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The Ethanol Production from Sugar Beet Pulp Supported by Microbial Hydrolysis with *Trichoderma viride*

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Abstract: Despite the significant progress in the research, the problem of finding an efficient method for producing bioethanol from renewable lignocellulosic waste materials remains unresolved. Our investigation aimed to assess the efficacy of ethanol production from sugar beet pulp (SBP) utilising various approaches, including pretreatment variations, enzymatic processes, and microbial hydrolysis. Our research involved using the post-cultivation concentrate of *T. viride* LOCK 0588 grown in the SBP-based medium as a source of enzymes. The SBP hydrolysis process was carried out for 48 h at 50 °C. The quantity of sugar released, up to 61 g dm^{-3}, through the utilisation of this extract proved to be on par with the outcomes achieved by the application of the commercial Cellic Ctec2 preparation. The final yields of the ethanol production with the use of the coculture of *S. cerevisiae* (Ethanol Red) and *Scheffersomyces stipitis* LOCK 0047 strain were in the range 5.1 ± 0.11 kg 100 kg^{-1} ÷ 5.38 ± 0.11 kg 100 kg^{-1}. These results provide a solid basis for improving larger-scale industrial procedures that involve converting SBP into bioethanol using a cost-efficient approach of microbial hydrolysis with *T. viride* and a blend of pentose and hexose fermenting yeast.

Keywords: sugar beet pulp; bioethanol; *Scheffersomyces*; Ethanol Red; ethanol fermentation

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1. Introduction

Approximately 15 million tons of sugar beet are harvested annually in the European Community, about 50% of global production. The main product of more than ninety existing sugar factories is crystalline sucrose, but during this process, two by-products are also created: sugar beet pulp (SBP) and molasses. Molasses, containing around 50% sucrose, is the desired raw material in biotechnological processes, obtaining ethanol, fodder production, and baking. One ton of sugar beet processing leads to receiving about 500 kg of wet sugar beet pulp (100–125 kg of dry matter), 150–170 kg of sucrose, and 38–41 kg of molasses [1,2].

Sliced thin wedges of sugar beet root, after sucrose extraction with hot water, are called sugar beet pulp. It is typically compressed or dehydrated for use in animal nutrition. The yearly production of SBP in Europe reaches around 7–8 million tons of pressed and about 2–3 million tons of dried product [2].

In the strict world of economic demands, sugar factories are seeking various methods of molasses and SBP valorisation. In this task, biotechnologists offer valuable support in facilitating the conversion of lignocellulose-containing sugar beet pulp into bioethanol. Despite the continuous development of alternatives to carbon-containing fuels for transportation, bioethanol remains one of the most demanded forms of energy used in cars and planes [3–5].

Beet pulp can be transformed into ethanol for fuel purposes after combining physicochemical and enzymatic hydrolysis, followed by biochemical transformations in yeast cells starting from mono-sugar assimilation towards ethanol and carbon dioxide formation. SBP primarily comprises polysaccharides, including cellulose (20–30%), hemicelluloses (20–30%), lignins (1–2%), and pectins (35–60%), which together are up to 85% of the dry matter [6,7]. Before ethanolic fermentation, biopolymers from pulp must be degraded into fermentable mono-sugars. To enable easier accessibility of enzymes to the cellulose chains, the raw material is usually subjected to physicochemical pretreatment, leading to the disruption of lignin and the crystalline structure of the raw material matrix. Cellulose, hemicellulose, and pectins can be broken down into monosaccharides through the action of cellulases, hemicellulases, and pectinases or by acids, primarily sulfuric, nitric, or hydrochloric. Glucose, galactose, and mannose released from lignocellulosic raw materials are pretty easily fermented to ethanol by most distiller strains of *Saccharomyces cerevisiae*. Still, pentoses can be fermented to ethanol by fewer strains, e.g., *Scheffersomyces stipitis* [8–13]. Combined fermentations carried out by the yeasts *S. cerevisiae* and *Scheffersomyces stipitis* are often used in bioconversion processes of lignocellulosic feedstocks to ethanol. These strains are used simultaneously or added to media containing a mixture of hexoses and pentoses sequentially to convert the available sugars to ethanol as efficiently and rapidly as possible [14–17]. While acid treatment at high temperatures is the most economical method of lignocellulose hydrolysis, it faces limitations due to stricter environmental regulations. As a result, newer techniques are being developed to reduce chemical usage and incorporate their circulation within the process, which comes with challenges.

In contrast, enzymatic methods are widely considered eco-friendly; however, the financial feasibility of utilising enzymes has been questioned. As a result, significant attention has been directed towards the microbial saccharification of raw materials, which involves the on-site production of lignocellulose-degrading enzymes by selected microorganisms. Microbiological hydrolysis appears to be the golden mean between economical yet non-ecological physicochemical and efficient but costly enzymatic hydrolysis methods. Microbiological hydrolysis, known for centuries (e.g., in koji production) [18], is gaining new applications in recent years concerning the hydrolysis of lignocellulosic materials. As microorganisms for this method, mould strains capable of extracellularly secreting a wide range of enzymes, including cellulases, are most commonly used [19–22]. This results in the liberation of monosaccharides from the polysaccharide substrate. The chief disadvantage of employing this treatment is the reduction in polysaccharides for the proliferation of cellulose-producing organisms. Nevertheless, utilising cost-efficient raw materials renders it a promising technique for the hydrolysis of lignocellulosic materials [4,9,11,23].

The purpose of our study was to assess the efficiency of producing ethanol from SBP by utilising different approaches to pretreatment, enzyme, and microbial hydrolysis assisted by the *Trichoderma viride* strain, along with fermentation using *Saccharomyces cerevisiae* and *Scheffersomyces stipitis* yeast.

2. Materials and Methods

Raw sugar beet pulp in thin strips (approx. $2 \times 2 \times 30 \div 60$ mm) was obtained from the Dobrzelin Sugar Factory (Dobrzelin, Poland) and stored at -20 °C until used.

Cellulose content was determined according to the Kürschner-Hoffer [24], hemicellulose according to Araschimovich and Ermakov [25], and lignin content according to the method recommended by the National Renewable Energy Laboratory (NREL) [26]. The sugar beet pulp was analysed using methods recommended for the sugar industry [27]. The dry matter content (DM) was measured using a Radwag (Radom, Poland) WPS-30S weighing dryer. Reducing sugars and total sugars (after mild hydrolysis at 72 °C with HCl) were determined according to the standard Miller method [28] and expressed in g of inverted sugar per kg of SBP dry matter. The sucrose content in sugar beet pulp was determined by subtracting the amounts of total and reducing sugars, using a conversion factor of 0.95.

To increase the yield of sugar released, the thermo-chemical pretreatment (PRE) was performed according to three methods:

"AA" (Acid Autoclaved)—3 dm^3 of 2% of sulfuric acid was mixed with 2 kg of SBP and autoclaved at 121 °C for 60 min;

"AB" (Acid Boiled)—3 dm^3 of 2% sulfuric acid was mixed with 2 kg of SBP and boiled at 100 °C for 60 min;

"WB" (Water Boiled)—3 dm^3 of water was mixed with 2 kg of SBP and boiled at 100 °C for 60 min.

After the pretreatment, the mixtures were cooled down, and the pH was adjusted to 5.3–5.5 using 30% NaOH.

Enzymatic hydrolysis (E) was performed in two options: "Eh" with the use of enzymatic preparation Cellic CTec2 and "Mh" with post-cultivation effluent of *Trichoderma viride* LOCK 0588 in SBP medium obtained as described below. In both methods (Eh and Mh), the final cellulase dose was 5 FPU/g of cellulose, and the process was carried out for 48 h at 50 °C.

To initiate the microbial production of cellulase with the use of *Trichoderma viride* LOCK 0588 for Mh hydrolysis, a similar procedure to that described by Zakkpa et al. [22] was performed, but using the dried SBP instead of corncobs. Potato dextrose agar (PDA) with chloramphenicol (500 mg cm^{-3}) to suppress bacterial growth was used as an activating/storage medium. The inoculated slants were incubated for 6 days at 25 °C. The SBP medium was prepared and consisted of the following per litre: $(NH_4)_2SO_4$ 1.4 g, $CaCl_2$ 0.3 g, KH_2PO_4 2.04 g, urea 2.1 g, $MgSO_4 \cdot 7H_2O$ 0.3 g, citric acid 0.25 g, Tween 80 2 cm^3, peptone 1 g, beet pulp powder 10 g, and trace mineral stock solution 1 cm^3. The trace mineral stock was prepared by mixing 500 cm^3 with $MnSO_4 \cdot H_2O$ 0.93 g, $FeSO_4$ 2.55 g, $Co(NO_3)_2 \cdot 6H_2O$ 1.25 g, $ZnSO_4 \cdot H_2O$ 1.78 g, and concentrated HCl 5 cm^3. The beet pulp was oven-dried at 120 °C for 24 h and then ground and sieved (0.2–0.5 mm) to obtain the powder. A total of 100 cm^3 of SBP medium was transferred to each of the 12 Erlenmeyer flasks (total capacity 250 cm^3 each). *T. viride* inoculum was prepared by flooding potato dextrose agar (PDA) slants with 10 cm^3 sterile distilled water. Conidia were scratched with an inoculation loop, and the tubes were shaken vigorously to obtain a homogeneous suspension. The spores were counted using a haemocytometer. Approximately $1 \div 1.2 \times 10^7$ spores of each isolate were inoculated into a conical flask containing 100 cm^3 of autoclaved SBP under laminar flow of sterile air. The inoculated flasks were incubated on a shaker at 150 rpm at room temperature (25 ± 2 °C) for 14 days. The liquid phase of the post-cultivation medium was then filtered using a 0.45 um bacteriological filter and then concentrated in a rotary vacuum evaporator (45 ± 1 °C; 110–130 mbar; 20 rpm) to 10% of its original volume, and such a concentrated solution (stored at −20 °C before hydrolysis) was used as a cellulase source in our experiments.

Before fermentation, hydrolysates were enriched with nitrogen and magnesium compounds. Nitrogen was supplemented by diammonium hydrogen phosphate $(NH_4)_2HPO_4$ (0.3 g dm^{-3}). Magnesium was added as $MgSO_4 \cdot 7H_2O$ at 0.05 g dm^{-3} of medium. The pH was then adjusted (pH = 5.2 ± 0.1)

Fermentation (at 32 ± 1 °C for 96 h) was carried out in 2 dm^3 flasks (without agitation) containing 1 dm^3 of the medium using 2 variants: 1) using only the preparation of Ethanol Red (ER) dry distillery yeast (*S. cerevisiae*) (Fermentis Division S.I. Lesaffre, France) at a dose of 0.5 g DM dm^{-3} of hydrolysate; 2) using a mixed culture of Ethanol Red yeast (0.3 g DM dm^{-3} of hydrolysate) and *Scheffersomyces stipitis* (*SS*) LOCK 0047 (LOCK 105, Lodz University of Technology, Poland) (1 g DM dm^{-3} of hydrolysate) applied simultaneously at the beginning of the fermentation.

The concentrations of glucose (GLU), galactose (GAL), fructose (FRU), xylose (XYL), rhamnose (RHA), arabinose (ARA), sucrose (SAC), raffinose (RAF), cellobiose (CEL), and ethanol in the media were analysed using High-Performance Liquid Chromatography (HPLC) with an Agilent 1260 Infinity system (Agillent Technologies, Santa Clara, CA, USA) and a Hi-Plex Ca column (7.7 × 300 mm, 8 μm) (Agilent Technologies, USA) equipped

with a refractive index detector (RID) operating at 55∘C. The column temperature was maintained at 80 °C. HPLC-grade water served as the mobile phase, flowing at a rate of 0.6 mL/min, and a sample volume of 20 µL was used. Prior to analysis, samples were mixed with zinc sulfate solution to achieve a final concentration of 10% for protein precipitation. Sediments were removed through centrifugation at 7000 rpm for 10 min, and before analysis, samples were filtered using 0.45 µm Teflon membranes.

The ethanol gain was quantified as the absolute ethanol amount obtained from 100 kg of wet SBP. The fermentation yield (F_y) was calculated for the assayed fermentable sugars and expressed as a percentage of the theoretical yield according to the following formula: $F_y = (E_c \times 100\%)/(F_s \times 0.51)$, where E_c is the ethanol concentration in the fermented medium [g/L]; F_s is the sum of assayed fermentable sugars; and 0.51 is the constant representing the theoretical yield of ethanol from glucose and xylose.

All assays were carried out in triplicate. Statistical analysis (variance analysis, SD determination, Student's *t*-test at significance level a = 0.05) was carried out using the Origin 7.5 computer program.

3. Results and Discussion

3.1. Parameters of Sugar Beet Pulp

The dry matter content of the SBP used in our experiments was equal to 243.4 ± 8.8 g/kg. Dominant parts of this were hemicellulose (388.4 ± 15.8 g/kg of DM), cellulose (336.9 ± 16.6 g/kg of DM), and sucrose (134.8 ± 10.52 g/kg of DM). Some mono-sugars expressed as inverted sugar and lignin were also present in SBP dry matter (10.1 ± 0.48 g/kg of DM and 1.3 ± 0.02 g/kg of DM, respectively). The remaining, not specified, constituents of analysed SBP dry matter were probably pectines and mineral compounds. These results (Table 1) correspond with the results reported in many papers describing the properties of sugar beet pulp [2,6,11,29–32]. Some fluctuations are apparent, depending on the sugar beet variety, cultivation method, harvesting, extraction technology, and storage conditions of SBP.

Table 1. Chemical composition of sugar beet pulp.

Parameter	Value
Dry matter [g/kg]	243.4 ± 8.8
pH	5.8 ± 0.1
Reducing sugars as inverted sugar (g/kg DM)	10.1 ± 0.48
Sucrose (g/kg DM)	134.8 ± 10.52
Raffinose (g/kg DM)	2.5 ± 0.04
Cellulose (g/kg DM)	336.9 ± 16.6
Hemicellulose (g/kg DM)	388.4 ± 15.8
Lignin (g/kg DM)	1.3 ± 0.02

3.2. Sugar Content in the SBP Hydrolysates

The first step of these investigations was to assay sugar content in sugar beet pulp hydrolysates obtained using six combinations of pretreatment and main hydrolysis. The sugar content in the obtained hydrolysates is shown in Table 2.

The general pool of simple sugars in the analysed hydrolysates, obtained in the sequence of various pretreatments and hydrolysis with the use of the commercial enzyme or obtained with the use of *T. viride* in our experiment, was dominated by glucose. Its content varied from 8.35 ± 0.35 g dm^{-3} (WB/Eh sample) to 28.72 ± 0.44 g dm^{-3} for the AA/Eh combination of the pretreatment and hydrolysis method. As for other saccharides found in more significant amounts, rhamnose was found in the hydrolysates, and its concentrations started from 1.03 ± 0.011 g dm^{-3} (for WB/Eh hydrolysis), and the highest was spotted for the AA/Mh sample 8.27 ± 0.22 g dm^{-3}. Fructose concentration ranged from 2.74 ± 0.18 to 5.28 ± 0.21 g dm^{-3} of the WB/Eh and AA/Mh hydrolysates, respectively. Arabinose values varied from 0.31 ± 0.02 g dm^{-3} for the WB/Eh sample to 4.92 ± 0.17 g dm^{-3} for the AA/Mh hydrolysis sequence. Xylose ranged from 00.9 ± 0.008 g dm^{-3} (WB/Eh) to

4.92 ± 0.16 g dm^{-3} (AA/Mh). Sucrose's lowest value was noted for the AA/Eh sample (1.57 ± 0.08 g dm^{-3}), the highest of the WB/Mh sample (2.45 ± 0.11 g dm^{-3}). Comparing the influence of prehydrolysis and hydrolysis sequence efficiency on sugar content, it is seen ($p < 0.001$) that if the more intensive pretreatment was applied to the sample (higher temperature and acid added), the higher concentration of specific sugar in the hydrolysate was observed, and the "sum" value at the bottom of each column depicts it. Also, when comparing the microbial and enzymatic hydrolysis for the same pretreatment method, the final concentrations of specific sugars showed a slight difference but were not always statistically significant ($p > 0.05$). The samples hydrolysed with the post-cultivation extract of *T. viride* usually contained more specific sugar than those obtained with enzyme hydrolysis. It may be elucidated that *T. viride* preparation probably included additional enzymes supporting the cellulases in the decomposition of the pretreated SBP matrix. For sucrose, the lowest concentration was spotted for the AA/Eh method (1.57 ± 0.008 g dm^{-3}). The highest was for the WB/Mh treated sample (2.45 ± 0.11 g dm^{-3}), and it was the generally reverse trend in comparison to other sugars, but it can be elucidated by the fact that sucrose, during more intensive pretreatment (temperature or acid present), decomposes partially into glucose and fructose, while the other sugars are formed from polysaccharide decomposition where, to some extent, a more intensive method results in more efficient hydrolysis. The content of specific sugars in the hydrolysates closely correlates to those presented by Berłowska et al. [11,29] and Patelski et al. [33]. According to the literature, rhamnose in sugar beet roots is bound with galacturonic acid by α-1-4-glycosidic bonds, which form long "smooth" regions. In contrast, α-1-2-glycosidic bonds create branched regions of the pectin chains [34]. The rest of the rhamnose is linked by α-1-5-glycosidic bonds with arabinose chains, built into the branch at position 3 of 1-4 bound β-galactans with a low degree of polymerisation and highly branched galactans with 1-3 and 1-6 bonds [35]. According to the literature, the sugars mentioned above can be found in sugar beet roots, which explains their presence in the analysed substrates [7,36].

Table 2. Sugar content in SBP hydrolysates obtained with different methods.

Sugar	PRE/H Combination; Sugar Concentration [g dm^{-3}]					
	WB/Eh	**WB/Mh**	**AB/Eh**	**AB/Mh**	**AA/Eh**	**AA/Mh**
SAC	2.34 ± 0.1	2.45 ± 0.11	1.93 ± 0.09	1.86 ± 0.07	1.57 ± 0.08	1.82 ± 0.09
FRU	2.74 ± 0.18	2.95 ± 0.19	3.66 ± 0.19	3.72 ± 0.21	5.48 ± 0.21	5.28 ± 0.21
GLU	8.35 ± 0.35	8.97 ± 0.33	18.43 ± 0.37	19.13 ± 0.24	26.53 ± 0.64	28.72 ± 0.44
GAL	2.33 ± 0.42	3.12 ± 0.38	4.91 ± 0.12	4.83 ± 0.18	6.63 ± 0.21	6.21 ± 0.18
ARA	0.31 ± 0.012	1.21 ± 0.12	2.71 ± 0.13	2.42 ± 0.11	4.12 ± 0.18	4.92 ± 0.17
RHA	1.03 ± 0.011	1.82 ± 0.06	3.84 ± 0.37	3.63 ± 0.31	6.63 ± 0.42	8.27 ± 0.22
XYL	0.09 ± 0.008	0.99 ± 0.009	3.23 ± 0.12	3.43 ± 0.15	4.13 ± 0.17	4.92 ± 0.16
sum	17.19	21.51	38.71	39.02	55.09	60.14

3.3. Sugar Uptake during Fermentation

Hydrolysates, enriched in necessary microelements, were fermented using Ethanol Red as the monoculture or coculture with the *Scheffersomyces stipitis* strain. The results of sugar remaining after fermentation are presented in Tables 3 and 4.

Table 3. Sugar content in SBP hydrolysates after 96 h of fermentation with Ethanol Red yeast.

Sugar	PRE/H Combination; Sugar Concentration [g dm^{-3}]					
	WB/Eh	**WB/Mh**	**AB/Eh**	**AB/Mh**	**AA/Eh**	**AA/Mh**
SAC	0.19 ± 0.005	0.35 ± 0.003	0.35 ± 0.002	0.82 ± 0.003	0.27 ± 0.006	0.82 ± 0.002
FRU	0.21 ± 0.008	0.11 ± 0.003	0.46 ± 0.004	0.46 ± 0.003	0.21 ± 0.004	0.21 ± 0.005
GLU	0.26 ± 0.011	0.73 ± 0.011	0.83 ± 0.01	1.01 ± 0.008	0.12 ± 0.002	0.62 ± 0.009
GAL	1.53 ± 0.06	2.82 ± 0.08	2.32 ± 0.12	2.32 ± 0.04	2.12 ± 0.08	1.87 ± 0.07
ARA	0.29 ± 0.01	1.23 ± 0.09	2.53 ± 0.04	2.42 ± 0.03	4.11 ± 0.12	4.88 ± 0.07
RHA	0.94 ± 0.01	1.73 ± 0.02	3.81 ± 0.09	3.64 ± 0.09	6.54 ± 0.14	8.14 ± 0.11
XYL	0.09 ± 0.008	0.94 ± 0.003	3.11 ± 0.04	3.33 ± 0.04	4.14 ± 0.07	4.81 ± 0.04
sum	3.51	7.91	13.41	14	17.51	21.35

Table 4. Sugar content in SBP hydrolysates after 96 h of fermentation with the mixture of Ethanol Red and *Scheffersomyces stipitis* yeast.

Sugar	PRE/H Combination; Concentration [g dm^{-3}]					
	WB/Eh	**WB/Mh**	**AB/Eh**	**AB/Mh**	**AA/Eh**	**AA/Mh**
SAC	0.17 ± 0.005	0.25 ± 0.003	0.26 ± 0.002	0.62 ± 0.003	0.37 ± 0.006	0.32 ± 0.002
FRU	0.16 ± 0.008	0.31 ± 0.003	0.26 ± 0.004	0.36 ± 0.003	0.31 ± 0.004	0.21 ± 0.005
GLU	0.21 ± 0.011	0.23 ± 0.011	0.73 ± 0.006	1.11 ± 0.008	0.32 ± 0.002	0.42 ± 0.009
GAL	0.62 ± 0.06	0.55 ± 0.03	0.84 ± 0.03	0.82 ± 0.02	0.57 ± 0.002	0.63 ± 0.02
ARA	0.13 ± 0.006	0.33 ± 0.007	0.64 ± 0.004	0.56 ± 0.009	0.78 ± 0.004	0.14 ± 0.009
RHA	0.32 ± 0.008	0.52 ± 0.005	0.49 ± 0.009	0.43 ± 0.009	0.32 ± 0.004	0.65 ± 0.07
XYL	0.08 ± 0.008	0.34 ± 0.005	0.51 ± 0.004	0.66 ± 0.005	0.49 ± 0.008	0.34 ± 0.008
sum	1.69	2.53	3.73	4.56	3.16	2.71

The sugar uptake during fermentation carried by a mixture of the *S. cerevisiae* strain (Ethanol Red), and *Scheffersomyces stipitis* (Table 4) was more efficient than fermentation carried only by the *S. cerevisiae* strain (Table 3). As a non-pentose fermenting yeast, Ethanol Red yeast only uses fructose, glucose, sucrose, and a slight amount of galactose. This strain can ferment galactose, which is uncommon for distillers' strains of *S. cerevisiae* [37,38]. Changes in the 5-C sugar content varied around the barrier of statistical significance; slight decreases of pentose concentration in a hydrolysate fermented with the *S. cerevisiae* strain may be elucidated by possible microbial contaminations with some bacteria. The sum of the sugar left after fermentation with the Ethanol Red strain is higher for hydrolysates obtained with intense pretreatment (autoclave and 2% of sulfuric acid), and this is the result of the increase in pentoses content, released more efficiently from efficiently pretreated raw material, e.g., the xylose content after the fermentation of water pretreated biomass with use of *T. viride* enzymatic extract (WB/Mh) was equal to 0.94 ± 0.003 g dm^{-3}, while for acid/autoclave pretreated biomass with use of the same enzyme source (AA/Mh), it was equal to 4.81 ± 0.04 g dm^{-3}; thus, the sum of left sugars was more than twice as high as the AA/Mh hydrolysate (21.35 g dm^{-3}) than WB/Mh (7.91 g dm^{-3}); see Table 3.

Contrary to the results in Table 3, the sugars from hydrolysates fermented with pentose fermenting *Scheffersomyces stipitis* were far more depleted. For glucose, the levels varied from 0.21 ± 0.011 g dm^{-3} (WB/Eh sample) to 1.11 ± 0.008 g dm^{-3} for the AB/Mh hydrolysate (Table 4), while the starting concentrations of glucose were respectively equal to 8.35 ± 0.35 g dm^{-3} and 19.13 ± 0.24 g dm^{-3} for these hydrolysates (Table 2). For xylose, the levels varied from 0.08 ± 0.008 g dm^{-3} (WB/Eh sample) to 0.66 ± 0.005 g dm^{-3} for the AB/Mh hydrolysate (Table 4), while the starting concentrations of xylose were, respectively, equal to 0.09 ± 0.008 g dm^{-3} and 3.43 ± 0.15 g dm^{-3} for the same hydrolysates (Table 2).

Also, the sum of the remaining sugars after the fermentation presented in Table 4 depicts far more efficient exploitation by the coculture of hexose and pentose fermenting strains than for only hexose fermenting Ethanol Red experiments. As a result, hydrolysates

fermented by the mixture of *S. cerevisiae* and *Scheffersomyces stipitis* cells contained no more than 4.56 g dm^{-3} of sugars (Ab/Mh sample—Table 4). In contrast, the same hydrolysate fermented solely with Ethanol Red contained 14 g dm^{-3} of sugars expressed as a sum of assayed values (Table 3).

Xylose and arabinose, generally present in lignocellulosic materials, should be utilised to make lignocellulosic biomass processing economically reasonable [39]. Contrary to this, *Saccharomyces cerevisiae*, responsible for the dominant part of industrial ethanol production, uses only hexose sugars but cannot ferment any pentose [8]. *S. cerevisiae* cannot grow on xylose and does not produce ethanol with this sugar, but it can generate only modest quantities of xylitol. The explanation for this may be that the transport of xylose is two times slower than glucose under anaerobic conditions [40]. Some pentose fermenting yeast, like *Scheffersomyces stipitis*, can ferment xylose and hexoses [9,12] but exhibit low tolerance to ethanol concentrations over 30–35 g dm^{-3}. As a result, their metabolism is then inhibited significantly [41,42]. The ability of such strains to metabolise xylose depends also on the oxygen concentration in the medium [13,43].

Conducting separate hydrolysis and fermentation (SHF) leads to elevated sugar concentrations. However, this exposes the yeast to osmotic stress and may potentially induce substrate inhibition, which may be a potential explanation for our findings, particularly concerning the substantial quantity of the sum of the remaining sugars (1.69–4.56 g dm^{-3}—Table 4) that were present after the fermentation with the coculture of *S. cerevisiae* and *Scheffersomyces stipitis*. This obstacle may be removed by simultaneous saccharification and fermentation (SSF) since sugars are released in such a process and consumed almost simultaneously [44,45].

3.4. Ethanol Yield from Sugar Beet Pulp

The most critical parameter of the whole experiment was the yield of ethanol from sugar but pulp hydrolysates. The results of ethanol gain were expressed as kg of pure ethanol from 100 kg of raw (wet) SBP, and the results are presented in the Table 5.

Table 5. Ethanol gain (kg of pure ethanol) from 100 kg of wet SBP (approx. 24% of DM).

Yeast	PRE/H Combination; Ethanol Yield [kg × 100 kg^{-1} SBP]					
	WB/Eh	**WB/Mh**	**AB/Eh**	**AB/Mh**	**AA/Eh**	**AA/Mh**
ER	1.48 ± 0.04	1.53 ± 0.03	2.79 ± 0.13	2.71 ± 0.08	3.88 ± 0.13	3.97 ± 0.16
ER + SS	1.73 ± 0.044	2.08 ± 0.083	3.72 ± 0.17	3.95 ± 0.14	5.33 ± 0.18	5.1 ± 0.11

To evaluate the fermentation process, we also decided to present the fermentation yield as a percentage of the theoretical value (based on the amount of sugar measured and the conversion ratio of sugar to ethanol of 0.51). The results are shown in Table 6.

Table 6. Fermentation yield (% of theoretical value possible to obtain from fermentable sugars).

Yeast	PRE/H Combination; Fermentation Yield [%]					
	WB/Eh	**WB/Mh**	**AB/Eh**	**AB/Mh**	**AA/Eh**	**AA/Mh**
ER	67.5	55.8	56.5	54.5	55.2	51.8
ER + SS	78.9	75.8	75.4	79.4	75.9	66.5

The lowest ethanol gain from 100 kg of wet SBP, equal to 1.48 ± 0.04 kg 100 kg^{-1} of pure ethanol for the WB/Eh sample, was pretreated by boiling at 100 °C with subsequent enzymatic hydrolysis with the use of commercial Cellic Ctec2 enzyme preparation, and fermentation was carried by the single culture of only-hexose fermenting strain ER. There were no significant differences ($p > 0.05$) between the results obtained for the same pretreatment with the use of different enzyme sources (commercial CellicCtec2 or *T. viride*

post-cultivation extract obtained by us). The only one exception of this rule was spotted for WB/Eh and WB/Mh samples fermented with a mixed culture of hexose and pentose fermenting strains (ER + SS) when the ethanol yield for WB/Eh was significantly lower ($p < 0.05$) than the WB/Mh sample. The more significant impact on ethanol gain was the method of sample pretreatment. The notable differences ($p < 0.05$) between the results for samples pretreated using WB, AB, and AA were noted. However, the highest absolute value of ethanol gain equal to 5.33 ± 0.18 kg 100 kg^{-1} was noted for the AA/Eh pretreated sample in autoclave with the subsequent enzymatic hydrolysis with the use of commercial CellicCtec2 enzyme preparation, and fermentation was carried out by the coculture of the ER and SS strains. It must be underlined that after a statistical comparison of this result with the value 5.1 ± 0.11 kg 100 kg^{-1} obtained for the AA/Mh sample, it turned out that these two results were not statistically different ($p > 0.05$). The fermentation yield expressed as a percentage reflects the economic dimension of the processes less well but shows the degree of attenuation of the individual hydrolysates. The highest attenuation of 79.4% of the theoretical yield was achieved for the AB/Mh hydrolysate fermented by the coculture of the ER and SS strains. The lowest fermentation percentage yield of 51.8% was determined after the fermentation of the AA/Mh hydrolysate by the ER strain (*S. cerevisiae*).

The results obtained in our experiment are closely correlated to those obtained by Berłowska et al. [11,29]. Looking from the economic point of view—costs of the enzymes, it may be stated that the best option for economic conversion of SBP into ethanol in our experiments was AA/Mh method when the enzymatic concentrate after *T. viride* cultivation in SBP medium was obtained and used as the enzyme source. It supports some literature data containing conclusions that, in many cases, microbial "in–situ" cellulose-obtaining is the promising way for efficient saccharification of lignocellulosic hydrolysates [4,7,8,38,39,46–49].

4. Conclusions

The current state of industrial trials and scientific research shows that finding an effective method for producing bioethanol from renewable lignocellulosic waste materials is still challenging. Our study aimed to compare the efficiency of ethanol formation from sugar beet pulp with some variants of pretreatment, enzyme, and microbial hydrolysis. In our research, as one of the enzyme sources, the post-cultivation concentrate of *T. viride* LOCK 0588 in an SBP-based medium was used. The highest sugar-to-ethanol conversion ratio, equal to 79.4% of the theoretical yield, was observed for the AB/Mh hydrolysate fermented by the coculture of the ER and SS strains. The lowest fermentation percentage yield of 51.8% was determined after the fermentation of the AA/Mh hydrolysate by the ER strain (S. cerevisiae). The final ethanol gain using the coculture of *S. cerevisiae* (Ethanol Red) and *Scheffersomyces stipitis* LOCK 0047 strain exceeded 5.1 kg from 100 kg of wet SBP. Enzyme costs are one of the main obstacles to the industrial development of lignocellulosic materials' conversion into SCP or bioethanol. The best option for the economic conversion of SBP into ethanol in our experiments was the AA/Mh method, where the enzymatic concentrate after *T. viride* cultivation in the SBP medium was obtained and used as the enzyme source. Those results are a promising starting point towards the optimisation of larger-scale industrial processes of SBP valorisation by bioethanol production with the cost-effective microbial hydrolysis with *T. viride* and the fermentation carried out by cocultures of pentose and hexose fermenting yeast.

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