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Integrated Bioethanol Fermentation/Anaerobic Digestion for Valorization of Sugar Beet Pulp

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Abstract: Large amounts of waste biomass are generated in sugar factories from the processing of sugar beets. After diffusion with hot water to draw the sugar from the beet pieces, a wet material remains called pulp. In this study, waste sugar beet pulp biomass was enzymatically depolymerized, and the obtained hydrolyzates were subjected to fermentation processes. Bioethanol, biomethane, and biohydrogen were produced directly from the substrate or in combined mode. Stillage, a distillery by-product, was used as a feedstock for anaerobic digestion. During biosynthesis of ethanol, most of the carbohydrates released from the sugar beet pulp were utilized by a co-culture of *Saccharomyces cerevisiae* Ethanol Red, and *Scheffersomyces stipitis* LOCK0047 giving 12.6 g/L of ethanol. Stillage containing unfermented sugars (mainly arabinose, galactose and raffinose) was found to be a good substrate for methane production (444 dm^3 CH_4/kg volatile solids (VS)). Better results were achieved with this medium than with enzymatic saccharified biomass. Thermal pre-treatment and adjusting the pH of the inoculum resulted in higher hydrogen production. The largest ($p < 0.05$) hydrogen yield (252 dm^3 H_2/kg VS) was achieved with sugar beet stillage (SBS). In contrast, without pre-treatment the same medium yielded 35 dm^3 H_2/kg VS. However, dark fermentation of biohydrogen was more efficient when sugar beet pulp hydrolyzate was used.

Keywords: sugar beet pulp; hydrolysis; bioethanol; stillage; methane; hydrogen

1. Introduction

Biomass is considered a potential substitute for fossil fuels, and has attracted a great interest from governments and industry. It is the fastest-growing renewable energy source in the European Union [1]. Large amount of waste biomass are generated in sugar factories from the processing of sugar beet pulp. After diffusion with hot water to draw the sugar from the beet pieces, a wet material called pulp remains. Around 660 kg of sugar beets are required to produce 100 kg of sugar, with 330 kg of wet pulp and 25 kg of molasses generated as by-products [2]. The pulp mostly contains polymeric saccharides such as cellulose (22–30%), hemicelluloses (24–32%), lignin (1–2%) and pectin (38–62%), which constitute up to 75–85% of the dry matter [3].

Products released when the carbohydrates in biomass are broken down can be converted by microorganisms into valuable compounds. Easily degradable biomass resources are often used to produce bioethanol or biogas. Waste products from the agricultural industry, such as straw, sugar beet pulp, sugar beet silage [4], and beet leaves containing lignocellulosic complex in their structures could also be used. The monomers released from lignocellulosic substrates are a mixture of hexoses and pentoses. The main problem with using such biomass-hydrolyzates is the limited number of microorganisms which are able to utilize both kinds of saccharide simultaneously. Xylose, galactose,

and arabinose derived from lignocellulose are less effective carbon sources for fermentation processes than glucose [5]. The presence of glucose often prevents the use of secondary carbon sources [6]. Commonly-used conventional strains of *Saccharomyces cerevisiae* are unable to assimilate arabinose [7]. Most commercial strains, particularly of distilling yeasts, cannot metabolize pentoses. These carbohydrates remain as biomass waste, in the form of stillage, following ethanol fermentation [8].

Stillage is the main by-product of bioethanol production. The properties of stillage differ according to the substrate used and the process operating conditions [1]. However, this acidic and corrosive wastewater is generally characterized by a high chemical oxygen demand (COD). The waste management of stillage is an important economic and environmental consideration for the alcohol industry [9]. Digestion of stillage could not only improve the energy balance, but also add value to the residues, which could be used as fertilizer. Anaerobic digestion of bioethanol vinasse is considered a very promising solution [8]. Almost all organic biodegradable substances are converted into biogas during anaerobic digestion [10]. Moreover, the methane obtained is a second-generation fuel, which can be transformed into heat and electricity. This could be used to power alcohol fermentation processes that require thermal and electric energy [10], creating a well-balanced system.

Taking into consideration reported in literature studies on the ethanol fermentation and anaerobic digestion of sugar beet derived streams including sugar beet stillage (SBS) we formulated the goal of this research. Our aim was to assess the complementarity of anaerobic digestion and bioethanol fermentation, with a particular focus on the monomers released during hydrolysis. The liquid fraction derived from hydrolysis of sugar beet pulp was subjected to alcoholic fermentation, while the remaining solid residue and stillage was used as a substrate for methane or hydrogen production. Integrated fermentation and anaerobic digestion is common industrial practice in the ethanol industry, however sugar beet pulp is still a relatively new substrate for this solution. Furthermore, our investigation focused also on the intensification of biosynthesis of hydrogen as the newest generation fuel.

2. Results and Discussion

2.1. Hydrolyzate Characterization

To assess the potential of sugar beet pulp-based hydrolyzates for use in second-generation biofuel (ethanol) production, the physicochemical parameters of obtained hydrolyzates were analyzed (see Table 1). Extract content, indicating the concentration of dissolved solids (mostly sugars), ranged from 9.18 ± 0.58 to 11.36 ± 0.76°Blg. The smallest amount of reducing sugars was detected in the hydrolysate obtained from briquetted pulp, while the largest was found in the hydrolyzate from wet pulp (season 2) ($p < 0.05$). During pre-treatment of sugar beet pulp, weak acids (such as acetic acid) may also be released, which can have a negative effect on yeast growth and fermentation [11,12]. In most strains of *S. cerevisiae*, volatile acids (including acetic acid) are not metabolized by glucose-repressed yeast cells, and enter the cells in a non-dissociated form by simple diffusion [13,14]. The toxic effects of this undissociated form of acid translate into an exponential inhibition of growth and fermentation rates [15,16]. The content of volatile acids in the hydrolyzates was therefore also determined. The values varied widely, from 1.05 ± 0.05% *w/v* (for briquetted pulp-based hydrolysate) to 1.94 ± 0.23% *w/v* (for wet pulp-based hydrolysate, season 2). The briquetted pulp-based hydrolysate showed the highest pH value, while the wet pulp-based hydrolysate (season 2) had the lowest ($p < 0.05$).

The dry mass of sugar beet pulp is composed mainly of polysaccharides, with small amounts of fat, protein, ash, and lignin [17]. Some differences in the physicochemical composition of sugar beet pulp-based hydrolyzates can be explained by the varieties of sugar beet processed in the sugar factory, by the different conditions of sugar beet cultivation and by the technologies used for processing (Table 1). Raffinose, which can be found in sugar beet roots [18], was also detected in

both the sugar beet pulp and the obtained hydrolyzates. The highest concentrations of hexoses, especially glucose and galactose, as well as pentoses, i.e. arabinose, were observed in the hydrolyzate from wet pulp (season 2) ($p < 0.05$). It is interesting to note the relatively high concentration of raffinose in the hydrolyzates, ranging from 13.79 ± 0.59 g/L (for briquetted pulp-based hydrolysate) to 21.24 ± 2.60 g/L (for wet pulp-based hydrolysate, season 2). The content of raffinose can differ in various locations. Low level of raffinose is usually observed for sugar beets with high sucrose concentration. A proportional effect on the level of raffinose has also the content of nitrogen in used fertilizer [19]. An important parameter in the composition of fermentation media is nitrogen content. Nitrogen is necessary for the proper fermentative activity of yeast cells [20]. The tested hydrolyzates contained relatively low amounts of total nitrogen (0.17 ± 0.03 to 0.27 ± 0.06% *w*/*v*). The media were therefore supplemented with nitrogen salts.

Table 1. Physicochemical parameters of hydrolyzates obtained from different types of sugar beet pulp.

Physicochemical Parameters	Type of Hydrolyzate		
	Briquetted Pulp	**Wet Pulp (Season 1)**	**Wet Pulp (Season 2)**
Extract (°Blg in 20 °C)	9.71 ± 0.55a	9.18 ± 0.58a	11.36 ± 0.76b
Reducing sugars (g/100 mL)	3.02 ± 0.22a	4.71 ± 0.26b	6.14 ± 0.34c
Total sugars as invert sugar (g/100 mL)	3.93 ± 0.26a	5.07 ± 0.34b	6.60 ± 0.52c
Volatile acids (% *w*/*v*)	1.05 ± 0.05a	1.71 ± 0.17b	1.94 ± 0.23b
pH	4.42 ± 0.03c	4.20 ± 0.04b	3.70 ± 0.02a
Nitrogen (% *w*/*v*)	0.27 ± 0.06a	0.17 ± 0.03a	0.22 ± 0.04a

Results expressed as mean values ± SE ($n = 3$); mean values in rows with different letters are significantly different ($p < 0.05$).

The carbohydrate fraction of the sugar beet pulp consists mainly of glucose, a monomer of cellulose, as well as xylose, glucose, mannose, galactose, and arabinose as building blocks of hemicelluloses [21]. Glucose with arabinose and galacturonic acid, together with smaller amounts of galactose and rhamnose, also build sugar beet pectin [18,22,23]. All these monomers were determined in the studied hydrolyzates (Table 2).

Table 2. Composition of carbohydrates obtained from different types of the sugar beet pulp hydrolyzate.

Carbohydrate (g/L)	Type of Hydrolyzate		
	Briquetted Pulp	**Wet Pulp (Season 1)**	**Wet Pulp (Season 2)**
Glucose	7.75 ± 0.70a	9.79 ± 0.54a	12.74 ± 1.19b
Fructose	5.76 ± 0.40a	5.90 ± 0.29a	5.46 ± 0.60a
Mannose	1.04 ± 0.14a	0.97 ± 0.17a	1.84 ± 0.45b
Arabinose	3.67 ± 0.50a	5.99 ± 0.87b	11.67 ± 0.82c
Galactose	4.52 ± 0.90a	7.19 ± 0.39b	14.18 ± 0.31c
Raffinose	13.79 ± 0.59b	18.61 ± 0.71a	21.24 ± 2.60a
Rhamnose	0.88 ± 0.59a	1.75 ± 0.08b	2.26 ± 0.30b
Xylose	0.31 ± 0.05a	0.36 ± 0.08a	0.70 ± 0.05b
Galacturonic acid	0.28 ± 0.04a	6.51 ± 0.44c	4.43 ± 0.19b

Results expressed as mean values ± SE ($n = 3$); mean values in lines with different letters are significantly different ($p < 0.05$).

2.2. Ethanol Fermentation of Sugar Beet Pulp Hydrolyzate

2.2.1. Selection of Strains Suitable for Fermentation of Hydrolyzates

Effective utilization of sugar beet pulp biomass-derived media presents several challenges. These relate mainly to the specific composition of the carbohydrates and to the fermentation abilities of used microoganisms [3,21,24]. In our study yeast were chosen for use in further experiments based on their assimilation and fermentation abilities (Table 3). Hydrolyzates of celluloses and hemicelluloses consist mainly of monomeric hexoses and pentoses. The glucose and xylose released can be fermented by *S. cerevisiae* and *Sch. stipitis*, respectively [25]. In our study, as well as the widely-used *S. cerevisiae* strain, five unconventional yeast strains were tested for their potential to utilize pentoses. Wild-type strains of *S. cerevisiae* are unable to utilize xylose. However, it has been reported that several genetically-modified *S. cerevisiae* strains ferment xylose, synthesizing ethanol. Native yeast species able to ferment xylose include Scheffersomyces and Candida species, as well as some strains of *K. marxianus* [26]. Due to its natural ability to assimilate pentose and hexose sugars, *K. marxianus* could provide an alternative option to conventional yeasts for second-generation ethanol fermentation [27]. The metabolic diversity of their various strains makes these species interesting for several potential applications [28].

Table 3. Fermentation abilities and carbohydrate assimilation profiles of tested yeast strains.

Yeast Strain	***Sch. stipitis* 1541 NCYC**			***O. angusta* 495 NCYC**			***K. marxianus* 179 NCYC**			***S. cerevisiae* Ethanol Red**			***K. marxianus* LOCK0026**			***Sch. stipitis* LOCK0047**		
Time (h)	**24**	**48**	**96**	**24**	**48**	**96**	**24**	**48**	**96**	**24**	**48**	**96**	**24**	**48**	**96**	**24**	**48**	**96**
Glucose	*	*	*	-	*	*	*	**	***	***	***	***	*	*	*	*	*	*
	−	−	+	−	−	−	+	+	+	+	+	+	−	+	+	−	+	+
Galactose	*	*	*	*	*	*	*	**	***	-	***	***	*	*	*	*	*	*
	−	−	+	−	−	−	+	+	+	+	+	+	+	+	+	−	+	+
Arabinose	*	*	*	-	*	*	-	*	*	-	-	-	-	-	-	*	*	*
	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+
Xylose	-	-	-	-	-	-	-	*	*	-	-	-	-	-	-	-	*	*
	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+
Rhamnose	*	*	*	*	*	*	-	*	*	-	-	-	-	-	-	*	*	*
	−	−	−	−	−	−	−	−	−	+	+	+	−	−	−	−	+	+
Mannose	*	**	**	*	*	*	*	***	***	***	***	***	*	*	*	*	**	**
	−	−	+	−	−	−	+	+	+	+	+	+	−	−	−	−	−	+

- No changes in optical density [0–0.5 MCF]; * Minimal changes in optical density [0.6–2 MCF]; ** Significant changes in optical density [3–7 MCF]; *** Strong changes in optical density [8–12 MCF]; − No gas in Durham tube; + Gas in Durham tube.

The metabolic abilities of the yeast strains were determined based on the profiles of saccharide monomers released from the beet pulp. All of the tested strains utilized glucose, galactose, and mannose for biomass proliferation. CO_2 production was observed mainly for *K. marxianus* 179 NCYC and *S. cerevisiae* (Table 3). Arabinose was assimilated by four of the tested strains, but only *Sch. stipitis* LOCK 0047 was able to ferment this carbon source, metabolizing xylose for this purpose. These strains were therefore selected for use in further experiments.

2.2.2. Ethanol Fermentation of Sugar Beet Pulp-Based Hydrolyzates

In the next stage of the investigation, the fermentation abilities of the selected yeast strains were verified in the actual media, i.e., in sugar beet pulp-based hydrolyzates. Fermentation of the prepared media was conducted using the commercial yeast *S. cerevisiae* and other yeast species, i.e., *K. marxianus* and *Sch. stipitis*. The yeast strain *S. cerevisiae*, commonly used in the distilling industry, is recommended for the fermentation of hexose sugars (including glucose) in hydrolyzates from starchy raw materials. According to the literature [29–32], *K. marxianus* is able to utilize various substrates, such as xylose, arabinose, cellobiose, glycerol, xylitol, lactose, and inulin. This is an advantage for the conversion of feedstock containing mixed carbon sources. Moreover, *K. marxianus* is able to utilize pentose and hexose sugars for cell biomass generation, fermenting glucose to ethanol, and pentose to xylitol [33]. *Sch. stipitis* reveal the ability to ferment many sugars, such as

glucose, galactose, mannose, xylose, and cellobiose, along with mannan and xylan oligomers [34]. Despite the fact, that a large number of yeast species can metabolize xylose, only 1% of strains ferment xylose to ethanol [35]. It is important to find the most efficient yeast species for the alcoholic fermentation of pentose sugars, including xylose, the main hemicellulosic sugar. Thus, *Sch. stipitis* is an attractive option for use in ethanol production from hemicellulose.

As shown in Figure 1, the highest production of ethanol was observed media fermented with *S. cerevisiae* and *Sch. stipitis* yeast strains, especially in briquetted sugar beet pulp-based hydrolyzates (12.6 g/L). Fermentation using single cultures of *S. cerevisiae* distillery yeast and *K. marxianus* NCYC179, as well as mixed cultures of these yeast strains (applied sequentially), resulted in lower yields of ethanol ($p < 0.05$).

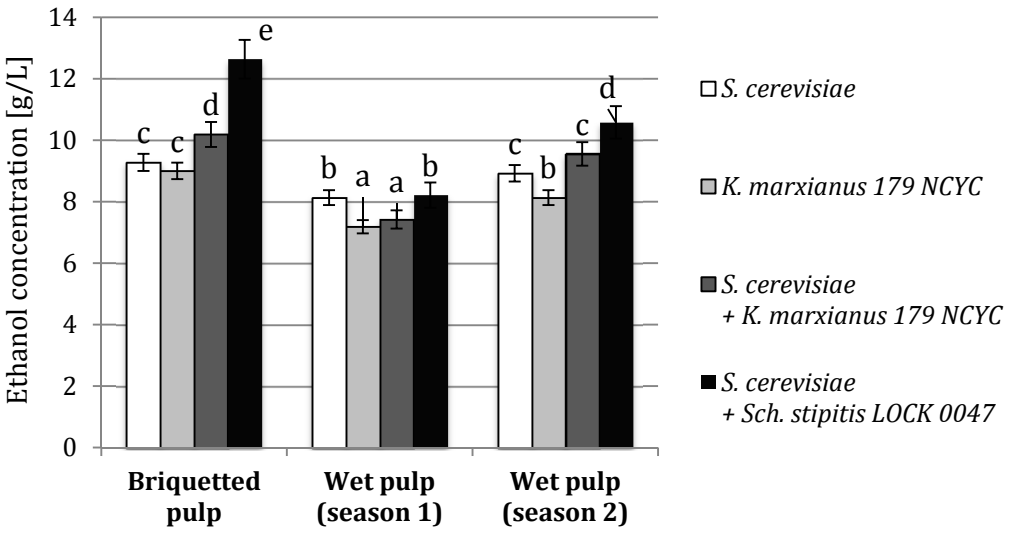

Figure 1. Effect of yeast strain on ethanol production in sugar beet pulp-based hydrolyzates. Mean values with different letters are significantly different ($p < 0.05$).

The highest ethanol yields were produced from briquetted pulp (see Figure 1), and as a consequence of the intake of sugars during the alcoholic fermentation was also highest for these worts (see Figure 2). The lowest intake of sugars was observed in fermentation trials from wet pulp (season 2), although ethanol concentrations in these hydrolyzates were higher than in the trials with wet pulp (season 1). This can be explained by the fact that the hydrolyzates from wet pulp (season 2) contained significantly higher ($p < 0.05$) concentrations of fermenting hexoses (i.e., glucose and galactose) and pentoses (i.e., arabinose and xylose) than the hydrolyzates of either briquetted pulp or wet pulp (season 1) (see Table 2).

A high content of fermenting sugars is advantageous from a technological point of view, because it helps to provide a high yield of ethanol from the raw material [36]. However, it requires the use of yeast strains that are resistant to the multiple stresses affecting the process, including ethanol stress and the osmotic stress that results from high sugar concentrations [37,38]. Moreover, yeast cells have specific growth requirements, leading to imbalances or limitations which can result in incomplete fermentation. These requirements include specific amounts of nitrogen, carbon, vitamins, oxygen, and metal ions [39]. In view of these facts, it may be supposed that higher intakes of sugars than those attained in our experiments could be achieved from the tested hydrolyzates using complex nutrients for yeasts.

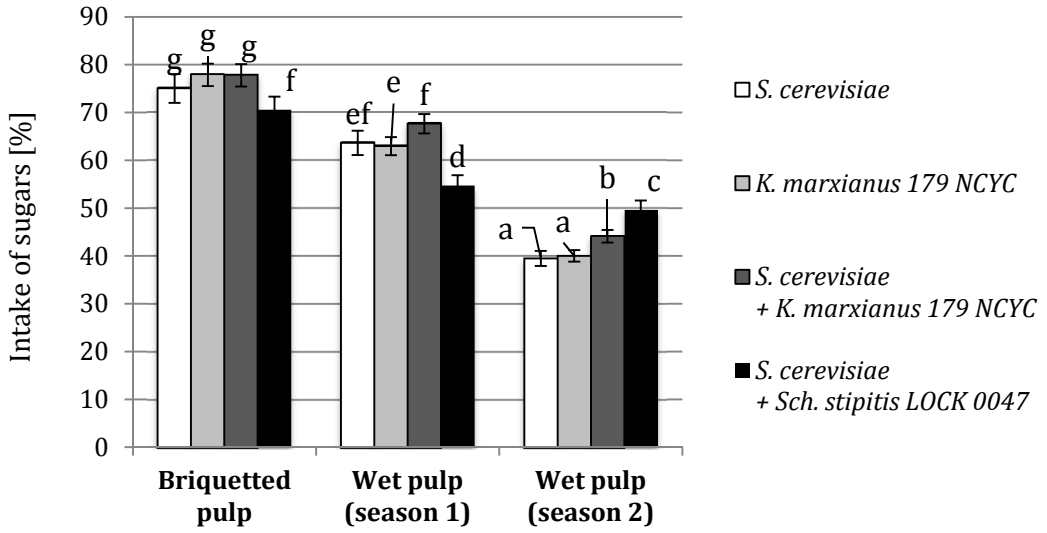

Figure 2. Intake of sugars in sugar beet pulp-based hydrolyzates fermented using different yeast strains. Mean values with different letters are significantly different ($p < 0.05$).

Ethanol production from hexoses such as glucose is a well-established industrial process. However, using pentoses such as xylose still presents some challenges [25]. Unconventional strains such as *K. marxianus* possess the useful potential to assimilate a wide variety of substrates, although difficulties may arise. *K. marxianus* has been tested for growth on a number of substrates, including xylose. Cultured in an oxygen-limited environment, five environmental isolates of *K. marxianus* showed the ability to consume xylose and produce ethanol [26]. *K. marxianus* can present different characteristics in terms of galactose utilization. Some studies have shown that ethanol production in media containing galactose was not as high as when glucose was the carbon source, while other research found galactose to be a better carbon source than glucose for ethanol production [28].

The carbohydrate utilization profiles of the tested yeast strains after fermentation of sugar beet pulp hydrolyzates (see Table 4) revealed that hexose sugars (i.e., glucose and mannose) were consumed in the largest amounts (78–100%) by all the tested yeast strains, including monocultures and co-cultures. Relatively low utilization was observed in the case of fructose. The addition of monocultures and co-cultures of *S. cerevisiae* and *K. marxianus* caused utilization of fructose, ranging widely from 17–46%, whereas fermentation with *S. cerevisiae* and *Sch. stipitis* resulted in full (100%) consumption of this sugar. It can be supposed that the *Sch. stipitis* yeast strain is able to consume fructose effectively, but it is not known whether this monosaccharide is metabolized exclusively to ethanol or to other products. Carbon catabolite repression usually takes place in media containing various sugars [40]. A potential solution to this important problem may be to ferment the hydrolyzates using mixed complementary cultures of conventional and unconventional strains. In parallel hexose and pentose sugar utilization, the lower utilization of pentoses by pentose-fermenting yeast strains presents a major problem. These strains prefer glucose over xylose as a source of carbon, and use glucose in the initial phase of fermentation [5]. For these reasons, we inoculated the fermentation medium with the pentose-fermenting strain after 24 h, once most of the hexoses had been utilized. Mixed populations generally showed the potential to utilize a broader spectrum of the sugars released from the beet pulp than the yeast monocultures (see Table 4). Especially in the case of galactose, but also in the cases of arabinose, xylose, rhamnose, and galacturonic acid, the intakes were highest in fermentation trials using *S. cerevisiae* and *Sch. stipitis* co-cultures.

Table 4. Carbohydrate utilization profiles of the tested yeast strains for fermentation of sugar beet pulp hydrolyzates.

Strain	Compound Utilization (%)								
	Glucose	**Fructose**	**Mannose**	**Arabinose**	**Galactose**	**Raffinose**	**Rhamnose**	**Xylose**	**Galacturonic Acid**
Briquetted pulp hydrolyzate									
S. cerevisiae Ethanol Red	99	46	100	0	0	12	0	0	0
K. marxianus 179	100	44	100	0	0	0	0	0	0
S. cerevisiae Ethanol Red + *K. marxianus* 179	100	39	100	0	0	34	0	0	0
S. cerevisiae Ethanol Red + *Sch. stipitis* LOCK0047	100	100	78	93	92	36	62	89	81
Wet pulp (1) hydrolyzate									
S. cerevisiae Ethanol Red	100	40	100	16	2	46	0	0 *	3
K. marxianus 179	100	38	100	8	23	51	0	0 *	0 *
S. cerevisiae Ethanol Red + *K. marxianus* 179	100	37	100	11	7	56	0	0 *	0 *
S. cerevisiae Ethanol Red + *Sch. stipitis* LOCK0047	99	100	94	90	89	52	54	67	81
Wet pulp (2) hydrolyzate									
S. cerevisiae Ethanol Red	99	17	95	8	9	37	0	0 *	0 *
K. marxianus 179	99	0	95	0	0	0 *	0	0 *	0 *
S. cerevisiae Ethanol Red + *K. marxianus* 179	100	29	100	15	15	40	0	0 *	0 *
S. cerevisiae Ethanol Red + *Sch. stipitis* LOCK0047	100	100	80	71	91	49	52	86	70

* Increase in carbohydrate concentration.

Using mixed cultures of complementary strains for utilizing carbon sources, as well as the controlled inoculation of *S. cerevisiae* and non-Saccharomyces yeasts represents a feasible way of improving the complexity and productivity of fermentation processes. *S. cerevisiae* is known to be rather ineffective at fermenting lignocellulosic hydrolyzates. However, other yeasts assimilate a broader spectrum of carbon compounds. Yeast strains belonging to Pichia (Schefersomyces) genera could also be used in co-cultures with conventional yeasts.

Fermentation of sugars other than glucose (galactose, mannose, arabinose, xylose) has been reported after inoculation of sugar beet hydrolyzate (SBH) with *Schefersomyces stipitis* [3]. However, compared to *Saccharomyces cerevisiae*, *Schefersomyces stipitis* is known to be less effective for the production of ethanol. *Schefersomyces stipitis* has also been reported to consume 45% of the xylose and 0% of the arabinose content in SBH [25].

2.3. Hydrogen and Methane Production from Suger Beet Residues

Distilleries usually produce 13–20 L of stillage for every liter of ethanol [9]. Stillage is a waste product that currently remains unused, with significant economic and ecological costs. The average composition of the stillage obtained after fermentation of sugar beet pulp hydrolyzate is shown in Table 5.

Table 5. Composition of carbohydrates in stillage obtained after ethanol fermentation of sugar beet pulp hydrolyzate.

Medium	Compound (g/L)								
	Glucose	Fructose	Mannose	Arabinose	Galactose	Raffinose	Rhamnose	Xylose	Galacturonic Acid
Sugar beet stillage	0.41 ± 0.03ab	0.47 ± 0.05b	0.36 ± 0.05a	7.80 ± 0.60f	7.42 ± 0.62f	14.45 ± 0.9g	6.04 ± 0.56e	4.20 ± 0.40d	3.29 ± 0.29c

Mean values with different letters are significantly different ($p < 0.05$).

Increasing the yield of renewable energy from biomass could be achieved by producing a combination of bioethanol and biogas. Integrating these bio-processes can improve both the energy and mass yields of the individual processes [1]. This is particularly important when using media rich in saccharides, such as sugar beet pulp hydrolyzates. As shown in Figure 2, the pool of non-fermented compounds in the hydrolyzates tested in our study varied from 22–60% of the total carbohydrates. These values depended on the type of yeast strain, the mode of fermentation, and the composition of the hydrolyzate. The use of mixed cultures improved fermentation efficiency. However, in the case of hydrolyzate from season 2, the amount of non-utilized carbohydrates (mainly raffinose) was relatively high, while xylose and galacturonic acid concentrations increased during fermentation. This shows that further hydrolysis of the sugar beet pulp, oligo- and polisaccharides had occurred. The compounds released, as non-fermented carbon sources, could provide an important raw material for subsequent biotechnological process, leading to biogas or hydrogen.

The SBH, SBS, and sugar beet pulp residues (SBPR) were subjected to anaerobic digestion to assess their potential for use in the production of methane and hydrogen. The results are summarized in Table 6 and Figures 3–5.

Hydrogen production was a very intensive process, with an extremely slow lag phase of around 0.5 h. Most hydrogen production was achieved within the first two days with all of the tested materials. The largest hydrogen yield (252 dm^3 H_2/kg volatile solids (VS)) was achieved from SBS with thermal pretreatment and pH adjustment. In contrast, SBH with thermal pre-treatment, and pH adjustment yielded 229 dm^3 H_2/kg VS. The corresponding value for SBPR was only 150 dm^3 H_2/kg VS. In the production of hydrogen through dark fermentation, saccharide-rich substances are depolymerized in anaerobic conditions by hydrogen-producing facultative anaerobes and obligate anaerobe. SBH is rich mainly in glucose (9.79–12.74 g/L) (Table 2), and might be expected to provide a high hydrogen yield. In comparison to SBH, SBS contains lower amounts of carbohydrates, and therefore might be expected to be a worse substrate for the generation of bio-hydrogen (Table 6). However, in practice there were no significant differences between the hydrogen yields from the substrates. A possible explanation for this may be the presence of yeast biomass in SBS, which supplies this substrate with missing components such as carbohydrates, nitrogen or phosphorus.

Table 6. Parameters for batch digestion tests with sugar beet stillage (SBS), sugar beet hydrolyzate (SBH) and, sugar beet pulp residues (SBPR).

Parameter	Unit	SBPR	SBS	SBH
Mass of substrate	G	24a	154c	134b
Substrate Volatile Solids (VS)	g/kg	126.34 ± 8.56b	34.25 ± 1.11a	39.62 ± 1.34b
Mass of inoculum	G	500a	500a	500a
Inoculum VS	g/kg	21.21 ± 1.20a	21.21 ± 1.20a	21.21 ± 1.20a
Duration time	D	10a	10a	10a
		without pretreatment		
Specific methane production (SMP)	dm^3 CH_4/kg VS	188.14 ± 5.68bB	444.91 ± 7.25cB	2.27 ± 0.24aA
Specific hydrogen production (SHP)	dm^3 H_2/kg VS	77.61 ± 1.87cA	35.15 ± 2.63aA	46.90 ± 1.67bA
		thermal pretreatment and pH adjustment		
Specific methane production (SMP)	dm^3 CH_4/kg VS	2.53 ± 0.36aA	58.62 ± 2.14cA	17.99 ± 1.02bB
Specific hydrogen production (SHP)	dm^3 H_2/kg VS	150.10 ± 2.61aB	252.39 ± 1.95cB	229.24 ± 4.31bB

Results expressed as mean values ± SE (n = 3); mean values in lines with different letters are significantly different ($p < 0.05$); mean values of SMP (without and with pre-treatment) and SHP (without and with pre-treatment) in columns with different capital letters are significantly different ($p < 0.05$).

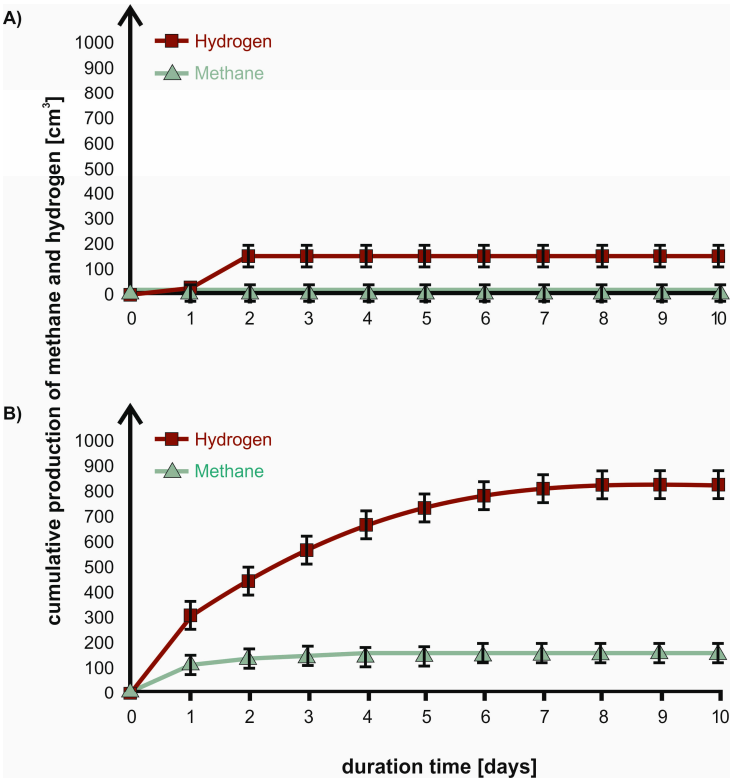

Figure 3. Cumulative methane and hydrogen production from sugar beet stillage (SBS) (**A**)—without pretreatment; and (**B**)—with thermal pretreatment and pH adjustment in batch experiments.

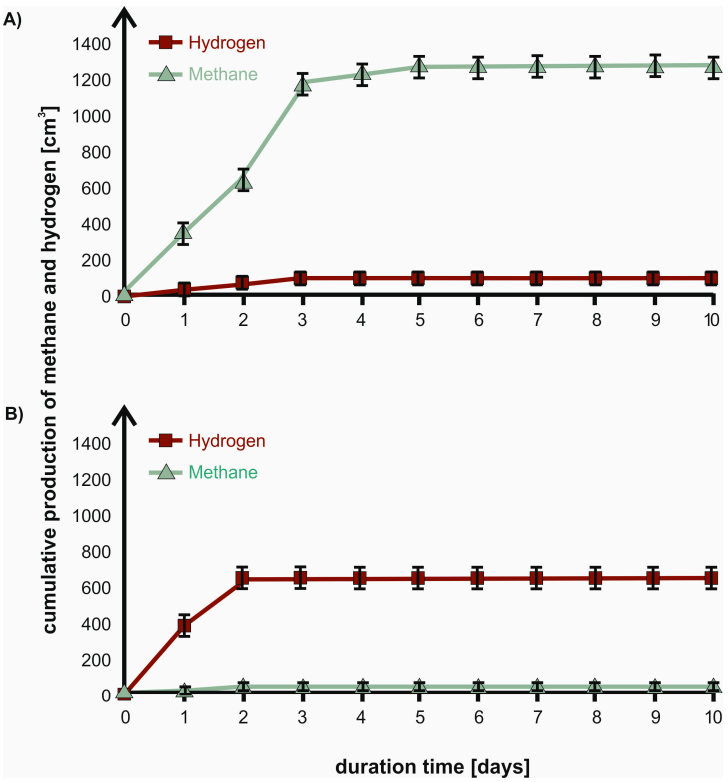

Figure. 4. Cumulative methane and hydrogen production from sugar beet hydrolyzate (SBH) (**A**)—without pretreatment; and (**B**)—with thermal pretreatment and pH adjustment in batch experiments.

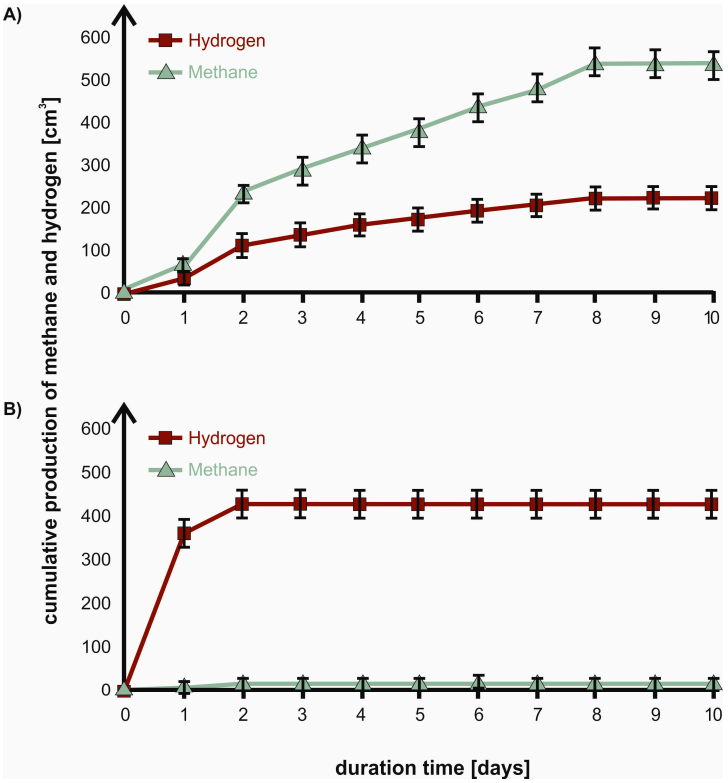

Figure 5. Cumulative methane and hydrogen production from sugar beet pulp residues (SBPR) (**A**)—without pretreatment; and (**B**)—with thermal pretreatment and pH adjustment in batch experiments.

Lignocellulosic biomass often requires treatment before processing. Zieminski et al. [41] showed that the partial enzymatic degradation of sugar beet pulp biomass enabled biogas production to be increased considerably. Liu et al. [8] reported that after the distillation of sugarcane bagasse, the components of the residual stillage were more available for anaerobic digestion. Sequential bioethanol and biogas production improved the yield and diversity of the products. According to Moshi et al. [42], both biomass-derived methane and ethanol production could be economically viable, depending on the geography and the existing infrastructure. In our opinion, the integration of these processes would be optimal, especially in the case of second-generation ethanol production, since this strategy combines energy production with the management of by-products.

2.4. Mass and Energy Balance

Figure 6 and Table 7 shows the complete mass and energy balance for the proposed integrated system.

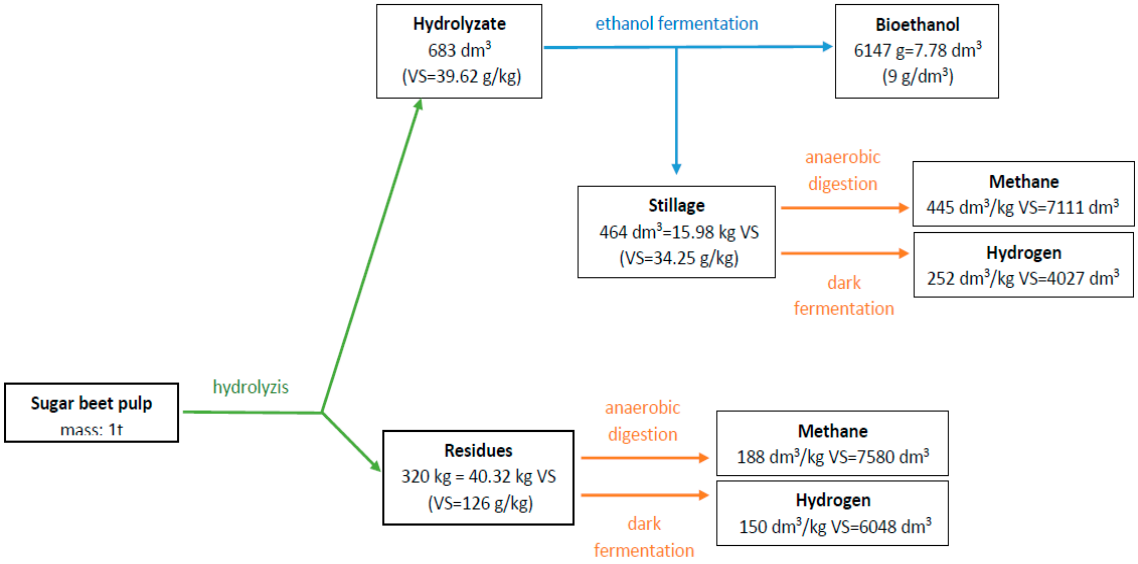

Figure 6. Mass balance.

Table 7. Energy balance.

Fuel	Volume (dm³)	Energy Yield Per Volume (kWh/m³)	Theoretical Energy Yield (kWh)	Energy Yield (% of Total)
Bioethanol	7.78	6570	51.11	21.57
Methane (stillage)	7111	10.20	72.53	
Methane (residues)	7580	10.20	77.32	
∑Methane			149.85	63.23
Hydrogen (stillage)	4027	3.58	14.42	
Hydrogen (residues)	6048	3.58	21.65	
∑Hydrogen			36.07	15.22

As described in an earlier study [43], one ton of the sugar beet pulp subjected to hydrolysis gave 683 dm³ of the liquid fraction (hydrolyzate), and 320 kg of beet pulp residues. The average specific bioethanol yield from the hydrolyzate was 9 g/dm³, giving a total bioethanol volume of 7.78 dm³. A residue of bioethanol production is stillage, which represented approximately 68% of the hydrolyzate. As shown in Figure 6, the total methane and hydrogen yields from the stillage were 7111 dm³ and 4027 dm³, respectively. Slightly greater methane and hydrogen yields were obtained from the anaerobic digestion of SBPR. Based on the energy balance calculations (Table 7), a total of 237 kWh (minus 10–20% as losses) could theoretically be achieved from 1 ton of fresh sugar beet pulp, with two-thirds of this energy in the form of methane (from both the stillage and SBPR), while

bioethanol would contribute around 22% of the total energy yield. Hydrogen would provide approximately 15% of the energy balance.

3. Materials and Methods

3.1. Biological Material

3.1.1. Yeast Strains

Five collection strains were used: *Scheffersomyces stipitis* (syn. *Pichia stipitis*) NCYC1541; *Ogataea angusta* (syn. *Pichia angusta*) NCYC495; *Kluyveromyces marxianus* NCYC179 obtained from the National Collection of Yeast Cultures (Norwich, UK); and, *Kluyveromyces marxianus* LOCK0026, *Scheffersomyces stipitis* (syn. *Pichia stipitis*) LOCK0047 from Lodz Culture Collection (Lodz, Poland), The commercial strain *Saccharomyces cerevisiae* Ethanol Red was also used (Fermentis Division S.I. Lesaffre, Marcq-en-Barœul, France).

3.1.2. Methane and Hydrogen Fermentation Inoculums

Anaerobic sludge was collected from an anaerobic mesophilic digester at the Municipal Wastewater Treatment Plant in Lodz, Poland. This was used as inoculum for the batch experiments. The inoculum had total and VS concentrations of 32.25 g TS/kg and 21.21 g VS/kg, respectively.

3.2. Feedstock: Sugar Beet Pulp Hydrolyzate

Fresh sugar beet pulp hydrolyzate was obtained from the Dobrzelin Sugar Factory (Dobrzelin, Poland). The sugar beet pulp, both briquetted and wet, was suspended in plain warm water to achieve a dry matter concentration of around 12% (*w*/*v*). The biomass was saccharified in a 3 m^3 reactor for 16 h at 50 °C, using a mixture (1:1) of two multi-enzyme preparations made by Novozymes: Viscozyme® (Bagsvaerd, Denmark) and Ultraflo® Max (Bagsvaerd, Denmark) (0.03 L/kg of sugar beet pulp dry weight). Saccharification was stopped by heating (80 °C for 10 min) [44].

The physicochemical parameters of the hydrolyzates are shown in the sections Results and Discussion.

3.3. Carbohydrate Fermentation Test

Glucose, galactose, arabinose, xylose, rhamnose, and mannose were tested as the main carbon sources for assimilation and fermentation processes. Sterile tubes of sugar broth containing 2% of one of the carbohydrates and 0.5% yeast extract were inoculated with the appropriate yeast suspension and incubated for 96 h. Assimilation of the tested carbohydrates was assessed in terms of changes in the optical density of the liquid sugar broth.

3.4. Propagation of Yeast Strains

Two-step propagation was performed. In the first, stationary stage, inoculum cultures were grown for 24 h at 30 °C in 100 mL Erlenmeyer flasks filled with 50 mL of liquid Yeast extract-peptone-glucose (YPG) medium, supplemented with xylose (1%). The inoculum was transferred into 1 L flasks with 100 mL YPG medium. Propagation was carried out on a rotary shaker (150 r.p.m.) for 48 h at 30 °C. The biomass obtained was washed twice with sterile physiological saline. The biomass yield was determined by drying the sample to a constant weight at 105 °C.

3.5. Ethanol Fermentation Using Sugar Beet Pulp Hydrolyzate

Fermentation experiments were carried out in 1 L glass flasks, each containing approximately 0.5 L of a medium based on sugar beet pulp hydrolyzate. The hydrolyzates were supplemented with $(NH4)_2HPO_4$ (0.3 g/L).

Fermentation was carried out in two modes: using a monoculture of *S. cerevisiae* Ethanol Red dry distillery yeast, or *Kluyveromyces marxianus* NCYC179, or mixed cultures applied sequentially, i.e., *S. cerevisiae* Ethanol Red and *Kluyveromyces marxianus* NCYC179 or *Scheffersomyces stipitis* LOCK0047. The process was initiated by Ethanol Red yeast and after 24 h the samples were inoculated with a second strain. The yeast slurry was added to the medium at a ratio of 2 g of yeasts dry mass/L. In the case of dry *S. cerevisiae* Ethanol Red, the yeast was hydrated (15 min incubation in water) and acid-washed (25% *w/w* sulfuric acid, pH 2.5). The flasks were closed with stoppers equipped with fermentation pipes, filled with glycerol, and kept in a thermostat-regulated room at 30 °C. Fermentation was conducted for 24 h, and the samples were then re-inoculated with one of the non-conventional yeast strains. Fermentation was continued for the next 48 h, with the entire process time amounting to 72 h. The process was controlled gravimetrically (a decrease in mass caused by the liberation of carbon dioxide). Finally, samples were collected to determine the concentrations of ethanol, hexose and pentose sugars.

Evaluation of Total Sugar Intake

The total sugar intake (percentage consumption of total sugars during fermentation) was calculated as the ratio of sugars used to their content in the fermentation medium prior to fermentation, expressed as a percentage.

3.6. Anaerobic Digestion of Stillage

3.6.1. Methane Fermentation

Batch experiments were carried out to determine the biochemical methane and biogas potential of the individual substrates. Biomethane potential (BMP) tests were performed in 1 dm^3 glass bottles, each of which was connected to a 1 dm^3 gas collecting tank to provide anaerobic conditions and to measure the biogas yield (by the water displacement method). Anaerobically-digested sewage sludge, collected from the Municipal Wastewater Treatment Plant in Lodz (Poland), was used as inoculum. Each reactor was initially filled with a 500 cm^3 batch of the inoculum, and the substrate was then added to achieve an inoculum-to-substrate ratio of 2:1 based on the content of VS. An inoculum-to-substrate (I/S) ratio of 2 is suggested as mandatory for standardized BMP tests, since it has not been found to inhibit methane production [45].

The headspace of each reactor was purged with nitrogen prior to closing, and then connected to the biogas collecting tank. The batch reactors were maintained at 35 °C using a thermostat. The batch reactors were shaken twice each day, and the volume of biogas produced was recorded every day. The experiments were ended at the point when biogas production stopped completely. All batch experiments were performed in triplicate. Three blanks with only 500 cm^3 of inoculum were also studied, to determine the gas productivity of the inoculum.

3.6.2. Dark Fermentation

The amount of hydrogen generated by the microbial consortia in an inoculum is affected by differences in the fermentative metabolisms of the bacteria and by interactions between them. Two experimental patterns were established for sugar beet residue (SBR), SBS, and SBH. In the first approach, the substrates were mixed in inoculum and subjected to anaerobic digestion, as described in Section 3.6.1 (control group). In the second approach, the substrates with inoculum were first treated with a 20% H_2SO_4 solution, to adjust the pH value to 5.5, and then heated using a laboratory dryer at 80 °C for 1.5 h. These operations were performed in order to deactivate hydrogen-consuming bacteria and methanogens, and to provide optimal conditions for hydrogen

generation. Experiments were then performed according to the procedure described in Section 3.6.1. All tests (6 reactors each for SBR, SBS, and SBH) were conducted in triplicate, and the results expressed as averages. The dark fermentation trials ran for 10 days.

3.7. Analytical Methods

3.7.1. Analysis of Hydrolyzate

The sugar beet pulp hydrolyzates were analyzed following the methods recommended for the sugar industry [46]. The total extract in the tested hydrolyzates was measured using an areometer with a scale in Balling degrees (°Blg), referring to the concentration of dissolved solids, mostly sugar, as the percentage weight of saccharose or maltose. Total nitrogen was determined using the Kjeldahl method [46]. Reducing sugars (after inversion with hydrochloric acid) were determined spectrophotometrically according to the Miller method. Both were expressed in g of invert sugar per 100 mL [46]. Volatile acids (expressed as acetic acid) were assayed using steam distillation. pH was measured using a digital pH-meter.

3.7.2. Monosaccharide Content

The monosaccharide profiles of the sugar in the beet pulp hydrolyzates (before and after fermentation) and stillage were analyzed using UV-spectrophotometry and Megazyme Kits (Megazyme, Inc., County Wicklow, Ireland). For glucose, mannose, and fructose, we used a K-MANGL kit; for arabinose, the K-ARGA kit; for galacturonic acid, the K-URONIC kit; for xylose, the K-XYLOSE kit; for raffinose, the K-RAFGA kit; and, for rhamnose, the K-RHAMNOSE kit [21]. The monosaccharide profiles of the sugar in stillage obtained after distillation of ethanol from the fermented medium were analyzed using the same procedures.

3.7.3. Ethanol concentrations

Prior to analysis, samples of the hydrolyzates were mixed with $ZnSO_4$ to final concentrations of 10% to induce protein precipitation. The solid debris was removed by centrifugation at 4000 rpm for 20 min. All samples were then filtered through 0.45 μm PES (polyethersulfone) membranes. The concentrations of ethanol in the media and in post-fermentation effluents were determined using HPLC (Agilent 1260 Infinity, Santa Clara, CA, USA) on a Hi-PlexCa column (7.7 mm × 300 mm, 8 μm) (Agilent Technologies, Santa Clara, CA, USA) equipped with a refractive index detector (RID) at 55 °C. Column temperature was maintained at 80 °C. HPLC grade water was used as a mobile phase with a flow rate of 0.6 mL/min and a sample volume of 20 μL.

3.7.4. Methane and Hydrogen Potential

The substrates were analyzed for total and volatile solids (TS, VS) and pH, based on the Standard Methods for the Examination of Water and Wastewater (APHA, 2012). Biogas yield was monitored on a daily basis using the water displacement method, as described in the literature [47]. The composition of the biogas was analyzed using a portable gas analyzer GA-21 plus (Madur Electronics, Vienna, Austria), equipped with electrochemical sensors to measure the following gases: O_2, CO_2, CH_4, H_2, H_2S.

3.8. Statistical Analysis

All samples were prepared and analyzed in triplicate. The results were tested statistically by analysis of variance (one-way ANOVA), at a significance level $p \leq 0.05$ using STATISTICA 10.0 software (StatSoft, Tulsa, OK, USA) to indicate differences. If statistical differences were detected ($p < 0.05$), the means were compared using Tukey's test (with a significance level of 0.05).

4. Conclusions

This article has presented a concept for the management of waste sugar beet pulp biomass, delivering three valuable bio-products: ethanol, methane, and hydrogen. The medium obtained after enzymatic hydrolysis is a source of carbohydrates that can be metabolized by ethanol-synthesizing yeast, or by bacterial communities to produce hydrogen. Fermentation can be considered as an effective pre-treatment of sugar beet pulp hydrolyzate as a raw material for methane biosynthesis. The resulting stillage is 200 times more suitable for this purpose than unfermented hydrolyzate, while remaining still a good substrate for hydrogen production. The integration of ethanol fermentation and anaerobic digestion allows for comprehensive utilization of the tested raw material with an improved energy balance.

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