste in the world, including around 8.9 million tons of cereal waste, such as wheat, rice and barley [4,5]. There is a remarkable interest in the use of straw obtained in large quantities, which constitutes an important biomass resource that could be used for

the production of bioethanol. Currently, this straw is usually burned on site, producing unwanted emissions, or it is used as animal feed. However, the latter use suffers from a low nutritional value and low digestibility [6]. It is estimated that one third of barley straw (BS) remains in the fields and another third is used for other purposes, such as livestock farming [7]. In 2019, according to the Food and Agriculture Organisation (FAO), barley was an extensive cereal with an annual production of 159 million tons, of which 60% was in Europe. In the same year, Spain was one of the main barley producers with 7.7 million tons, representing 8% of the European production [8]. The main residue or by-product of barley is straw, with a ratio of up to 0.53 kg of straw/kg of grain [9] which implies a high amount of BS (up to 4 million tons/year in the case of Spain). Therefore, BS is considered a promising raw material for second-generation bioethanol, due to its abundance and lignocellulosic composition.

Beyond the production of biofuels such as ethanol, the generation of simple sugars from lignocellulosic biomass in general—and from barley straw in particular—can be the first stage in the production of platform chemicals for obtaining a wide range of products. In 2004, Werby and Petersen identified 12 building blocks that could be obtained from sugars contained in biomass, through biochemical routes. A number of transformation routes have been commercially developed, with each process representing a substitution for fossil resources [10].

The lignocellulosic nature of BS, which contains cellulose, hemicellulose and lignin, in a biochemical working scheme, constitutes a complex composition that is difficult for microorganisms to break down into low molecular weight sugars. For this reason, BS requires a pretreatment stage to break down and modify the fibre structure and make carbohydrates available for hydrolysis and fermentation [11]. Consequently, the process for obtaining second-generation bioethanol consists of the following stages: pretreatment, enzymatic hydrolysis (EH) and fermentation.

Various authors have investigated a wide variety of pretreatments of BS as a raw material for obtaining sugars: Uncatalysed steam explosion (SE) [12], ethanosolv [13], ionic liquids [14], dilute acid [15,16], twin-screw reactors [17], twin-screw reactors with alkaline or enzymes [18], NaClO and H_2O_2 combined [19], autohydrolysis [20], N_2 explosive decompression [21], organosolv [22], alkaline [23] and ultrasound [24]. In this study, SE has been proposed as an efficient pretreatment of lignocellulosic materials due to its effective biomass disruption characteristics for hardwoods and agricultural residues, low use of chemicals and low energy consumption, all of which has made SE one of the most thoroughly investigated pretreatments. In addition, it has been developed on a commercial scale [12]. Some disadvantages in SE can include: partial degradation of hemicellulose-derived sugars; solubilisation and alteration of the lignin compounds to chemicals that can hinder subsequent processes; and the possibility that the extractives break down during the pretreatment, resulting in potent inhibitors, even in low concentrations [12].

In this present work, phosphoric acid was used as a catalyst for SE pretreatment. Although it is more expensive than other acids—such as hydrochloric or sulfuric—phosphoric acid is less toxic and corrosive, which reduces equipment costs [25]. Moreover, phosphoric acid presents a high capacity for solubilising the hemicellulose fraction at low concentrations, with the added advantage of being a source of phosphorus, a nutrient for the microorganism in the fermentation of the prehydrolysates [26]. To the best of our knowledge, phosphoric acid has never been used as a catalyst in the SE pretreatment of BS for obtaining bioethanol.

From an environmental and economic point of view, it is more interesting to process all the pretreated biomass together (also known as slurry) in the subsequent stages of enzymatic hydrolysis (EH) and fermentation. The type and concentration of final inhibiting compounds varies depending on the lignocellulosic biomass, the pretreatment conditions (mainly time and temperature) and the presence or absence of acid catalyst (nature and concentration) [27]. For this reason, the slurry obtained after pretreatment by SE is filtered and washed in some studies, thus separating the solid fraction, which is

rich in cellulose and lignin (water-insoluble solid, WIS), from the liquid fraction (liquor or prehydrolysate), which is rich in hemicellulose, lignin, degraded sugars, acetic acid and other compounds [28]. These other compounds may promote an inhibitory impact on the microorganisms involved in the overall process; therefore, detoxification treatments are often implemented to reduce their concentration. These are based on physical, chemical or biological procedures. Supplementing fermentations with a small amount of sodium metabisulfite also reduces toxicity [29]. However, the benefits of including a detoxification step imply additional expenses in the process [30].

After the pretreatment of the biomass has been performed, EH and fermentation can be carried out in different process configurations: separate (SHF) and simultaneous (SSF). Following the path of integration of biotechnological processes, working in the SSF configuration process is a good choice since it is carried out in one reactor and end-product inhibition is minimised, allowing higher solid levels [31]. The main disadvantage of the SSF compared to the SHF configuration is that it is usually conducted at operation temperatures inferior to those at which optimal activity of cellulolytic enzymes is achieved. Additionally, there is the possibility of carrying out a preliminary step to SSF, presaccharification, which reduces the viscosity of the slurry at high substrate loading [31].

Ethanol yield can be also improved by using a microorganism capable of co-fermenting the kinds of sugars most commonly present in lignicellulosic biomasses, such as hexoses (glucose and galactose) and pentoses (xylose, arabinose and mannose). Taking this into account, this work deals with the effects of these parameters on ethanol production in different process configurations; namely, separate hydrolysis and co-fermentation (SHCF); simultaneous saccharification and co-fermentation (SSCF); and presaccharification and simultaneous saccharification and co-fermentation (PSSCF).

In this present work, *Escherichia coli* SL100 was chosen to co-ferment the hexoses and pentoses present in BS [32]. Previous results with this microorganism have shown high yields of ethanol production from other pretreated lignocellulosic materials, such as eucalyptus, where a yield of 0.24 g ethanol/g biomass was achieved [33], and sorghum, where 10,600 L of ethanol per hectare was obtained, comparing favourably with other reports [34].

The main objective of the present work was to assess the production of second-generation bioethanol by *E. coli* SL100, from BS pretreated with SE catalysed with phosphoric acid, following different process configurations: SHCF, SSCF and PSSCF.

2. Materials and Methods

2.1. Raw Material

The BS (*Hordeum vulgare*) used in this study was kindly supplied by a local farmer of Huelma (Jaén, southern Spain). The air-dried BS was milled using a laboratory hammer mill (Retsch, SM2000), sieved for a particle size of 1 cm and stored at room temperature. The moisture and the composition of the raw material were determined according to the standard procedures of the National Renewable Energy Laboratory of USA [35] (see Section 2.3).

2.2. Process Description

The raw material was pretreated in a SE batch reactor as described elsewhere [36] in a 4.5 L reaction vessel. The optimal pretreatment conditions were determined previously [37] based on the maximal production of sugars (including the glucose obtained from enzymatic hydrolysis of the pretreated solids and the xylose from the liquid fraction or prehydrolysate) and minimal generation of fermentation inhibitors. The BS was soaked overnight in 2 L of 2.88% *w*/*v* phosphoric acid, filtered using a mechanical press and then introduced directly into the steam explosion vessel for pretreatment at 160 °C for 30 min. The 30 min process time was initiated once the target temperature (160 °C) was reached.

After each pretreatment batch, and depending on the work scheme chosen (as shown in Figure 1), the slurry obtained (containing 6% *w*/*v* solid to liquid) was stored in plastic bags below 5 °C, or in other cases, was separated in two fractions, solid and liquid, by cake filtration. In the latter cases, the fraction filtrated WIS was washed with distilled water

until a neutral pH was obtained. As the WIS fraction showed a high moisture content and there was a risk of proliferation of contamination and degradation of the sugars, the WIS was oven-dried at 38 °C. Some authors have suggested that this may cause irreversible pore collapse in the micro-structure of the lignocellulosic biomass, thus hindering access and saccharification by cellulases [38]. Nevertheless, prior to the present study, enzymatic hydrolysis tests with the same oven-dried WIS resulted in high glucose yields, up to 90%, (data not shown); therefore, the oven-dried strategy was followed. Moreover, the liquid fraction, also called the prehydrolysate, was separated into two batches on which two different fermentation strategies were applied: one of them was not subjected to any detoxification and the other fraction was subjected a detoxification with ammonium hydroxide (see Section 2.5).

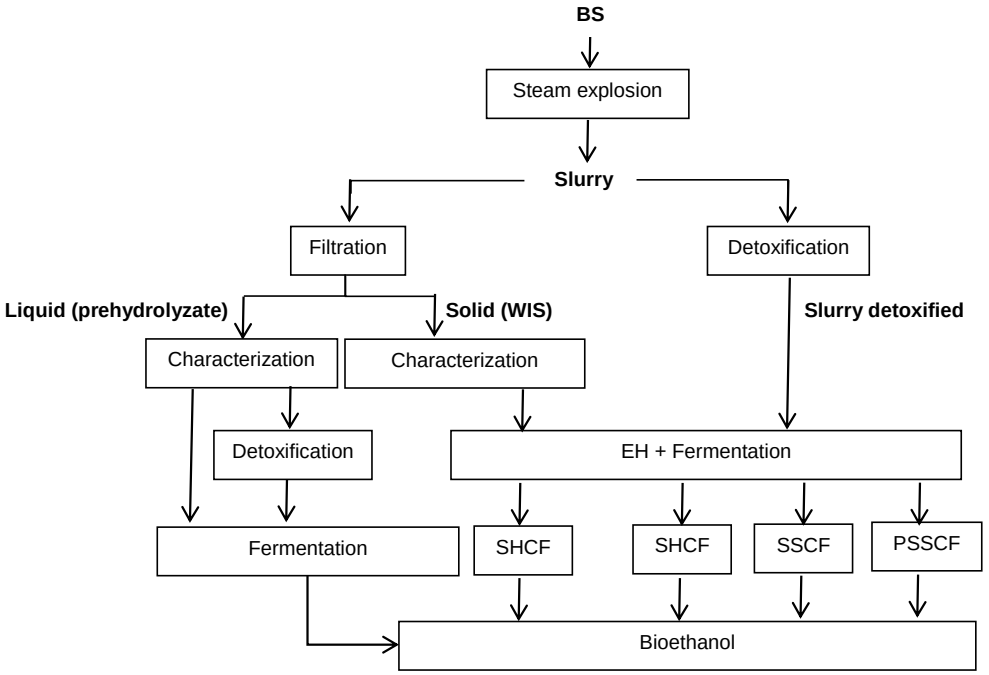

Figure 1. Work scheme for bioethanol production from BS.

2.3. Analytical Methods

The composition of the BS used as the raw material in this study was determined by the NREL laboratory analytical procedure for the quantification of structural carbohydrates and lignin in biomass [35]. Prior to other determinations, and according to this procedure, milled BS was subjected to a two-step extraction; firstly with water, and subsequently with ethanol. Afterwards, the monomer content of the extracted raw material was measured following a double acid hydrolysis step, to separate the lignocellulosic structures. The liquid resulting from this acid hydrolysis was then analysed for sugar content by HPLC as described below. The remaining solid was considered as acid-insoluble lignin (AIL).

Following pretreatment, the composition of WIS was determined as described above for the raw material except that no two-step extraction was used.

HPLC (Waters, Milford, MA, USA) was used for the determination of the sugar content (glucose, xylose, arabinose, mannose and galactose) of the double acid hydrolysis carried out with the raw material, and with the WIS and prehydrolysates obtained from pretreatment. The HPLC was equipped with a refractive index detector (model 2414) and a column CARBOSep CHO-782 Pb (Transgenomic, Inc., Omaha, NE, USA) working with ultrapure water as the mobile phase at a flow of 0.6 mL/min and at constant temperature of 70 °C. In addition, the prehydrolysates were also analysed for the following inhibitory compounds: Acetic acid, formic acid, furfural and hydroxymethylfurfural (HMF). For the samples of prehydrolysate, EH and the fermentation test, the measurement of inhibitors

and ethanol was performed with an HPLC system (Agilent Technologies 1260 model, Santa Clara, CA, USA) with a refractive index detector and an Aminex HPX-87H column (Bio-Rad Hercules, CA, USA), operating at 65 °C with 5 mM H_2SO_4 solution as eluent at a flow of 0.6 mL/min.

2.4. Microorganism and Growth Conditions

A bacterium able to ferment pentoses and hexoses was used; specifically, ethanologenic *Escherichia coli* SL100. Inocula were prepared in AM1 medium following the composition described previously [39], composed of (in g/L) xylose (16), glucose (10), $(NH_4)_2HPO_4$ (2.63), $NH_4H_2PO_4$ (0.87), $MgSO_4 \cdot 7H_2O$ (0.246), KCl (0.149), betaine (0.117), citric acid (0.1) and trace elements (different concentrations).

The growth conditions were 37 °C and 200 rpm for 24 h on a rotary shaker (AG CH-4103 Bottmingen, Infors HT Ecotron, Germany).

2.5. Detoxification and Fermentation of the Prehydrolysate

The detoxification procedure utilised for the prehydrolysate consisted of incorporating a certain amount of NH_4OH (5 N) solution to achieve pH 9, keeping identical requirements (agitation, time and temperature) to those described in previous works [29,32]. Subsequently, the detoxified prehydrolysate was subjected to centrifugation for 5 min at 5000 rpm to remove precipitated solids. The concentrated ammonium hydroxide solution favoured minimal changes to the final volumes.

For fermentation of prehydrolysates, Dasgip Bioblock equipment (Eppendorf AG, Hamburg, Germany) was used, including four fermenters of 1 L capacity, whose system control and operating parameters have been described in a previous study [29]. Specifically, the fermentation tests were carried out in duplicate and the fermenters were prepared for each of the two types of liquor obtained (with and without detoxification using ammonium hydroxide), adding 0.5 L of liquor and the AM1 medium components, excluding the ammonium salts [38]. It has been demonstrated that sodium metabisulfite is efficient in lowering the toxicity of the fermentation medium, as well as enhancing xylose metabolism by *E. coli* [40]. Therefore, sodium metabisulfite (1.5 mM) was added just upstream of the microorganism. Initially the pH was adjusted to 6.5 with 96% *v*/*v* sulfuric acid and a cell suspension of 4% *v*/*v* culture of inocula was added. The fermenters were placed inside block fermentation equipment, maintaining a temperature of 37 °C. An automatic pH adjustment system with 2 M potassium hydroxide was used. For sampling, 1 mL of sample was taken from each fermenter and centrifuged at 10,500 rpm for 10 min and then passed through 0.45 µm filters. The composition was then determined by HPLC analysis.

Fermentation tests carried out in this study were terminated once all the glucose and xylose present in the medium were consumed.

2.6. Characteristics of the Configurations Adopted for the Fermentation of the WIS and Slurry

In each configuration of the fermentation process with the WIS and slurry (see Figure 1), all experiments were performed in duplicate in 1-L fermenters Dasgip Bioblock equipment (Eppendorf AG, Germany) with a working volume of 0.5 L. The initial concentration of *E. coli* SL100 was 0.25 g/L. Before inoculation, the components of the AM1 medium and 1.5 M sodium metabisulfite (in a ratio of 1 mL/L sample) were added and the pH was adjusted to 6.5. Table 1 summarises the conditions fixed for each fermentation for the following parameters: temperature, agitation and pH. For periodic monitoring and analysis of the ethanol and sugar content, 1 mL samples from each fermenter were collected, centrifuged at 10,500 rpm for 10 min and passed through a 0.45 µm filter before being introduced into the HPLC system.

Table 1. Process variables and equipment used for the different configurations assayed on the WIS and slurry resulting from SE pretreatment.

Configuration	Fraction	Number of Steps	Equipment	Variables
SHCF	WIS	(1) EH [1]	Rotary shaker	50 °C 150 rpm pH 4.8
		(2) Co-F [2]	Dasgip Bioblock	37 °C 150 rpm pH 6.5
	Slurry	(1) EH	Rotary shaker	50 °C 150 rpm pH 4.8
		(2) Co-F	Dasgip Bioblock	37 °C 150 rpm pH 6.5
SSCF	Slurry	(1) EH + Co-F	Dasgip Bioblock	37 °C 150 rpm pH 6.5
PSSCF		(1) PreS [3]	Dasgip Bioblock	50 °C 150 rpm pH 4.8
		(2) EH + Co-F		37 °C 150 rpm pH 6.5

[1] EH = enzymatic hydrolysis. [2] Co-F = co-fermentation. [3] PreS = presaccharification 6 h.

The slurries were detoxified with ammonium hydroxide (5 N NH_4OH) and its pH was adjusted to 9, with constant stirring at 150 rpm and at a temperature of 50 °C, for 30 min. In all cases, 96% *v*/*v* sulfuric acid was used to lower and adjust the pH to the conditions of each configuration (see Table 1). This process did not involve significant changes to the volume and concentration of solids in the slurry (<1%).

All fermentation tests carried out in this study were terminated once all the glucose and xylose present in the medium were consumed.

2.6.1. Sequential Hydrolysis and (Co-)Fermentation (SHCF) of the WIS and Slurry

The SHCF process was applied to both the whole slurry and the WIS obtained from the pretreatment. The process was composed of two steps, i.e., enzymatic hydrolysis (EH) and fermentation. For the first step, the EH was performed using the enzyme complex Cellic CTec2, kindly provided by Novozymes A/S (Denmark), with 15 FPU/g substrate enzyme loading. In addition, 15 UI/g substrate of β-glucosidase (Novozym 50010, Novozymes A/S) were supplemented. The solid to liquid ratio was 5% for WIS treatment and 6% in the case of the slurry. Duplicated EH tests were performed in 0.05 M sodium citrate buffer (pH 4) at 50 °C on a rotary shaker (AG CH-4103 Bottmingen, Infors HT Ecotron, Germany) for 72 h at 150 rpm agitation rate. Average results are reported (standard deviations were in all cases <3%). The second step of the SHCF process consisted of the fermentation of the liquid fraction, obtained after filtration of the EH resulting material. Inoculation of 4% *v*/*v* *E. coli* SL100 was performed in 0.5 L final volume, under the operational conditions described in Section 2.6.

2.6.2. Simultaneous Saccharification and (Co-)Fermentation (SSCF) of the Slurry

The SSCF process was carried out in duplicate experiments from 0.5 L of slurry obtained from SE pretreatment. The slurry, with 6% *w*/*v* solid to liquid ratio, was prepared following the same procedure described above for WIS (pH setting, adding AM1 components and 1 mL/L sodium metabisulfite 1.5 M). Next, the slurry was inoculated with *E. coli* SL100 at a proportion of 4% *v*/*v* (after 24 h of growth of a cell suspension at 35 °C). Enzyme loading in SSF experiments was the same as that used in SHCF tests.

2.6.3. Presaccharification and Simultaneous Saccharification and (Co-)Fermentation (PSSCF) of the Slurry

In this process configuration, in duplicate experiments, 0.5 L of the slurry obtained after SE pretreatment (6% *w*/*v* solid to liquid ratio) was submitted to presaccharification (EH) for 6 h (15 FPU/g substrate of cellulose, 15 UI/g substrate of β-glucosidase, 150 rpm and 50 °C) before the inoculation of the *E. coli* SL100. After this time period, the temperature was adjusted to 37 °C and a sample was collected. For adding the inoculum, in contrast to the procedure described above, the culture was centrifuged and resuspended with the presaccharified sample. During the process, Dasgip Bioblock equipment (Eppendorf AG, Germany) allowed the control of the fermentation process variables as shown in Table 1 (temperature, agitation and pH).

2.7. Calculation of Yields

The EH yield from WIS, referred to as $EH_{pretreated}$ and expressed as g glucose per 100 g glucose present in the pretreated material, was calculated using Equation (1):

$$EH_{pretreated} = \frac{([G] - [G]_0) \times 0.02 \times 100}{[G]_{pm}} \times 100 \quad (1)$$

where [G] stands for the concentration of glucose released by EH, $[G]_0$ is the concentration of glucose in the enzymatic solution used, 0.02 refers to the 5% w/v ratio and $[G]_{pm}$ is the amount of glucose available in 100 g of pretreated material.

Another way of calculating the yield from the EH of the WIS was considering the glucose present in 100 g raw material (instead of the glucose in 100 g of pretreated material). This yield is referred to as EH_{raw} and was determined with Equation (2) as follows:

$$EH_{raw} = \frac{([G] - [G]_0) \times 0.02 \times 44.5}{[G]_{raw}} \times 100 \quad (2)$$

where 44.5 is the total gravimetric recovery (g per 100 g raw material) and $[G]_{raw}$ is the content of glucose in 100 g of raw material.

Fermentation yields were determined for SHCF, SSCF and PPSCF by using Equation (3):

$$Y_E = \frac{[E]}{[G] + [X]} \times 100 \quad (3)$$

where [E] is the concentration of ethanol in the fermentation broth and [G] and [X] are the initial concentrations of glucose and xylose in the substrate, either the prehydrolysate, the WIS or the slurry.

Additionally, the yield of ethanol as a percentage of the maximum attainable yield was calculated as shown in Equation (4):

$$Y_{Emax} = \frac{Y_E}{51.1} \times 100 \quad (4)$$

where 51.1 (g ethanol per 100 g sugars) is the stoichiometric coefficient of the ethanol fermentation process.

3. Results

3.1. Raw Material and Composition after SE Pretreatment

The composition of the BS used in the present study is shown in Table 2. It is known that the chemical composition of BS varies according to location, type of soil, season, growing location, farming practices and analytical procedure [41].

Based on the carbohydrate analysis carried out in this study, the total sugar fraction of the dry biomass was 74.52% (excluding the fraction of free glucose present in the extracts), of which 57% corresponds to hexoses and the remaining 43% to pentoses. Glucose, which is derived from cellulose present in both the BS fibres and the plant cell wall, is the major component (42.27%), followed by xylose (30.23%), which is the major hemicellulose constituent. Other compounds present in the BS to be considered are complex compounds, such as lignin (16.26%), and non-structural compounds, such as extractives (18.57%). Other components, such as some organic compounds (uronic acid and acetates), and certain trace elements, such as minerals, waxes, fats, starches, resins and gums, were not analysed but may be present in lower amounts [19]. The composition found in this study is similar to those reported by other authors in previously works [12,15–17,20,24] whose percentages ranged from 34–42% for glucose, 13–39% for xylose, 16–23% for lignin and 7–15% for extractives.

Table 2. Composition of BS.

Compounds	% Dry Weight
Cellulose	38.43 ± 1.75
as glucose	42.27 ± 1.75
Hemicellulose	28.55 ± 0.44
xylose	30.23 ± 0.26
arabinose	1.59 ± 0.12
galactose	0.43 ± 0.04
Lignin	16.26 ± 1.80
AIL [1]	14.22 ± 1.40
ASL [2]	2.04 ± 0.40
Extractives	18.57 ± 0.54
glucose	2.53 ± 0.02
Ash	1.97 ± 0.40
Acetyl groups	1.05 ± 0.09

Mean values and standard deviations of three determinations. [1] AIL = acid-insoluble lignin. [2] ASL = acid-soluble lignin.

This composition data for the glucan and hemicellulosic fraction indicates that BS is an appropriate material for the production of second-generation bioethanol [42]. Although the lignin and extractive fractions in BS may hinder the conversion of sugars to bioethanol, SE pretreatment should diminish these barriers, including the degradation of hemicellulose constituents, thereby improving enzyme accessibility and digestibility of the cellulose component [11,12].

After SE pretreatment, the average solid recovery was 44.5%. As shown in Table 3, this reduction in solid fraction was mainly due to the solubilisation and degradation of xylose and extractives. The matter loss primarily occurs at the expense of hemicellulose, of which xylose is the major component. This component is more thermally degradable, hence the change in biomass composition.

Table 3. Composition of WIS (% dry weight) from steam-exploded BS.

Compounds	Raw Material	WIS
Glucose	42.27 ± 1.75	65.11 ± 1.45 (68.33) [1]
Xylose	30.23 ± 0.26	3.72 ± 0.13 (5.75)
Lignin	16.26 ± 1.80	12.62 ± 0.88 (77.59)
Extractives	18.57 ± 0.54	5.2 ± 0.24 (12.32)

[1] Data in parentheses show the recovery per 100 g of compound in the raw material.

The obtained prehydrolysate (see Table 4) consisted of a mixture of hydrolysable sugars and degradation products. This is due to the action of the high temperature saturated water steam of the SE process generating autohydrolysis reactions, during which a proportion of the hemicellulose and lignin were converted into soluble compounds [43]. Analysis of the carbohydrates shows that xylose was the most abundant sugar in the liquid. 42.44% of the xylose initially present in the BS was found, while the values of glucose recovery were below 9%. In addition to sugars, other compounds such as acetic acid, formic acid, furfural and HMF were also present in the prehydrolysate. All these products have been identified previously in other prehydrolysates of herbaceous biomass [44] and have been described as potential inhibitors of fermentation [27]. Acetic acid (0.94 g/L), formic acid (0.65 g/L) and furfural (0.23 g/L) were the main degradation products found and their presence could be explained by the hydrolysis of hemicellulose, and more specifically by the degradation of xylose in the case of furfural. In addition, it is likely that some of the hemicellulose was lost by volatilisation of part of the furfural, a process observed by other authors during a study of the same pretreatment on olive tree wood [45]. The presence of formic acid is common in prehydrolysates as a product of sugar degradation [43].

Table 4. Composition (g/L) of prehydrolysate from steam-exploded BS steam.

Compounds	Prehydrolysate [1]
Glucose	1.68 ± 0.26 (8.77)
Xylose	12.85 ± 0.75 (42.44)
Acetic acid	0.94 ± 0.07
Formic acid	0.65 ± 0.05
Furfural	0.23 ± 0.02
HMF	0.08 ± 0.01

[1] Data in parentheses show the recovery of the compound (g/100 g) relative to the raw material.

3.2. Detoxification and Fermentation of Prehydrolysate

Figure 2 shows the effects of ammonium hydroxide detoxification on the sugar and inhibitory compound composition of the prehydrolysates analysed in this study. In general, the concentration of the initial sugars (sum of glucose, xylose, galactose, mannose and arabinose) were not significantly reduced, but there was an effect on furfural and HMF levels.

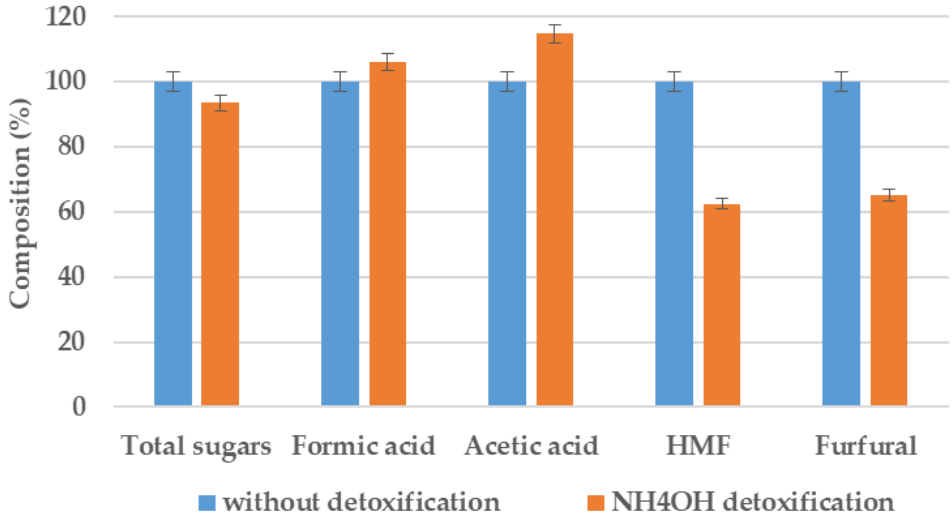

Figure 2. Normalised composition of prehydrolysates compared to initial.

Once detoxification was complete, the fermentation of the detoxified prehydrolysate was carried out. Another fermentation was carried out in parallel, without detoxification, as a control. *E. coli* SL100 was able to ferment the two prehydrolysates, producing similar final concentrations of ethanol—5.45 g/L (without detoxification) and 5.15 g/L (with ammonium hydroxide detoxification)—as shown in Figure 3. This means that the same yield of ethanol was achieved in both cases, 42%, which is 83% of the theoretical maximum.

From Figure 3, it can be observed that the ethanol yield obtained after 18 h of fermentation of the detoxified prehydrolysate was the same yield obtained after 54 h in the case of non-detoxified prehydrolysates. With detoxified prehydrolysates, this yield reached >90% of the final total yield achievable after 30 h, whereas the non-detoxified prehydrolysate took 67 h to reach the same yield. Considering the possible industrial application, shorter residence time in the fermenters and lower operating costs are key advantages. Therefore, economic analysis is required to verify any benefits of performing the detoxification process, in the terms applied in this work.

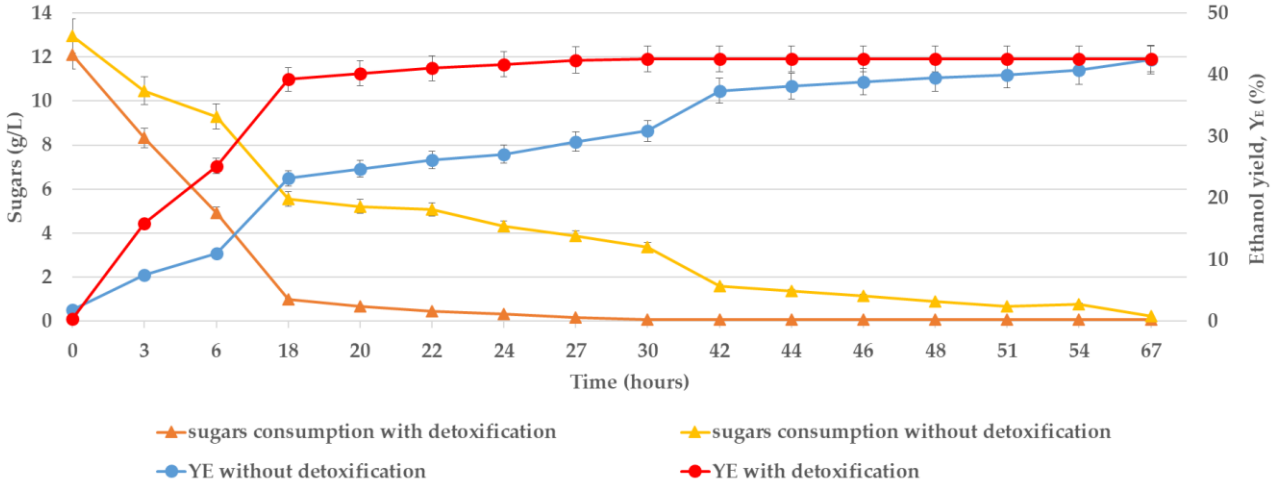

Figure 3. Total sugar consumption and ethanol yield of prehydrolysates during fermentation.

3.3. Sequential Hydrolysis and Co-Fermentation of the WIS

In this process configuration, the enzymatic hydrolysis of the WIS was first performed for 72 h. The WIS concentration was 5% *w*/*v* and the total volume was 0.5 L; enzyme dosage was the same as described in Section 2.6.1. The results are shown in Figure 4.

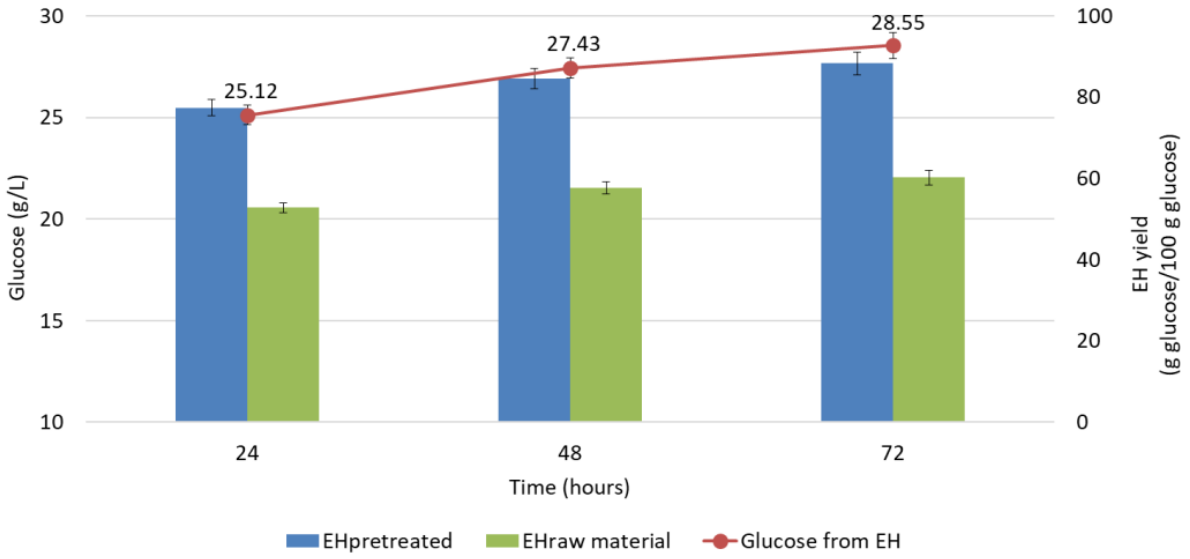

Figure 4. Glucose concentration (g/L) and yield (g glucose/100 g glucose) from EH of the WIS.

The final glucose concentration reached 28.6 g/L, which represented 88.3% of the potential glucose contained in the WIS (60.3% of the glucose contained in the raw BS).

Following the SHCF working scheme proposed, fermentation tests were carried out with *E. coli* SL100 on a 500 mL sugar solution, following the EH step. Figure 5 shows the average results obtained from fermentation experiments carried out in duplicate. The time required for to achieve the total conversion of sugars present in the fermentation media (consisting of 98% glucose/xylose) was 42 h, resulting in an ethanol concentration of 13 g/L, equivalent to an ethanol yield of 46.4% (89.1% of the theoretical maximum).

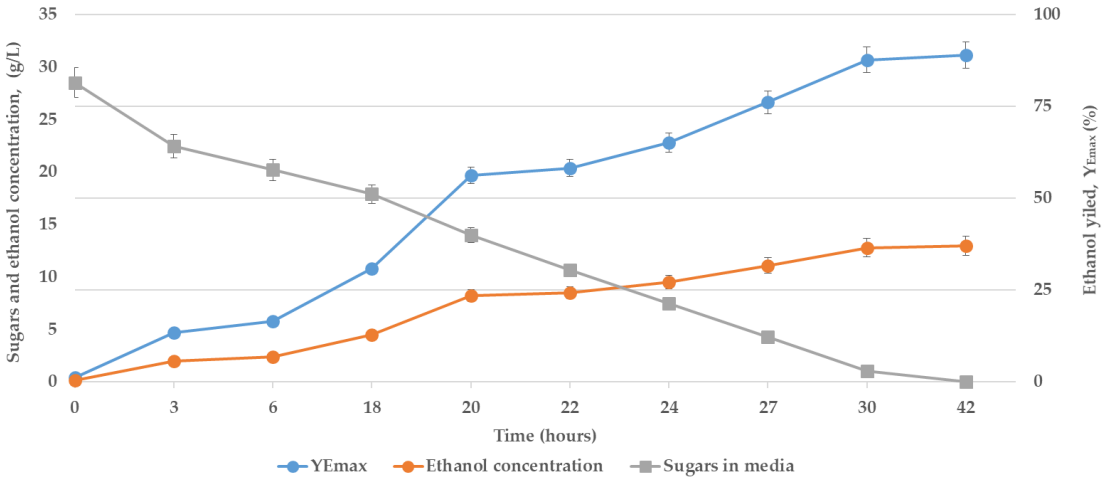

Figure 5. Sugar concentration (g/L) and ethanol yield from fermentation of the WIS.

3.4. Sequential Hydrolysis and Co-Fermentation (SHCF), Simultaneous Saccharification and Co-Fermentation (SSCF) and Presaccharification and Simultaneous Saccharification and Co-Fermentation (PSSCF) of the Slurry

In order to investigate the potential bioethanol yield of pretreated BS following the digestion and fermentation of the WIS and the prehydrolysate separately (as described previously), co-fermentation with *E. coli* SL100 was carried out directly on the slurry resulting from SE pretreatment with different levels of integration: SHCF, SSCF and PSSCF (Figure 1). From a techno-economic point of view, the use of high solid-loading slurries is recommended, but working with lignocellulosic slurries under these conditions often involves mixing and agitation difficulties, resulting in low conversions [46,47].

The greatest ethanol production was obtained using the PSSCF configuration (20.6 g/L), followed by the SHCF (18.45 g/L) and then the SSCF (15.1 g/L) configurations (Figure 6). In both SHCF and PSSCF, the highest amount of bioethanol (50% of the total, or more) was obtained in the first 24 h, indicating that the fermentation stage, as expected, was enhanced by the previous period of EH under optimal conditions. It should be noted that in SHCF, the fermenting microorganism completely consumed the sugars present within 42 h, as was the case in the experiments with the WIS fermentation (Section 3.3), while in the PSSCF process, the fermentation was slower and more progressive over time, reaching complete sugar consumption after 160 h. In the configuration of SSCF, bioethanol production was gradual over time and glucose and xylose were not completely consumed until 185 h. A slower increase in ethanol yield is observed with *E. coli* SL100, due to its capability to assimilate some hemicellulosic sugars, initially preferring glucose and then consuming xylose [32].

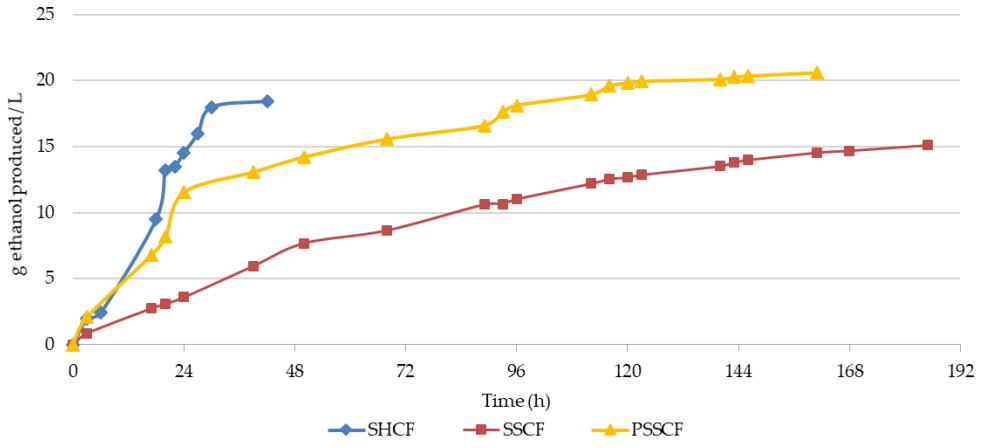

Figure 6. Ethanol produced from slurry with *E. coli* SL100.

The different ethanol yields are shown in Figure 7, which were calculated considering the maximum possible theoretical yield (51.1%). Again, the best configuration was SHCF with 78.8%.

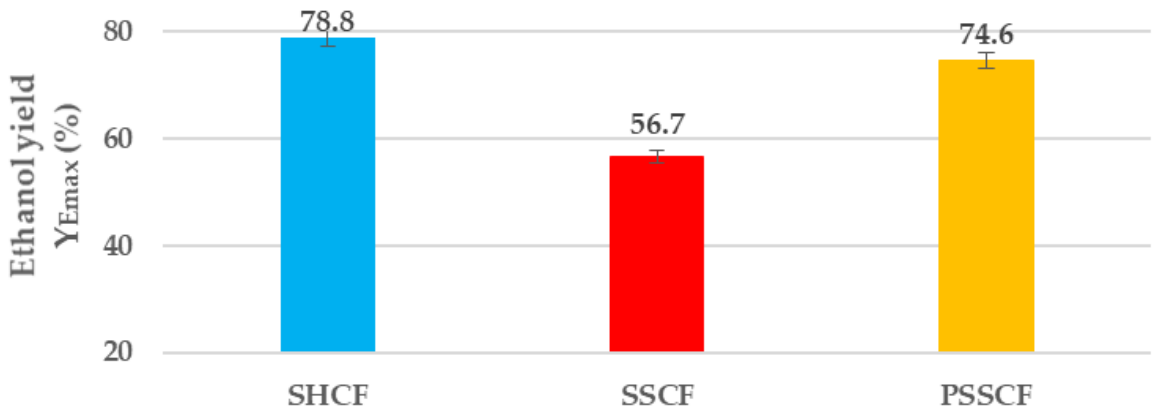

Figure 7. Ethanol yield from the slurry with *E. coli* SL100.

3.5. Mass Balance for the Production of Second-Generation Ethanol from BS Biomass

The results obtained in each step of the bioethanol production processes were used to estimate the mass balance for the different configurations proposed, based on 100 g of BS (dry weight basis).

Firstly, Figure 8 shows the ethanol potential produced by fermenting the two fractions (prehydrolysate and WIS) separately with *E. coli* SL100, both obtained after filtration of the SE slurry. It should be noted that 47 g of structural sugars were recovered from the two fractions following pretreatment, equivalent to 63% of the total sugars in the raw material, consisting of mainly glucose and a minor amount of xylose. The greatest reduction in sugars is due to the solubilisation of hemicellulose under the SE pretreatment conditions tested, which resulted in the recovery of ≈50% of the xylose present in the raw material. After separate fermentation of each fraction, 90% of the glucose and xylose present in the detoxified prehydrolysate, as well as in the solution obtained after EH of the WIS, are utilised. Overall, 19.43 g of bioethanol could be obtained from 100 g of BS, indicating that 50% of the structural sugars present in the raw material could be fermented.

According to the process integration schemes proposed in this study for obtaining bioethanol from BS, Figure 9 shows the mass balance based on 100 g when processing the obtained slurry directly after SE pretreatment. It is noted that after the SE carried out in this study, the solid/liquid ratio obtained in the slurry was 6% w/v and that the content of potentially fermentable sugars was almost 42 g, which represented 57% of the sugars present in the raw material—clearly similar results to those observed above when separating the solid and liquid fractions (63%). In the SHCF process, the 78% yield obtained in the EH step indicates that the fermentation medium contained 48% of the sugars present in the raw material, from which 14.30 g bioethanol was obtained by fermentation, 38% of the maximum ethanol attainable. More bioethanol was obtained when the process scheme followed the PSSCF strategy, after which nearly 16 g bioethanol resulted from fermentation (42% of the maximum theoretical possible), while the lowest amount of bioethanol generated (12.13 g) was obtained with the SSCF scheme (32% of the maximum theoretical possible).

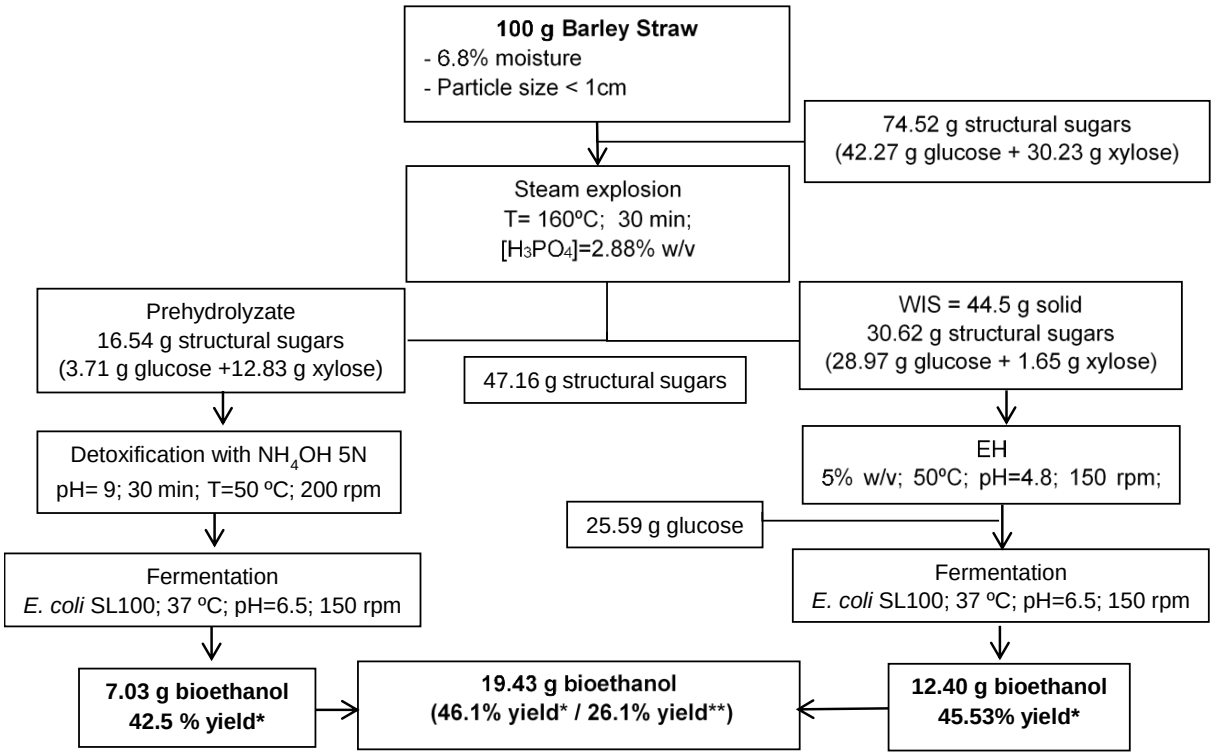

Figure 8. Mass balance of bioethanol production from BS for the SHCF of the WIS and fermentation of prehydrolysates. * relative to glucose and xylose in the prehydrolysate and WIS after EH; ** relative to glucose and xylose in the raw material.

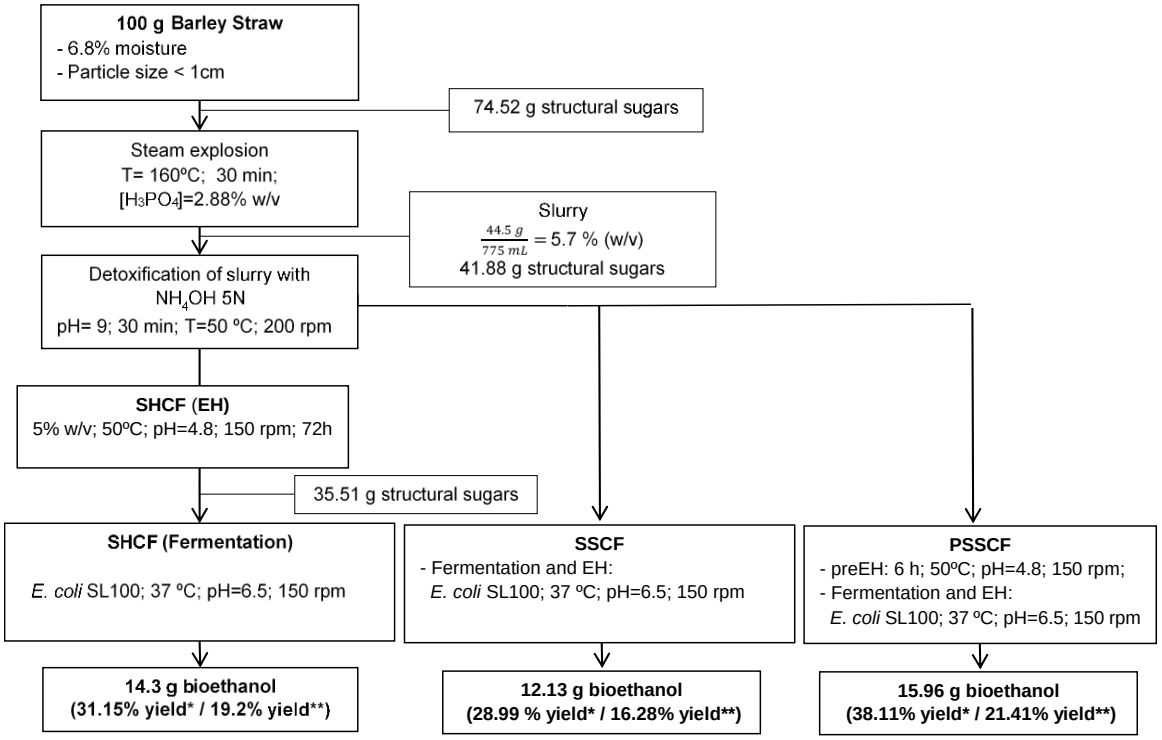

Figure 9. Mass balance of bioethanol production from the slurry of pretreated BS for SHCF, SSCF and PSSCF configurations. * relative to glucose and xylose in the slurry; ** relative to glucose and xylose in the raw material.

Therefore, from this comparison it can be concluded that the highest amount of bioethanol produced from 100 g of BS (19.43 g) would be obtained by detoxification, hydrolysis and then fermentation of the two fractions (prehydrolysate and WIS) separately. The disadvantages of working with separate fractions should be noted; mainly, the higher number of stages and equipment to be used, combined with the time and cost involved. The PSSCF option would be the most promising in this aspect, considering the results obtained (15.96 g of ethanol/100 g BS) under the conditions tested.

4. Discussion

Following the pretreatment of BS proposed in this study and then analysing the WIS obtained, it is noted that glucose content increased in relation to untreated material from 42% to 65%, while xylose decreased from 30% to 4%. Table 5 shows that the composition of the WIS is similar to that reported by other authors with the same raw material and pretreatment method. The xylose composition in the WIS in the present study is the lowest of the compared studies, which is reasonable since the conditions used were selected in a previous work to maximise the xylose content in the prehydrolysate [37].

Table 5. Composition of the WIS (%) from steam-exploded BS.

Reference	Conditions	Glucose	Xylose	Lignin
This study	160 °C 30 min [H_3PO_4] [1] 2.88% *w*/*v*	65.11 (68.33) [2]	3.72 (5.75)	12.62 (77.59)
[47]	180 °C 30 min	61.38 (89.4)	10.85 (21.9)	30.7
[42]	120 °C 30 min [H_2SO_4] 3% *v*/*v*	66.0	5.3	30.0
[31]	210 °C 5 min	64.46	7.0	21.6 [3]
[48]	190 °C 10 min 2% NaOH	69.3	21.47	n.r. [4]

[1] Impregnation overnight and soaked. [2] In parentheses, recovery relative to 100 g in raw material. [3] Acid-insoluble lignin. [4] n.r. = not reported.

The composition of the prehydrolysate was similar to that found by other authors when they pretreated BS with SE with the objective of maximising the presence of hemicellulosic sugars and increasing the yield of the solid fraction by EH [43] and acid hydrolysis, with the aim of obtaining the highest recovery of xylan [49]. Recently, Moraes et al. [16] obtained a prehydrolysate with higher concentration of xylose (20.92 g/L) when the authors subjected the raw material to acid hydrolysis. In this study, the sole purpose was to optimise the extraction efficiency of xylose into prehydrolysates.

Ammonium hydroxide detoxification of the prehydrolysate reduced the concentration of furfural and HMF by almost 40%, but was not as efficient in the removal of other inhibitor compounds, which raised the concentrations of acetic acid (14%) and formic acid (6%). Other authors also subjected the obtained BS prehydrolysates to detoxification after acid pretreatment, specifically concentration and active charcoal, which reduced the levels of toxic compounds and almost completely removed furfural and HMF [16].

When fermenting this prehydrolysate, yields of 83% of the theoretical maximum were obtained, and this good result may be because high concentrations of acetic acid are not a barrier to the action of *E. coli* SL100, since this strain is highly resistant to this inhibitor, and can even use acetate as a carbon source [50] or may even be stimulated by this compound [28]. No studies have been found where BS prehydrolysates are subjected to ethanolic fermentation, although these results are similar to those found by other authors, who also obtained a higher ethanol yield from different lignocellulosic biomass prehydrolysates, such as from corn stover, which reached 72% of the theoretical maximum [51]; or olive tree pruning, where the highest yields (80–82%) were obtained by ethanolic fermentation after detoxification with ammonium hydroxide, or overliming [29,52].

As shown in Figure 3, the main difference between the two fermentations carried out (with and without detoxification) was the efficiency of the process, since the maximum yield was reached in 30 h when the prehydrolysate was previously detoxified, which is less than half the time required when not detoxified. The duration of the process is similar to that reported by other authors, e.g., in the ethanolic fermentation of corn stover, the prehydrolysates were submitted to ammonium hydroxide detoxification for 36 h [51]. Therefore, despite the fact that the detoxification procedure does not remove inhibiting compounds, such as acetic and formic acid, the productivity of the fermentation with *E. coli* SL100 is benefited. Others authors attributed this greater efficiency to the removal of phenols or other toxic compounds between pH 6.8 to 8 [51], while others studies [53] suggested a greater effectiveness of NH_4OH detoxification owing to its ability to eliminate furanic and phenolic compounds. In a study on the techno-economic model for ethanol production from corn stover by dilute-acid pretreatment with a recombinant *Zymomonas mobilis* strain, it was concluded that that the ammonium hydroxide conditioning was a more economical alternative to overliming, despite the higher cost of ammonia and the need to redesign the wastewater treatment section [54].

In relation to the EH carried out on the WIS, the results achieved (88.34%) are an improvement on those obtained by other authors [31], who reached 80.2% of the glucose contained in the WIS (from EH at 5% *w*/*v*) after pretreating BS with SE (210 °C, 5 min without previous acid impregnation). The same authors reached the highest glucose recovery (86.7%) when they dosed EH at 2% *w*/*v*. A slightly lower glucose yield (83.5%) was obtained by Alriksson et al. [53] in the WIS of BS, also after SE pretreatment (180 °C, 30 min, without previous acid impregnation) with EH at 10% *w*/*v*.

Therefore, previous impregnation in SE pretreatment of the BS catalysed with a solution of phosphoric acid exerts a positive effect on the release of glucose by EH. In this sense, Person et al. [48] obtained an EH glucose conversion of 80–90%, after SE pretreatment catalysed by spraying NaOH solution on the BS. If other pretreatments of BS alternative to SE are considered, similar results were obtained by Kim et al. [13], who achieved a yield 87.83% using ethanosolv pretreatment (170 °C, 60 min, 1% sulfuric acid and 50% ethanol concentration); Vargas et al. [20] obtained 83% using autohydrolysis pretreatment; Salapa et al. [22] reached a 83.9% of yield with an organosolv pretreatment (140 °C, 0 min and 35 mol/m^3); and Ibarra-Díaz et al. [23] achieved a maximum 82% conversion of cellulose to glucose using peroxide-alkaline pretreatment. It should be noted that Sáez et al. [14], working with ionic liquids (110 °C and 60 min), also obtained high EH yields of glucose, greater than 90% (even of 100%), although this pretreatment currently has limitations for large scale application.

Following the SHCF working scheme proposed for the WIS, almost 90% of the glucose present in the raw material was made available. This positive effect of the phosphoric acid and the fact that the fermentation stage was performed under optimal conditions (typical of the SHCF process), such as temperature, agitation and pH, were confirmed by the high ethanol yields (89.1%). As displayed in Table 6, this yield compares favourably with those obtained by other authors who carried out SHF on WIS of BS after SE and other pretreatments and then fermented with different microorganisms, such as *S. cerevisiae* and *K. marxianus*.

Considering of the high fermentation yield achieved in fermenting the prehydrolysate and the WIS separately, the slurry obtained directly from the SE pretreatment was subjected to SHCF, SSCF and PSSCF, reaching the concentrations showed in Figure 6 and the ethanol yields summarised in Table 6. To be able to compare the three configurations of the process in economic terms, the ethanol concentration following saccharification and fermentation is key, and should be as high as possible in order to minimise the energy costs of evaporation and distillation [56]. Therefore, the best ethanol production was obtained for the PSSCF (20.6 g/L) followed by the SHCF (18.45 g/L) and then SSCF (15.1 g/L).

Table 6. Results of different fermentation process with slurry and WIS from BS pretreated.

Author	Substrate	Microorganism	Configuration	Y_{Emax} [1]
This study	WIS	*E. coli* SL100	SHCF	89.1
	slurry		SHCF	78.8
			SSCF	56.7
			PSSCF	74.6
[31]	WIS	*K. marxianus*	SHF	56.8
			SSF	67.4
			PSSF	56.8
[47]	WIS	*S. cerevisiae*	SHF	76.0
			PSSF	75.1
[21]	WIS	*S. cerevisiae*	SHF	72.1
[19]	WIS	*S. cerevisiae*	SHF	40.5
[49]	WIS	*S. cerevisiae DKIC*	SHF	64.3
			SSF	76.5
[55]	WIS	*S. cerevisiae* Ethanol Red	SSF	97.0
			PSSF	97.0
[20]	WIS	*S. cerevisiae*	SSF	93.0
[17]	WIS	*S. cerevisiae*	SSF	93.0
[18]	WIS	*Yeast CelluxTM 4*	PSSCF	53.5

[1] Percentage of theoretical maximum (51.1%).

These results could not be compared with other studies on the slurry fermentation of BS because slurry has never been used as a substrate in ethanolic fermentation, to the best of our knowledge. However, they could be compared with other works where fermentations were carried out in different configurations on the WIS of BS after SE pretreatment (Table 6). Comparing the yield obtained in the SHCF slurry process (78.8%) with that obtained in other studies on the WIS with the same configuration, this is the highest value, being similar to that reported by Álvarez et al. [47], who used steam-exploded BS and *S. cerevisiae*, which confirms that SE enhances access to the sugars present in the raw material and they also represent improvements in the integration of the process steps, as it is no longer necessary to separate the prehydrolysate in advance.

Operating in SSCF, the yield obtained from the slurry (56.7%) is lower than that found by other authors on the WIS, which suggests that, despite carrying out detoxification of the slurry with NH_4OH, the presence of the prehydrolysate inhibits fermentation. Furthermore, this limitation is reduced if the PSSCF process is used, with previous EH for 6 h, since the yield increases to 74.6%. This is below the highest yield found in the literature (97.0%) [55], also pointing out that this value is relative to the initial cellulose in the BS, whereas in our study it was operated in co-fermentation.

Concerning the mass balance, Han et al. [17] achieved 14.4 g of bioethanol/100 g of BS following pretreatment with a continuous twin-screw reactor and an SSF process at 10% *w*/*v* on the WIS, which compares favourably with the results from the SSCF conditions in this study (12.13 g) and is similar to the results obtained using the SHCF process with the slurry (14.3 g). In addition, Duque et al. [18] obtained 15.8 g of ethanol per 100 g of BS after pretreatment by combined soda and enzyme-catalysed extrusion, and PSSCF, results very close to those obtained in this study (15.96 g) after pretreatment of BS with SE after impregnation with phosphoric acid and PSSCF with *E. coli* SL100. Recently, Álvarez et al. [57] reported 12.6 g of ethanol/100 g BS following PSSF configuration in a process aimed at obtaining bioethanol and value-added products.

Finally, it is important to note that the proposed scheme for production of bioethanol from barley straw was mainly chosen based on the product yield and final concentration attainable. The techno-economic analysis of the different process configuration options, together with the environmental assessment, will be the key tools for finally selecting the actual operating conditions. Other aspects, such as by-product downstream operations (e.g., the further use of lignin fraction) or waste management will also play a relevant role in the final and complete process flow diagram.

5. Conclusions

The overall process of the conversion of phosphoric acid-soaked steam-exploded BS to bioethanol was studied in this work and the main conclusions obtained are the following:

1. BS can be used as a raw material for second-generation ethanol production, based on its sugar composition, following a process consisting of pretreatment, enzymatic hydrolysis and fermentation.
2. Using both C6 and C5 sugars, namely glucose and xylose, and appropriate process configurations, including sequential or simultaneous saccharification and fermentation schemes, may produce the best results in terms of ethanol production.
3. The SE pretreatment of phosphoric acid-soaked BS results in an enhanced release of glucose from the WIS fraction issued from the pretreatment.
4. Following the separate fermentations of the WIS and the prehydrolysate, an overall process bioethanol yield of 51.1% (compared to the theoretical maximum) was achieved.
5. Comparing the results among the three process configurations assayed, i.e., SHCF, SSCF and PSSCF, the best option proved to be PSSCF, based on the bioethanol yield of 41.9% (compared to the theoretical maximum) and based on the final bioethanol concentration obtained (20.6 g/L).
6. In terms of slurry fermentation productivity, the best option was SHCF, as the total fermentation time was 42 h, reaching a yield of 37.6% (compared to the theoretical maximum).

Based on the above points, the following scheme for the production of bioethanol from BS, focusing on the maximum total amount of bioethanol obtained, is suggested: SE pretreatment (overnight soaking of raw material with [H_3PO_4] = 2.88% w/v, 160 °C, 30 min), then filtration of the slurry followed by enzymatic hydrolysis and separate co-fermentations of two fractions obtained, following prior detoxification of the prehydrolysate with ammonium hydroxide (5 N). Under these conditions, the total amount of bioethanol that can be obtained from 100 g of BS is 19.43 g.

Further work will focus on increasing the xylose recovery following pretreatment, as only 50% of these sugars were recovered under the SE conditions carried out in this study. In addition, other slurry detoxification processes and conditions should be tested with a view to reducing fermentation times, including analysis of the economic aspects. A fed-batch configuration will also be studied, as a way of improving final conversion yields.

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