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Use of saccharose and structural polysaccharides from sugar beet biomass for bioethanol production**

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A b s t r a c t. In addition to saccharose, sugar beet root contains a lignocellulosic fraction, which is not used in the process of sugar production and remains in sugar beet pulp. There is a great interest in using the polysaccharides (cellulose, hemicellulose) present in this raw material for the production of bioethanol. The objective of this study was to assess the effect of the enzymatic treatment of sugar beet biomass on the hydrolysis of the cellulose and hemicellulose present in its cell walls, as well as its effect on the efficiency of alcoholic fermentation of saccharose and sugars liberated from structural polysaccharides. Its effect on the efficiency of the process of inoculating the fermentation medium with a monoculture or a co-culture of yeast strains fermenting hexose and pentose sugars was also investigated. Our results reveal that in order to enable the utilization of all fermentable sugars in the sugar beet root biomass (saccharose as well as monosaccharides bound in structural polysaccharides), initial enzymatic treatment should be applied, followed by alcoholic fermentation using sequential inoculation with a co-culture of *Saccharomyces cerevisiae* and *Pichia stipitis*. These conditions ensure the utilization of hexoses and pentoses (xylose) in alcoholic fermentation, thus enabling the production of 9.9±0.4 kg of ethanol from 100 kg of sugar beet biomass.

K e y w o r d s: sugar beet, saccharose, structural polysaccharides, alcoholic fermentation, *Saccharomyces cerevisiae, Pichia stipitis*

INTRODUCTION

The European Union (EU) is the world's leading producer of sugar beet, accounting for approximately 50% of global production (European Seed Association, 2019). The sugar beet industry plays a critical part in the European agricultural economy, but recently sugar beet producers have been adversely affected by low market prices and a series of changes in the sector. The International Sugar Organization recently forecast that the EU will produce 17.9 million t of sugar in the 2018/2019 season, down from 19.7 million t in the previous season. In October 2017, the EU scrapped sugar beet production quotas, allowing producers to grow as much beet as they wanted for the first time since 2006. This led to an increase in output. However, the current global surplus of sugar has pushed world sugar prices down to their lowest level for more than ten years, thereby throwing the industry into crisis. Moreover, the European Commission is forecasting that total sugar consumption in the EU will be reduced by 5% by 2030. The European Parliament must take many factors into consideration to maintain sustainable sugar beet production within the EU Member States (Sugar News & Reports, 2019). One possible option is to use this raw material for other purposes, including the production of bio-based fuels and chemicals (Berłowska *et al*., 2016). In addition to saccharose, sugar beet also contains a lignocellulosic fraction, which is not used in traditional sugar production and remains in the sugar beet pulp (SBP). The polysaccharides present in SBP could be used for the production of bioethanol. Sugar beet pulp consists mainly of polysaccharides 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 (Micard *et al*., 1996). Before fermentation, the cell-wall material must be degraded into fermentable sugars (Alvira *et al.,* 2010). Cellulose and hemicellulose can be hydrolysed down into simple sugars by cellulases or

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hemicellulases. Hexoses are fermented to ethanol by many naturally occurring microorganisms (Gong, 1983), including *Saccharomyces cerevisiae,* the most widely used yeast for ethanol production (Bai *et al*., 2008). However, *S. cerevisiae* is incapable of metabolizing xylose, and does not produce ethanol from it (Batt *et al*., 1986). Pentoses such as xylose and arabinose are fermented to ethanol by relatively few native strains, at relatively low yields (Gong, 1983). Some yeasts, such as *Candida shehatae, Candida tropicalis,* and *Pichia stipitis*, can ferment xylose and hexoses with relatively high yields (du Preez *et al.,* 1986). However, they have a low ethanol tolerance (Rouhollah *et al*., 2007).

The production of ethanol from sugar beet offers a promising solution for sugar factories interested in combining the production of sugar and bioethanol. Using sugar beet as a raw material for bioethanol production is an especially attractive possibility for distilleries located near sugar factories, as they could minimize transportation costs. Cooperation between these factories and distilleries could lead to increased production and exploit the capacity of both types of facilities.

The aim of the present study was to examine the effect of the enzymatic treatment of sugar beet biomass on the hydrolysis of cellulose and hemicellulose present in its cell walls, and on the efficiency of alcohol fermentation from saccharose and other sugars liberated from the structural polysaccharides. The effect of inoculating the fermentation medium with different yeast strains on the efficiency of the process was also evaluated.

MATERIALS AND METHODS

Fresh sugar beet sliced into thin chips was obtained from the Dobrzelin Sugar Factory (Poland) and stored at -20°C until used.

The sugar beet biomass was hydrolysed using an enzyme preparation, designated as IBT, originating from *Aspergillus niger,* containing cellulase (3.81 U ml^{-1}), xylanase (9.71 U ml^{-1}), and pectinase (29.17 U ml^{-1}) (Institute of Molecular and Industrial Biotechnology, Łódź University of Technology).

Fermentation was carried out using two variants: 1) using only a preparation of Ethanol Red dry distillery yeast (*S. cerevisiae*) (Fermentis Division S.I. Lesaffre, France) at a dose of 0.5 g d. m. l^{-1} of hydrolysate; 2) using a mixed culture of the *S. cerevisiae* strain (0.5 g d.m. l^{-1} of hydrolysate) and *Pichia stipitis* CBS 577 (ŁOCK 105, Łódź University of Technology, Poland) (3 g d. m. l^{-1} of hydrolysate), applied simultaneously or sequentially. With sequential inoculation, the process was initiated using Ethanol Red yeast (0.5 g d.m. l^{-1}) and after 36 h the hydrolysates were inoculated with *P. stipitis* (3 g d. m. l^{-1}). The yeast *Pichia stipitis* was cultivated under conditions described in our previous work (Berłowska *et al.,* 2016).

To prepare the hydrolysates for fermentation, sugar beet chips in the amount of 500 g were milled to obtain 0.8-1.0 mm particles, which were mixed with water in the ratio of 1:1 (w w^{-1}). The mixture was stirred (5 min) by an overhead stirrer (CAT, R50) to obtain a homogenous semi-liquid suspension. Next, the pH was adjusted to 4.8 using 25% (w w^{-1}) sulfuric acid and the suspension was subjected to the following types of processing (Table 1).

To determine the profile of fermentable sugars, depending on the enzyme preparation dose and hydrolysis conditions, a suspension was digested using 0.1 and 0.2 ml g^{-1} d.m. enzyme preparation. The samples were then maintained (without inoculation with yeast) under similar conditions as the fermented samples, *i.e.* at 35±2°C for 72 h, when simultaneous saccharification fermentation or co-fermentation (SSF, SSCF) took place, or at 50°C for 4 h or 24 h and then at 35±2°C for 72 h for processes carried out as separate hydrolysis/saccharification and (co-)fermentation (SHF, SHCF). In order to prevent microbial infections, the hydrolysates were supplemented with the antibiotics Penicillin G sodium salt (100 000 U kg^{-1} hydrolysate) and Streptomycin sulfate salt (0.1 g kg^{-1} hydrolysate).

Before fermentation, all trials were supplemented with $(NH_4)_2HPO_4$ (0.3 g kg^{-1}) as a source of nitrogen. Alcoholic fermentation was carried out in 2 l glass flasks, each containing approximately 1 kg of hydrolysate. Before being added to the hydrolysate, Ethanol Red distillery yeast (*S. cerevisiae*) was first hydrated and acid-washed (15 min incubation of cells suspended in water with the addition of 25% w w^{-1} sulfuric acid solution, pH 2.5, at room temperature). The yeast strain *P. stipitis* CBS 577 was added to the fermenting hydrolysates as a slurry without acid-washing. After inoculation with yeast, the flasks were closed with stoppers equipped with fermentation pipes, filled with glycerol, and stored in a thermostat-regulated room at 35±1°C. The process was monitored gravimetrically (to determine the decrease in mass caused by the liberation of carbon dioxide). When the fermentation process was complete (the entire process time for all samples did not exceed 72 h), the samples were collected to determine the concentrations of ethanol, saccharose, hexose, and pentose sugars.

The sugar beet root was analysed according to methods recommended for the sugar industry (AOAC, 1995). Dry matter was determined in a Radwag WPS-30S moisture analyser. Total nitrogen was determined using the Kjeldahl method and converted into protein content using factor 6.25. Reducing sugars were determined according to the Miller method (1959) and expressed in grams of invert sugar per kg of sugar beet pulp. The concentration of saccharose was calculated as the difference between the amounts of total sugars determined after acid hydrolysis with hydrochloric acid and reducing sugars (both determined according to the Miller method), taking into consideration a conversion coefficient of 0.95. Cellulose content was determined according to the Kürschner-Hoffer method (1933), hemicellulose content according to the Ernakow method (Arasimovich and Ermakov, 1987) and lignin content according to the method recommended by the National Renewable Energy Laboratory (NREL) (Templeton and Ehrman, 1995).

Table 1. Design of experiments for the enzymatic treatment and fermentation of sugar beet biomass

Processing method	Dose of enzyme preparation and digestion conditions	Yeast	Designation of the sample
Control sample	without enzymatic treatment	*S. cerevisiae*	Control S.c.
Simultaneous saccharification and fermentation (SSF)	0.1 ml g^{-1} d.m., hydrolysis during fermentation at 35±1°C	*S. cerevisiae*; an inoculation with yeast immediately after application of enzyme preparations	SSF 0.1 IBT S.c.
	0.2 ml g^{-1} d.m., hydrolysis during fermentation at 35±1°C		SSF 0.2 IBT S.c.
Separate hydrolysis/ saccharification and fermentation (SHF)	0.1 ml g^{-1} d.m., hydrolysis at 50°C for 4 h	*S. cerevisiae*; an inoculation with yeast after enzymatic hydrolysis	SHF 4h 0.1 IBT S.c.
	0.1 ml g^{-1} d.m., hydrolysis at 50°C for 24 h		SHF 24h 0.1 IBT S.c.
	0.2 ml g^{-1} d.m., hydrolysis at 50°C for 4 h		SHF 4h 0.2 IBT S.c.
	0.2 ml g^{-1} d.m., hydrolysis at 50°C for 24 h		SHF 24h 0.2 IBT S.c.
Simultaneous saccharification and co-fermentation (SSCF)	0.1 ml g^{-1} d.m., hydrolysis during fermentation at 35±1°C	*S. cerevisiae* + *P. stipitis;* simultaneous inoculation with co-culture of yeast immediately after application of enzyme preparations	SSCF 0.1 IBT S.c.+P.s. sim
	0.2 ml g^{-1} d.m., hydrolysis during fermentation at 35±1°C		SSCF 0.2 IBT S.c.+P.s. sim
	0.1 ml g^{-1} d.m.	*S. cerevisiae* + *P. stipitis;* sequential inoculation, initially with yeast *S. cerevisiae* (immediately after application of enzyme preparation) and next after 36 h of fermentation (when hexose sugars were almost entirely utilized) with *P. stipitis*	SSCF 0.1 IBT S.c.+P.s. seq
	0.2 ml g^{-1} d.m.		SSCF 0.2 IBT S.c.+P.s. seq
Separate hydrolysis/ saccharification and co-fermentation (SHCF)	0.1 ml g^{-1} d.m., hydrolysis at 50°C for 4 h	*S. cerevisiae* + *P. stipitis;* simultaneous inoculation with co-culture of yeast after enzymatic hydrolysis	SHCF 4h 0.1 IBT S.c.+P.s. sim
	0.1 ml g^{-1} d.m., hydrolysis at 50°C for 24 h		SHCF 24h 0.1 IBT S.c.+P.s. sim
	0.2 ml g^{-1} d.m., hydrolysis at 50°C for 4 h	*S. cerevisiae* + *P. stipitis;* sequential inoculation, initially with yeast *S. cerevisiae* (immediately after application of enzyme preparation) and next after 36 h of fermentation (when hexose sugars were almost entirely utilized) with *P. stipitis*	SHCF 4h 0.2 IBT S.c.+P.s. seq
	0.2 ml g^{-1} d.m., hydrolysis at 50°C for 24 h		SHCF 24h 0.2 IBT S.c.+P.s. seq

The pH was also measured (with a digital pH-meter). The contents of saccharose (SAC), glucose (GLC), fructose (FRU), galactose (GAL), xylose (XYL), arabinose (ARA), rhamnose (RHA), cellobiose (CEL), and raffinose (RAF) in the media before and after fermentation, as well as the content of ethanol after the completion of the fermentation process, were determined using HPLC as described by Berłowska *et al.* (2016).

Hydrolysis efficiency (HE) was calculated according to the following formula:

$$HY = \frac{C0.9}{RS + SAC + RAF + P} 100\%, \quad (1)$$

where: C is reducing pentose and hexose sugar concentration after hydrolysis (g kg^{-1}), RS is the reducing sugars in sugar beet biomass before hydrolysis (g kg^{-1}); SAC and RAF, are the saccharose and raffinose content (g kg^{-1}) respectively; and P is the polysaccharide (cellulose, hemicellulose) content (g kg^{-1}); 0.9 is the conversion coefficient

from polysaccharide (cellulose and hemicellulose) to pentose and hexose sugars (*i.e*., the molecular weight ratio of polysaccharide to hexose and pentose sugars).

The fermentation efficiency (*FE*) was calculated using a stoichiometric equation for hexose sugars (*i.e.* saccharose converted into invert sugar, glucose, fructose, and galactose) and pentose sugars (xylose) separately, and expressed as a percentage of the theoretical yield, according to the formula:

$$FE = \frac{E}{FS0.51}100\%, \quad (2)$$

where: E is the ethanol concentration in the fermented medium (g kg^{-1}), *FS* is fermentable sugars (in the samples fermented with *S. cerevisiae*, the fermentation capacity was calculated from the hexose sugars, while in the samples fermented with a mixed culture of *S. cerevisiae* and *P. stipitis,* the content of hexose and pentose sugars was used), and 0.51 is the constant which represents the theoretical yield of ethanol from glucose and xylose. The ethanol yield was expressed as the amount of absolute ethanol (A_{100}) obtained from 100 kg of sugar beet biomass.

All samples were prepared and analysed in triplicate. The results were tested statistically by analysis of variance (one-way ANOVA), at a significance level of 0.05, using STATISTICA 10.0 software (StatSoft, Tulsa, OK, USA) to indicate differences.

RESULTS AND DISCUSSION

The chemical composition of the sugar beet used in this study is shown in Table 2. The main sugar present in sugar beet root is saccharose. Its content in the tested raw material was 536.36±42.90 g kg^{-1} d.m. Small amounts of reducing sugars (21.89±1.31 g kg^{-1} d.m.) and raffinose (6.86±0.45 g kg^{-1} d.m) were also detected. Sugar beet biomass is also a source of structural polysaccharides, which have great potential to be used in second generation bioethanol production. A high content of cellulose and hemicellulose and a low content of lignin is advantageous from the technological point of view, since it promotes the production of high yields of fermentable sugars. However, the efficient hydrolysis and fermentation of these substrates depends on the type of pretreatment, the conditions of enzymatic hydrolysis, and on the microorganisms used for the fermentation of the released hexose and pentose sugars. Our results are similar to others reported in the literature (Wolak and Złocińska, 2012; Kühnel *et al.,* 2011). Any differences in the chemical composition of the sugar beet biomass can be related to the varieties of sugar beet and cultivation conditions.

Table 2. Chemical composition of sugar beet root biomass

Physicochemical parameters	Content
Dry mass (g kg^{-1})	297.0±12.5
pH	6.2±0.2
Reducing sugars as invert sugar (g kg^{-1} d.m.)	21.89±1.31
Saccharose (g kg^{-1} d.m.)	536.36±42.90
Raffinose (g kg^{-1} d.m.)	6.86±0.45
Cellulose (g kg^{-1} d.m.)	172.4±12.2
Hemicellulose (g kg^{-1} d.m)	214.5±17.2
Lignin (g kg^{-1} d.m.)	1.42±0.36
Protein (N x 6.25) (g kg^{-1} d.m)	18.2±2.5

Results expressed as mean values ± SE (n = 3).

Various doses of the enzyme preparation IBT and hydrolysis conditions were investigated in order to determine their effects on the profile of fermentable sugars. Due to the fact that the tested feedstock contained a relatively low lignin content, experiments were carried out without thermochemical pretreatment, which is recommended to remove most of the lignin and facilitate the activity of cellulases and hemicellulases on structural polysaccharides (Pessoa *et al.,* 1996, 1997). The aim of this stage of the study was to determine the amounts of sugars that could potentially be released under fermentation conditions (the samples were not inoculated with yeast).

The enzymatic hydrolysis of sugar beet-based medium with IBT preparation (at a dose of 0.1 ml g^{-1} d.m. sample I) under conditions prevailing during SSF and SSCF (*i.e.* a temperature of 35°C) resulted in the release of the following amounts of sugars (per kg of hydrolysate): 35.22 ± 1.75 g saccharose, 22.52 ± 1.13 g glucose, 21.73 ± 1.08 g fructose, 3.5 ± 0.18 g galactose, 13.5±0.68 g xylose, 0.64±0.03 g arabinose, 0.49 ± 0.02 g rhamnose, 0.71±0.04 g cellobiose, and 9.80 ± 0.49 g raffinose (Fig. 1). The yield from the hydrolysis of polysaccharides in this medium was 68.90±1.38% (Fig. 2). A two-fold increase in the dose of enzymatic preparations, to 0.2 ml g^{-1} d.m. (sample II), caused a relatively small rise (on average 15%) in the glucose and fructose concentrations, to 26.18±1.83 g kg^{-1} and 24.80 ± 1.74 g kg^{-1}, respectively. Conversely, saccharose concentration dropped to 31.15 ± 2.18 g kg^{-1}, most likely due to its hydrolysis to glucose and fructose as a result of the side activity of the enzyme preparation used. Moreover, the increase in the concentration of xylose, from 13.5 ± 0.95 g kg^{-1} to 14.7 ± 1.03 g kg^{-1}, was not significant ($p > 0.05$). In both samples, small amounts of galactose, arabinose, rhamnose, raffinose, and cellobiose were determined, as a result of the hydrolysis of non-starch polysaccharides (NSPs) (Lovegrove *et al.,* 2017).

The next stage of the investigation focused on whether carrying out separate enzymatic hydrolysis at 50°C for 4 h or 24 h before fermentation would improve the release of fermentable sugars. It was observed that 4 h of incubation at an elevated temperature (samples III and IV) significantly ($p < 0.05$) improved the liberation of monosaccharides.

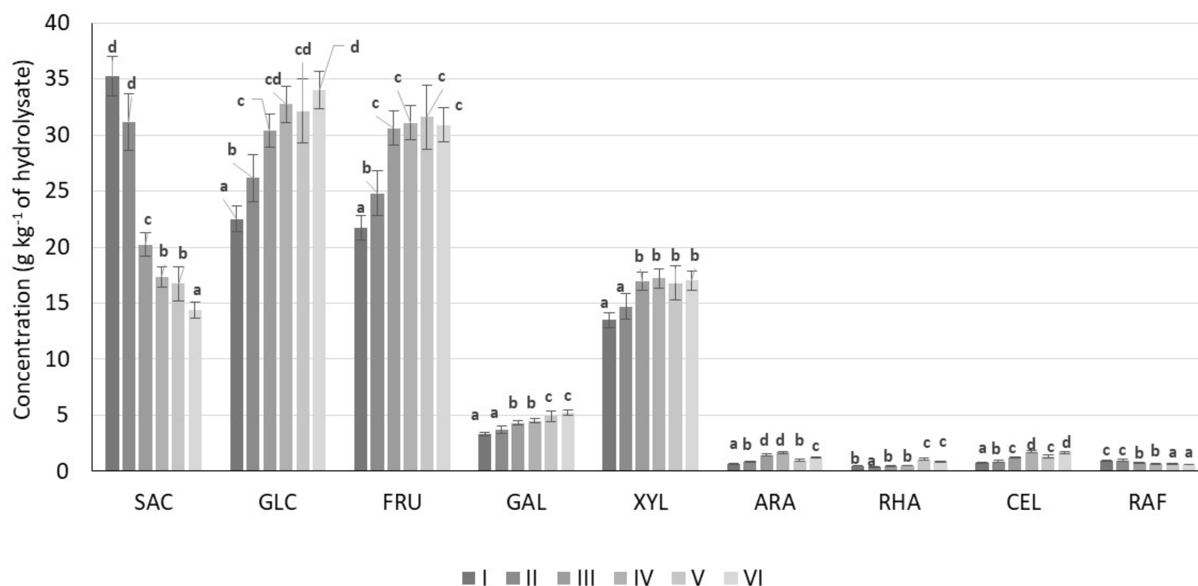

Fig. 1. Qualitative and quantitative composition of carbohydrates in sugar beet root biomass-based hydrolysates obtained after digestion of the feedstock with different doses of the enzyme preparation and under different conditions: I – 0.1 ml g^{-1} d.m., 35°C, 72 h; II – 0.2 ml g^{-1} d.m., 35°C, 72 h; III – 0.1 ml g^{-1} d.m., 50°C, 4 h and then at 35°C for 72 h; IV – 0.2 ml g^{-1} d.m., 50°C, 4 h and then at 35°C for 72 h; V – 0.1 ml g^{-1} d.m., 50°C, 24 h and then at 35°C for 72 h; VI – 0.2 ml g^{-1} d.m., 50°C, 24 h and then at 35°C for 72 h. Mean values with different letters for each sugar content are significantly different ($p < 0.05$).

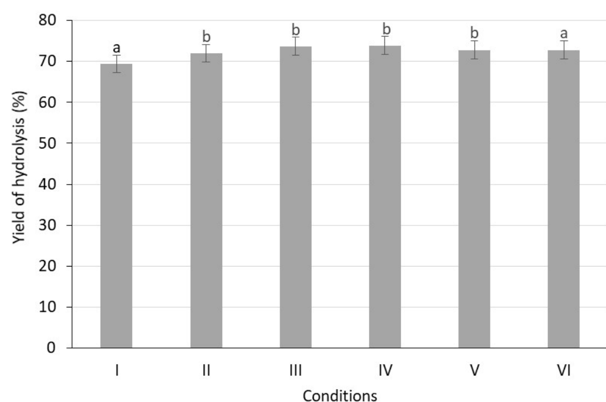

Fig. 2. Yield of hydrolysis of polysaccharides in sugar beet root biomass-based media. Explanations as in Fig. 1.

In turn, as with sample II, saccharose concentration in samples III and IV dropped to 20.24 ± 1.42 g kg^{-1} and 17.34 ± 1.21 g kg^{-1}, respectively. The yield of polysaccharide hydrolysis in the tested samples was similar and ranged from 73.82 ± 1.48% to 74.30 ± 49% ($p < 0.05$). However, irrespective of the enzyme dose, the concentrations of hexoses reached similar values and ranged from 30.39 ± 1.52 to 32.74 ± 1.64 g kg^{-1} hydrolysate for glucose and from 30.6 ± 1.53 to 31.1 g kg^{-1} hydrolysate for fructose ($p < 0.05$). This suggests that a 2-fold increase in the enzyme preparation dose does not cause a significant increase in the amounts of sugars liberated. Analogous observations were made with regard to other sugars, particularly xylose, which could be used by selected yeast strains in the process of alcoholic fermentation.

Prolonging the hydrolysis time at 50°C from 4 to 24 h did not result in a significant increase in the concentrations of fermentable sugars (samples V and VI). Also, saccharose concentrations did not show significant differences ($p < 0.05$) in relation to samples III and IV (Fig. 2). This may be due to the fact that when enzymatic hydrolysis is carried out as a separate step, it may lead to high concentrations of lower saccharides, which inhibit enzyme activity (Berłowska *et al*., 2016). In addition, it should be noted that increasing the time of hydrolysis at elevated temperatures generates higher energy inputs and raises the risk of microbial infections.

Based on our previous research, Ethanol-Red (*S. cerevisiae*) distillery yeast was applied to ferment the hexose sugars, while for the fermentation of pentoses (xylose) *Pichia stipitis* CBS 577 (ŁOCK 105, Łódź University of Technology, Poland) was used. In samples that were only fermented with *S. cerevisiae*, the lowest ethanol content (30.57 ± 0.53 g kg^{-1} hydrolysate) and fermentation efficiency (69.22 ± 0.73% of the theoretical yield) was obtained for the control sample (without enzymatic treatment). The addition of IBT enzyme preparation (0.1 ml g^{-1} d. m.) before inoculation with yeast resulted in an increase in ethanol content by approximately 2 g kg^{-1} hydrolysate, and as a consequence, fermentation efficiency rose to 73.57 ± 1.06% of the theoretical limit ($p < 0.05$). It was observed that enzymatic treatment resulting in the hydrolysis of structural polysaccharides also affects the liquefaction of sugar beet biomass, which facilitates the availability of sugars to yeast and, as a consequence, improves the efficiency of fermentation. A two-fold increase in the dose of enzyme preparation

followed by fermentation with *S. cerevisiae* did not cause a statistically significant improvement in the fermentation results ($p > 0.05$) (Table 3).

In the next stage of the study, the fermentation results were assessed for samples in which the separate hydrolysis of sugar beet biomass was performed with the enzyme preparation followed by fermentation with *S. cerevisiae* yeast (SHF). After 4 h of hydrolysis with a dose of 0.1 ml g^{-1} d.m. enzyme preparation, the ethanol concentration in the hydrolysate reached 38.21 ± 1.55 g kg^{-1} and the process efficiency was at $86.51 \pm 1.43\%$ of the theoretical yield. Neither prolonging the hydrolysis time from 4 to 24 h, nor doubling the enzyme preparation dose caused a significant improvement in the fermentation results ($p > 0.05$). However, there was a significant increase ($p > 0.05$) compared to the samples obtained using the SSF procedure (Table 3).

Two further series of experiments using the processing methods designated as simultaneous saccharification and co-fermentation (SSCF) and separate saccharification and co-fermentation (SHCF) were focused on the determination of the result of applying the hexose- and pentose-fermenting yeasts. Inoculation with the yeasts was carried out simultaneously or sequentially (*i.e*. initially with *S. cerevisiae* and then after 36 h, when the hexose sugars were almost entirely consumed, with *P. stipitis*). Two doses of enzyme preparation were also tested, as well as the use (or omission) of initially separate hydrolysis. It was observed that the fermentation results for hexose sugars were similar for the majority of samples, with simultaneous or separate hydrolysis having no statistically significant effect ($p > 0.05$). However, trials digested with 0.2 ml g^{-1} d.m. enzyme preparation and hydrolysed at 50°C for 4 h before sequential fermentation with *S. cerevisiae* and *P. stipitis*

Table 3. Fermentation factors of sugar beet root biomass-based hydrolysates

Designation of the sample*	Results for hexose sugars		Results for pentose sugars	
	Ethanol content of hydrolysate	Fermentation efficiency of the theoretical yield	Ethanol content of hydrolysate	Fermentation efficiency of the theoretical yield
	(g kg^{-1})	(%)	(g kg^{-1})	(%)
Control S.c.	30.57±0.53a	69.22±0.73a	–	–
SSF 0.1 IBT S.c.	32.53±0.89b	73.57±1.06b	–	–
SSF 0.2 IBT S.c.	33.45±0.85b	75.74±1.12b	–	–
SHF 4h 0.1 IBT S.c.	38.21±1.55c	86.51±1.43cd	–	–
SHF 24h 0.1 IBT S.c.	37.6±1.09c	86.53±1.13d	–	–
SHF 4h 0.2 IBT S.c.	38.5±0.94cd	87.17±1.02cd	–	–
SHF 24h 0.2 IBT S.c.	37.32±1.31c	85.89±1.36c	–	–
SSCF 0.1 IBT S.c.+P.s. sim	37.74±1.54cd	86.84±1.61cd	1.43±0.22a	20.43±0.31a
SSCF 0.2 IBT S.c.+P.s. sim	39.61±1.72cde	89.59±1.89def	2.07±0.18b	27.64±0.21b
SSCF 0.1 IBT S.c.+P.s. seq	39.58±1.79cde	89.51±1.88def	5.04±0.34d	73.21±0.36e
SSCF 0.2 IBT S.c.+P.s. seq	40.61±1.77de	91.84±2.03fg	5.68±0.37d	75.80±0.38f
SHCF 4h 0.1 IBT S.c.+P.s. sim	39.59±1.89cde	89.58±2.09de	2.84±0.26c	32.84±0.29c
SHCF 24h 0.1 IBT S.c.+P.s. sim	39.78±1.63cde	89.98±1.45e	2.94±0.24c	34.36±0.27d
SHCF 4h 0.2 IBT S.c.+P.s. seq	41.56±1.58e	93.12±1.68g	7.94±0.41e	90.52±0.46h
SHCF 24h 0.2 IBT S.c.+P.s. seq	41.74±1.66e	93.52±1.84g	7.55±0.44e	87.74±0.48g

*Detailed description of the samples – see Table 1. Results expressed as mean values ± SE (n = 3); mean values in columns with different letters are significantly different ($p < 0.05$).

yeast showed an increase in the efficiency of the process (up to 93.12±1.68% of the theoretical limit). On the other hand, prolonging the time of separate hydrolysis from 4 to 24 h did not improve the fermentation efficiency of hexose sugars ($p>0.05$), but rather entailed an unnecessary rise in thermal energy consumption and a greater risk of microbiological infections.

In the cases of both processing methods SSCF and SHCF, when a mixed culture of *S. cerevisiae* and *P. stipitis* was applied simultaneously, the lowest ethanol concentration (1.43±0.22 g kg^{-1} hydrolysate) produced from the pentose sugars was observed in the medium digested with 0.1 ml g^{-1} d.m. enzyme preparation SSCF. The fermentation efficiency calculated from xylose only reached 20.43±0.31% of the theoretical yield. A two-fold increase in the dose of enzyme preparation caused a statistically significant ($p>0.05$) increase in ethanol production, to 2.07±0.18 g kg^{-1} hydrolysate. However, the efficiency of xylose fermentation was still low and merely reached 27.6±0.21% of the theoretical value. Low ethanol contents, ranging from 2.84±0.26 g kg^{-1} hydrolysate (4 h hydrolysis) to 2.94±0.24 g kg^{-1} hydrolysate (24 h hydrolysis) ($p>0.05$), were determined in the samples subjected to separate hydrolysis and co-fermentation (SHCF), and when *S. cerevisiae* and *P. stipitis* were added simultaneously. As a consequence, the fermentation efficiency of xylose was also low and ranged from 32.84±0.29% to 34.36±0.27% of the theoretical yield.

The García-Cubero *et al*. (2009) report revealed that carrying out a separate hydrolysis and fermentation with a monoculture of yeast SHF or co-fermentation with a mixed culture of microorganisms SHCF led to the accumulation of sugars released at the enzymatic hydrolysis stage, which resulted in the product-inhibition of enzymes and lower ethanol output. However, in our study, the most favourable variant, enabling the highest ethanol production from xylose, was the short 4 h separate enzymatic treatment (0.2 ml g^{-1} d. m.) followed by fermentation with *S. cerevisiae* and *P. stipitis* applied sequentially. The ethanol concentration in this medium reached 7.94±0.41 g kg^{-1}, while the fermentation efficiency calculated from xylose was 90.52±0.46% of the theoretical yield. Similar fermentation factors were observed when enzymatic pre-hydrolysis was prolonged to 24 h ($p>0.05$) (Table 3). These results are in agreement with observations made by Berłowska *et al.* (2016) during studies concerning the alcoholic fermentation of sugar beet pulp, in which a 6 h enzymatic 'activation' before inoculation with yeast improved fermentation performance.

On the basis of the fermentation results in our study, we calculated the quantity of ethanol that could be obtained from 100 kg of sugar beet root biomass (Table 4). It was found that the lowest ethanol yield (6.1±0.1 kg from 100 kg sugar beet biomass) was obtained when fermentation was carried out without enzymatic treatment, with *S. cerevisiae*. The digestion of the sugar beet biomass using IBT enzymatic preparation, especially as a separate hydrolysis step at 50°C for 4 h, caused a statistically significant ($p<0.05$) increase in ethanol yield to 7.7±0.2 kg from 100 kg of sugar beet biomass. The only sugars utilized in these trials were hexoses. The application of a yeast able to ferment pentose sugars (especially xylose) liberated after enzymatic treatment resulted in the production of ethanol from xylose, and as a consequence an increase in its yield. The most favourable conditions for ethanol production established in our experiments are as follows: initial 4 h separate digestion of biomass with IBT enzymatic preparation at a dose of 0.2 ml g^{-1} d.m., and then fermentation with a co-culture of *S. cerevisiae* and *P. stipitis,* applied sequentially. Such conditions ensure that both hexoses and pentoses (xylose) are utilized in the fermentation process. It was found that 9.9±0.4 kg (*i.e.* 12.6 l) of absolute ethanol can be produced from 100 kg of sugar beet biomass under these conditions (Table 4). By comparison, Gumienna *et al.* (2014) reported that alcoholic fermentation of sugar beet root pulp with *S. cerevisiae* (Safdistil C-70; 30°C, 72 h) yielded up to 10.4 l (*i.e.* approximately 8.2 kg) of absolute ethanol from 100 kg of raw material. The differences in ethanol yield are most likely the consequence of the utilization of fermentable sugars liberated from structural polysaccharides in our experiments. The application of the proposed solution in industrial practice could contribute to an increase the efficiency of sugar beet biomass utilization and ethanol productivity, while also improving the nutritional value of distillery stillage resulting from the presence of yeast biomass. Another crucial issue is that biofuels, including bioethanol, are known to reduce the greenhouse gas (GHG) effect. The level of GHG emissions in the bioethanol life cycle depend on such factors as the raw material and production technology used. The usage of sugar beet-based ethanol is very favourable due to lowering GHG emission levels (Dziugan *et al.,* 2013).

CONCLUSIONS

1. Apart from saccharose, sugar beet biomass contains structural polysaccharides such as cellulose and hemicellulose, which makes it an attractive raw material for the production of bioethanol.

2. In order to increase the utilization of saccharose as well as monosaccharides linked in structural polysaccharides, it is advisable to apply initial enzymatic treatment to the biomass. In this study, it was found that the most favourable conditions, enabling the highest ethanol production from hexose and pentose sugars, was separate hydrolysis and fermentation, where 4 h digestion of biomass with 0.2 ml g^{-1} d.m. IBT enzymatic preparation at 50°C was applied.

Table 4. Ethanol yield (kg absolute ethanol) from 100 kg of sugar beet root biomass

Designation of the samples*	Ethanol yield (kg absolute ethanol)		
	from hexose sugars	from pentose sugars	sum
Control S.c.	6.1±0.1a	–	6.1±0.1a
SSF 0.1 IBT S.c.	6.5±0.2b	–	6.5±0.2b
SSF 0.2 IBT S.c.	6.7±0.2b	–	6.7±0.2b
SHF 4h 0.1 IBT S.c.	7.6±0.3c	–	7.6±0.3c
SHF 24h 0.1 IBT S.c.	7.5±0.2c	–	7.5±0.2c
SHF 4h 0.2 IBT S.c.	7.7±0.2c	–	7.7±0.2c
SHF 24h 0.2 IBT S.c.	7.5±0.3c	–	7.5±0.3c
SSCF 0.1 IBT S.c.+P.s. sim	7.5±0.3c	0.3±0.04a	7.8±0.3cd
SSCF 0.2 IBT S.c.+P.s. sim	7.9±0.3cd	0.4±0.04b	8.3±0.3de
SSCF 0.1 IBT S.c.+P.s. seq	7.9±0.3cd	1.0±0.07d	8.9±0.4ef
SSCF 0.2 IBT S.c.+P.s. seq	8.1±0.3d	1.1±0.07d	9.2±0.4f
SHCF 4h 0.1 IBT S.c.+P.s. sim	7.9±0.3cd	0.6±0.05c	8.5±0.4ef
SHCF 24h 0.1 IBT S.c.+P.s. sim	8.0±0.3cd	0.6±0.05c	8.5±0.4ef
SHCF 4h 0.2 IBT S.c.+P.s. seq	8.3±0.3d	1.6±0.1e	9.9±0.4g
SHCF 24h 0.2 IBT S.c.+P.s. seq	8.3±0.3d	1.5±0.1e	9.8±0.4g

*Detailed description of the samples – see Table 1. Results expressed as mean values ± SE (n = 3); mean values in columns with different letters are significantly different ($p<0.05$).

3. For the best possible results, the fermentation of hexose and pentose (xylose) sugars should be carried out using sequential inoculation with a co-culture of S. cerevisiae and P. stipitis yeasts. Such conditions allow 9.9±0.4 kg (*i.e.* 12.6 l) of absolute ethanol to be obtained from 100 kg of sugar beet biomass.

Conflict of interest: The Authors declare no conflict of interest.

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