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The use of β-xylanase for increasing the efficiency of biocatalytic conversion of crop residues to bioethanol

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ABSTRACT

Proteinaceous inhibitors of xylanase naturally occur in cereals where they are involved in various roles in the plant defence metabolism. This study focused on the inhibitors of xylanase present in local rye cultivars, and their influence on the efficiency of the fermentation processes during bioethanol production from rye residues in comparison with common wheat. Different origin xylanases from *Thermomyces lanuginosus* and *Trichoderma reesei* were the objects of the investigations. Kinetic studies of these xylanases in the presence of proteins with inhibitory activity indicated that *Th. lanuginosus* was found more sensitive to proteinaceous xylanase inhibitors presented in rye than *T. reesei*. The highest yield of xylose and arabinose was achieved by adding *T. reesei* to cell wall substrates, while *Th. lanuginosus* converted to arabinoxylans only into xylooligosaccharides and monosaccharide were not released. The activity of xylanase in composition with α-amylase and glucoamylase was selected to achieve a higher ethanol yield in the distillate. It improved the quality of bioethanol by increasing the content of ethanol and decreasing the concentrations of propanol, isobutanol, isoamyl and amyl alcohols and the methanol concentration. No significant differences were found between the contents of ethanol from different type of bran.

1. Introduction

Environmental issues, the growing demand for energy, political concerns and the medium-term depletion of petroleum has created the need for development of sustainable technologies based on renewable raw materials. Biofuels (biodiesel and bioalcohols) might help to meet the future energy supply demands as well as contributing to a reduction of green house gas emissions [1]. During the last few decades considerable attention has been given to the production of the first generation bioethanol from various energy crops including sugar cane, corn, wheat, maize, sugar beet and sweet sorghums [2]. The main disadvantage of this first generation bioethanol and other biofuels is that the conventional biofuel production process generally involves the use of 'food' crops. This issue has generated much controversy in a world where the a limited area of arable land and grain reserves will contribute to skyrocketing the food prices if such food crops will be used for biofuel production. That and other issues that arose related to deforestation, global warming and threats to biodiversity, in particular in developing countries encouraged the search for alternative technologies and feedstocks for biofuels production [1]. Alternative feedstocks, generally non-edible feedstocks, and/or technologies are starting to be developed in an attempt to overcome the major shortcomings of the production of the first generation biofuels. The second generation of bioethanol is usually produced from a range of alternative readily abundant and inexpensive, cellulosic biomass feedstocks including woody biomass, grasses, forestry and agricultural waste [3,4]. Cereal residues such as wheat and rye bran, produced in large quantities as by-product of the milling industry, herewith, different grains, spoiled and unusable for human consumption, should be potential energy stock [5].

Rye is the second important crop next to wheat in East Europe and is widely used in various fermentation processes such as bread and other food manufacturing processes. Therefore, there is a growing interest in rye residues as a potential feedstock for the second generation bioethanol production. Industrial bran usually accounts for 14–19% of the grain and comprises the outer coverings, the aleurone layer and the remnants of the starchy endosperm. It consists mainly of starch, hemicelluloses (arabinoxylans, β-glucans), cellulose, proteins and lignin [6].

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Table 1
Yields and molar sugar composition of rye and wheat cell wall materials.

Cereals	Cell wall material yield (g 100 g^{-1})	Molar sugar composition (mol%)							Carbo-hydrate content (g 100 g^{-1})	Ara:Xyl ratio	AX yield (g 100 g^{-1})
		Rha	Ara	Xyl	Man	Gal	Glc	UA			
Rye	15.3 ± 0.15	1.5	25.5	50	2	2	16.5	2	50.5 ± 0.71	0.53 ± 0.01	5.49 ± 0.22
Wheat	13.3 ± 0.12	2	24	47.5	1.5	4	18	3	37.5 ± 0.71	0.49 ± 0.02	3.26 ± 0.09

Data values are means ± SD (n = 2). Rha, rhamnose; Ara, arabinose; Xyl, xylose; Man, mannose; Gal, galactose; Glu, glucose; UA, uronic acid.

Hemicelluloses and cellulose can be converted to bioethanol. A major processing step in an ethanol plant is enzymatic hydrolysis of the hemicelluloses and cellulose fractions into their sugar components: pentoses and hexoses through treatment by enzymes; this step requires lengthy processing and normally follows a short-term pretreatment step [7]. Hemicelluloses are highly branched heteropolymers containing sugar residues such as hexoses (D-galactose, L-galactose, D-manose), methylpentoses (L-rhamnose, L-fucose), pentoses (D-xylose, L-arabinose) and uronic acid (D-glucuronic acid). The branched nature renders amorphous hemicelluloses, largely soluble in alkali, and as such, relatively easy to hydrolyze to its constituent sugars. These sugars can then be fermented into bioethanol [8]. Therefore, a mixture of enzymes including cellulases and hemicellulases different from those of the first generation bioethanol production should be employed in the hydrolysis step of cereal residues into fermentable sugars. The optimization of enzymatic hydrolysis of cereal residues also requires the complex evaluation of endogenous enzyme activities and selection of the commercial enzyme preparations resistant to their inhibitors.

In recent years, the interest in endoxylanases (EC 3.2.1.8) from microbial origin has increased due to their positive effect on the efficiency of the fermentation processes [9–11]. Xylanase functionality depends on biochemical properties of the enzyme, the substrate specificity of the hydrolysis pattern and the relative activity towards the water-unextractable and water-extractable arabinoxylans fractions [12,13]. However, efficiency of added commercial endoxylanases can vary depending on the cereal chemical composition and requires the optimization of dosage of enzyme used. It may be due to the levels of endogenous xylanases and endoxylanase inhibitors in cereals [14,15].

The optimization of the enzymatic hydrolysis of rye residues, herewith the increase of the efficiency of fermentation, cause the problems due to the lack of knowledge of the functionality of the endoxylanase inhibitors in rye. The problem requires the complex evaluation of the activity and the action of endoxylanase inhibitors.

The objective of the present work was to investigate the effect of the xylanase inhibitors possibly presented in rye on the functionality of different microbial endoxylanases usually applied to cereal-based processes. The method for enzymatic hydrolysis of polysaccharides in rye as well as in wheat bran to produce bioethanol has been described. The impact of the xylanase preparation on the quality of bioethanol produced from rye in comparison with wheat residues was also evaluated.

2. Experimental

2.1. Enzymes and enzyme activity measurements

Traditional in ethanol fermentation for starch hydrolysis and saccharification, commercial enzyme preparations of *Bacillus licheniformis* α-amylase (Termamyl 120L), containing 120 kilo Novo Units (KNU) g^{-1}, and *Aspergillus niger* glucoamylase (AMG 300L), containing 300 Novo Glucoamylase Units (AGU) ml^{-1}, were donated by Novozymes A/S (Bagsværd, Denmark). Xylanase (endo-1,4-β-xylanase) preparations Pentopan Mono BG and Ecopulp TX 200-A used to support the enzymatic degradation of non-starch polysaccharides was a kind gift from Novozymes A/S (Bagsværd, Denmark) and AB Enzymes (Ramajaki, Finland), respectively. Pentopan Mono BG is purified xylanase from *Thermomyces lanuginosus* (donor)/*Aspergillus oryzae* (host) standardized by manufacturer to 2500 Fungal Xylanase Units (FXU) g^{-1} (wheat soluble arabinoxylans). Ecopulp TX-200A is a thermostable xylanase preparation produced by *Trichoderma reesei* (anamorph *Trichoderma longibrachiatum*). The xylanase activity of the product was standardized by the manufacturer to 190,000 nkat g^{-1}.

The activity of both the Pentopan Mono BG and Ecopulp TX-200A xylanases was also determined in a reductometric assay according to Bailey et al. [16] with birchwood xylan as substrate and using colorometric dinitrosalicylic acid (DNS) reagent and D-xylose as standard (40 °C; pH 4.5). Xylanase activities of Pentopan Mono BG and Ecopulp TX-200A were determined having 8100 and 11,200 Xylanase Units (XU, 1 XU = 16.67 nkat) g^{-1} enzyme preparation^{-1}, respectively.

2.2. Testing of β-xylanase activities for degradation of cereal cell wall materials

The effectiveness of chosen commercial xylanase preparations were investigated on the cell wall materials extracted from rye and wheat wholemeal.

For the extraction of cell wall materials, 40 g of sample were dissolved in 400 ml Maleic buffer of pH 6.5 (0.01 M $C_4H_6O_5$, 0.01 M NaCl, 0.001 M $CaCl_2$, 0.05% NaN_3) and 2 mg of thermostable α-amylase from *B. licheniformis* (1500 Units mg^{-1}, Megazyme International Ireland Ltd., Wicklow, Ireland) was added and stirred for 2 h at 85 °C to form a gel. Subsequently the reaction mixture was cooled down on ice until 30 °C, and 80 mg of amyloglucosidase from *Rhizopus* sp. (11,600 Units g^{-1}, Sigma–Aldrich, Inc., St. Louis, MO, USA), 400 μl of pullulanase from *B. licheniformis* (75 Units mg^{-1}, Megazyme International Ireland Ltd., Wicklow, Ireland) were added, and treated enzymatically at 30 °C for 20 h in a water bath. After the incubation the reaction mixture was cooled down, and 1120 ml of C_2H_5OH (96%) was added and stirred for 24 h at 5 °C to dissolve the formed monomers and oligomers. Thereupon stirring was stopped and samples were left to stand for 2 h. The supernatant was removed by suction, sediments were centrifuged (10,000 × g, 15 min) and the retained pellet was repeatedly dissolved in Maleic buffer (pH 6.5) and all procedures described above were repeated one time. After the second centrifugation the sediments were filtered with a Buchner funnel, and rinsed 3 times with ethanol, and 3 times with acetone. The retained cell wall materials were left overnight to dry. Yield and composition of rye and wheat cell wall materials are presented in Table 1. The neutral sugar composition was determined by gas chromatography according to Englyst and Cummings [17] using inositol as internal standard. Uronic acid content of the cell wall material was determined by the automated colorimetric m-hydroxydiphenyl assay [18] using an autoanalyser (Skalar Analytical BV, The Netherlands).

For degradation of non-starch polysaccharides, 3 mg of cell wall materials were suspended in 1 ml of 50 mM $NaCOOCH_3$ buffer (pH 5) and incubated with the xylanase preparation (0.1%, w/v)

for 24 h at 60 °C. After the incubation the enzymatic reaction was stopped by heating of the reaction mixture at 100 °C for 10 min. The degree of non-starch polysaccharide degradation was determined by high-performance size-exclusion chromatography (HPSEC) and high-performance anion-exchange chromatography (HPAEC).

2.3. Testing of cereal inhibition activity against microbial β-xylanases

For the inhibition activity assay, 5 g of rye wholemeal were extracted in sodium acetate buffer (40 ml; 0.1 M; pH 4.5) for 1 h. The extract was centrifuged (10,000 × *g*; 4 °C; 25 min) in a Sorvall RC-5B centrifuge (Dupont Instruments, USA). The supernatant was filtered through a 0.45 μm Minisart filter (Sartorius, Germany) and stored at 4 °C temperature. The medium-viscosity (P-WAXYM) wheat arabinoxylans (arabinoxylan content 94–95% of the carbohydrates, the arabinose:xylose ratio 0.61) from Megazyme International Ireland Ltd. (Wicklow, Ireland). and birchwood xylan (poly(β-D-xylopyranose[1→4]); xylose residues ≥90%) from Sigma–Aldrich, Inc. (St. Louis, MO, USA) were used as substrates for the xylanase activity assay.

Xylanase inhibition activity was determined according to Elliot et al. [15] by comparing the reduction in xylanase activity in the presence of rye protein extract the with blank prepared with the absence of rye extract. The reaction mixture (1 ml) containing xylanase solution (0.1 ml) and rye extract (0.2 ml) in 0.1 M sodium acetate buffer (pH 4.5) was pre-incubated for 30 min at 40 °C. Further addition of substrate (0.5%, w/v; 0.05 ml) was performed, and the reaction mixture was incubated for 30 min under the same conditions. The reaction was terminated with DNS reagent. The endoxylanase inhibition activity was defined as the amount of inhibitor resulting in 50% reduction in absorbance at 540 nm under the experimental conditions. All measurements were performed at least in triplicate. The determination of the enzyme kinetic parameters was performed by the Lineweaver–Burk linearization procedure by using the Michaelis–Menten kinetic model. The reaction rate without and with the addition of rye extract as inhibiting agent was obtained as a function of the substrate concentration.

2.4. Enzymatic hydrolysis and fermentability test

A low-temperature technological process was used for the ethanol production under the laboratory conditions (Fig. 1). Crop residues (wheat and rye bran) were obtained from SC Kauno grudai (Kaunas, Lithuania). The material had a dry matter (DM) content of 86% and was stored in plastic bags at 4 °C. Rye or wheat bran was treated by a two-step enzymatic hydrolysis procedure consisting of the liquefaction and saccharification steps. Bran (100 g) was mixed with water previously heated to 90 °C in a ratio of 1:5 and kept at the same temperature in a waterbath for 30 min to reach partial degradation of polysaccharides and to decrease microbial activity.

Liquefaction of starch was carried out for 90 min at 65 °C temperature and a pH between 6.0 and 6.5 by adding a selected amount of α-amylase (Table 2). Further the simultaneous enzymatic liquefaction and saccharification step was performed in 120 min by a temperature between 55 and 60 °C and pH 5.0–6.0, and initial supplementation of a selected amount of glucoamylase and an appropriate amount of β-xylanase, according to Table 2, for the degradation of non-starch polysaccharides. The assays were carried out in stirred glass vessels. Samples were withdrawn after 0, 60, and 120 min for analysis of soluble dry matter (SDM) and fermentable carbohydrates (FC) content and acidity in the wort.

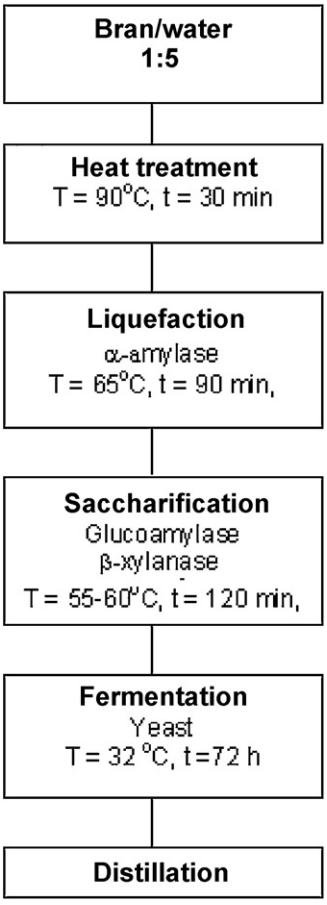

Fig. 1. Scheme of ethanol production.

300 ml of the wort was fermented at 30–33 °C temperature for 72 h using baker's yeast *Saccharomyces cerevisiae* (Lesaffre Polska S.A., Wołczyn, Poland), in a 500 ml glass flask, sealed with a rubber stopper and a tube was inserted into the stopper for removal of the carbon dioxide.

All fermentations were performed in duplicates. The fermented samples were analyzed for acidity, soluble dry matter (SDM) and non-fermented carbohydrates (NFC) content after 72 h. Fermented broth was filtered and the filtrate was subjected to quantitative and qualitative analysis of the alcohols, including the determination of ethanol as well as the determination of higher alcohols and other volatile substances.

2.5. Analysis

The SDM content was determined in the filtrate according to the standard AACC method [16]. Acidity analysis was performed by titration with 0.1 N NaOH. One degree (1°) of acidity corresponds to 1 ml of 1 N NaOH required to neutralise the acids present in 20 ml of

Table 2
Composition of raw material and enzyme preparation activity ranges per assay.

Raw material	Starch (%)	Total carbohydrate (%)	Total protein (%)	Xylanase (XU assay^{-1})	α-Amylase (KNU assay^{-1})	Glucoamylase (AGU assay^{-1})
Wheat bran	32.1	64.5	15.5	100–500	70	150
Rye bran	23.2	64.7	14.0	100–500	50	150

filtrate. FC in the wort and NFC after fermentation was determined by using the colorimetric Anthrone method for the determination of carbohydrates [19]. The analyses were performed in triplicates. The concentration of ethanol was determined using direct distillation and pycnometry [20].

Analysis of higher alcohols and other volatile substances was performed using gas chromatography (GC). A Hewlett Packard 5890 gas chromatograph equipped with a split-splitless injector and a FID detector was used in all measurements. The injection temperature was 200 °C and helium served as a carrier gas with a flow rate of 1.2 ml min^{-1}. Chromatographic separation was accomplished with a column Zebron ZB-WAX (30 m × 0.25 mm × 0.25 μm, 100% polyethylene glycol, Phenomenex, Torrance, CA, USA). The temperature program used was: initial temperature of 40 °C for 5 min, rising to 100 °C at a rate of 4 °C min^{-1}, and a 2 min hold. The detection temperature was set at 250 °C.

The enzymatic degradation of cell wall materials was followed by HPSEC. HPSEC was performed on three TSK gel columns (6.0 mm ID × 15 cm L per column) in series (SuperAW 4000, SuperAW 3000, SuperAW 2500; TosoHaas, Japan) and in combination with TSK SuperAW-L guard column (4.6 mm ID × 3.5 cm L; TosoHaas, Japan). Elution took place at 40 °C with 0.2 M $NaNO_3$ at 0.6 ml min^{-1}. Detection was performed using a Shodex RI71 refractive index detector (Showa Denko K.K., Japan). Calibration was performed using a pullulan standard.

Saccharide analysis was performed with high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD). An ICS3000 HPLC system (Dionex, Sunnyvale, USA), equipped with a CarboPac PA-1 column (2 mm ID × 25 cm; Dionex USA) in combination with a CarboPac PA guard column (2 mm ID × 2.5 cm L) and a ISC3000 ED PAD-detector (Dionex, USA) was used. The oligosaccharides in the samples taken after the fermentation were eluted (0.3 ml min^{-1}) with a combination of linear gradients of 0–150 mM $NaCOOCH_3$ in 100 mM NaOH during 10 min, then 150–450 mM $NaCOOCH_3$ in 100 mM NaOH during 25 min. Calibration was performed using arabinose, glucose, xylose glucuronic acid and a series of xylan oligomers.

The results were obtained by using a Microsoft Excel spreadsheet and a program Analyse-it was used for comparison of the means by one-way analysis of variance. The significant of the results from the data analysis was considered by $P < 0.05$.

3. Results and discussion

3.1. Application of β-xylanases from Th. lanuginosus and T. reesei for hydrolysis of rye and wheat cell wall materials

The higher yield of cell wall materials (Table 1) was isolated from rye (15.3%) in comparison with wheat (13.3%). The high amount of arabinose (24–25.5%), xylose (47.5–50.0%) and glucose (16.5–18%) in rye as well as in wheat showed that a substantial proportion of polysaccharides in the cell walls of these cereals consist of arabinoxylans (AX), cellulose and β-glucans. According the calculated arabinose (Ara)-to-xylose (Xyl) ratios (0.49–0.53), the structure of AX in rye and wheat are similar. The Ara/Xyl ratios determined in our analysis agreed reasonably well with literature data reported by other authors [21–24]. According to their studies, it varied from 0.46 to 0.60. On the contrary, glucose yield in analyzed cereal samples (15–18%) was higher than the yield (5–8%) that was presented by Rantanen et al. [21], Virkki et al. [22] and Pitkänen et al. [23]. It could be explained that a part of glucose may be released from undigested starch molecules or other cell wall polysaccharides, such as β-glucans. Further quantitative analysis of sugars showed that rye in comparison with wheat contained higher amounts of carbohydrates as well as AX.

Table 3
The yield (g 100 g^{-1}) of polymers ($M_w = 1 \times 10^6$ to 5×10^4 Da) present in rye and wheat cell wall materials after enzymatic degradation with different xylanases.

Cereals	*T. reesei* xylanase (Ecopulp)	*Th. lanuginosus* xylanase (Pentopan)
Rye	39.7 ± 1.2	56.9 ± 1.8
Wheat	32.5 ± 1.0	33.7 ± 0.8

The catalytic activities of xylanases from *Th. lanuginosus* (Pentopan) and *T. reesei* (Ecopulp) on hydrolysis of rye and wheat cell wall materials was followed by HPSEC (Table 3) and HPAEC (Fig. 2), some of the HPAEC elution-patterns are presented in Fig. 3.

The results of HPSEC analysis (Table 3) showed that the high molecular weight (M_w) compounds in rye cell wall materials appeared to be degradable quite well by both tested xylanases. *T. reesei* xylanase was able to degrade 60.3% and *Th. lanuginosus* xylanase – 43.3% of high M_w compounds in rye. Tested enzyme preparations were slightly more effective on wheat and released more oligomers (67.5% and 66.3%, respectively).

Furthermore, the mono- and oligosaccharides formed upon degradation of rye and wheat cell wall materials were analyzed by HPAEC (Figs. 2 and 3). The results of this analysis showed, that the yield of monosacharides and small linear xylooligosaccharides formed during enzymatic treatment of rye and wheat systems depended on the origin of xylanase preparations. The highest yield of xylose was achieved when *T. reesei* xylanase was added to rye and wheat cell wall materials. In this case, the final produced xylose in rye was 61.6 mg g^{-1} and in wheat–39.9 mg g^{-1}. Arabinose was released also in both cereal samples, and the final concentration reached 5.0–5.7 mg g^{-1}. On the contrary, *Th. lanuginosus* xylanase formed only xylobiose and xylotriose but could not convert arabinoxylans or xyloologosaccharides into xylose monomers. It appears that *T. reesei* xylanase is more effective for hydrolysis of cereal arabi-

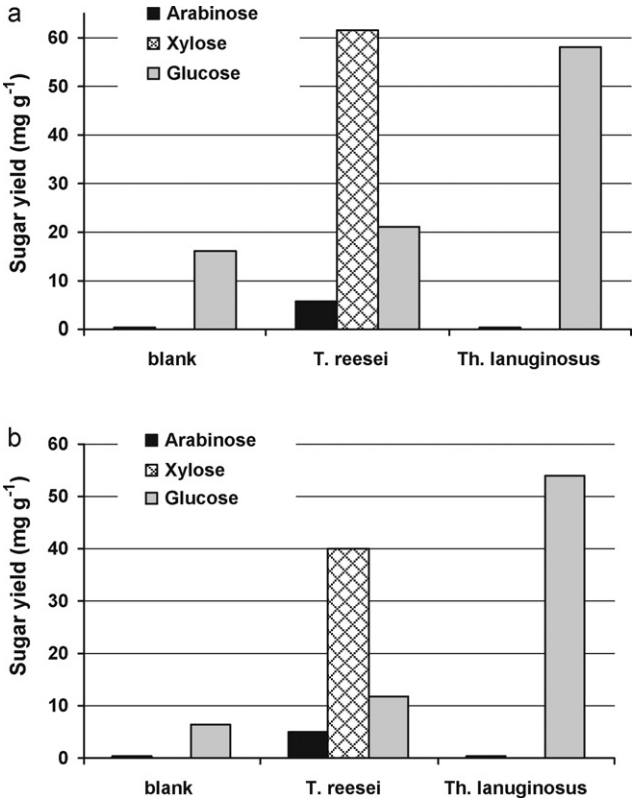

Fig. 2. Monosaccharides determined by HPAEC from rye (a) and (b) wheat cell wall materials treated with β-xylanase from *T. reesei* (Ecopulp) and *Th. lanuginosus* (Pentopan).

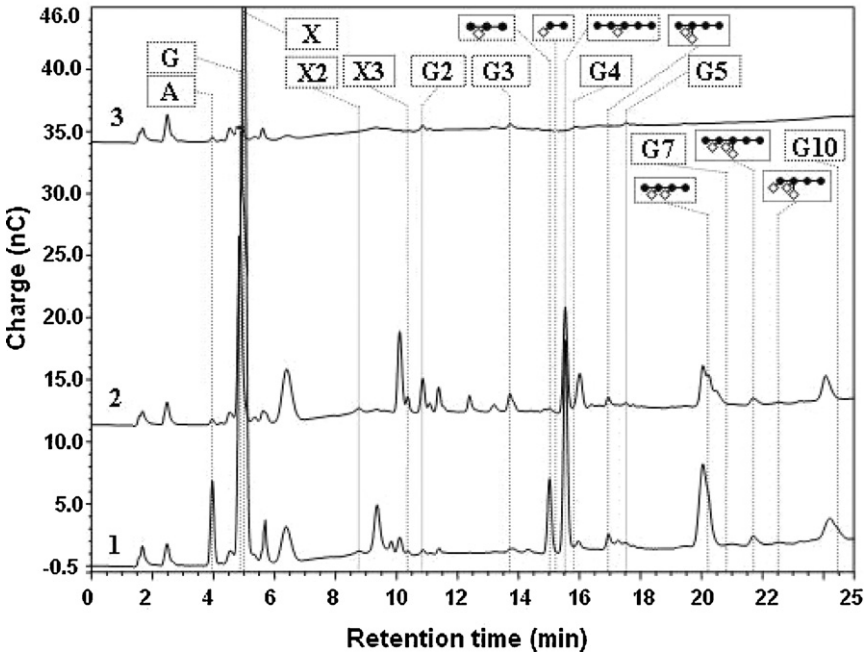

Fig. 3. HPAEC elution profiles of the mono- and oligosaccharides, obtained from rye cell wall materials treated with β-xylanase from and *T. reesei* (1) and *Th. lanuginosus* (2) (3: substrate before enzymatic degradation). Here: A, arabinose; X, xylobiose; X2, xylobiose; X3, xylotriose; G, glucose; G2, maltose; G3–G5, G7, G10, homologous series of hexoses; (●) β-D-Xyl*p*; (◊) α-L-Ara*f*; ( ) XA^{3}X = β-D-Xyl*p*-(1→4)-{[α-L-Ara*f*-(1→3)]-β-D-Xyl*p*-(1→4)}-D-Xyl; ( ) A^{3}X = α-L-Ara*f*-(1→3)]-β-D-Xyl*p*-(1→4)-D-Xyl; ( ) XXA^{3}XX = β-D-Xyl*p*-(1→4)-β-D-Xyl*p*-(1→4)-{[α-L-Ara*f*-(1→3)]-β-D-Xyl*p*-(1→4)}-β-D-Xyl*p*-(1→4)-D-Xyl; ( ) XA^{2+3}XX = β-D-Xyl*p*-(1→4)-{[α-L-Ara*f*-(1→2)]-[α-L-Ara*f*-(1→3)]-β-D-Xyl*p*-(1→4)}-β-D-Xyl*p*-(1→4)-D-Xyl; ( ) XA^{3}A^{2+3}XX = β-D-Xyl*p*-(1→4)-{[α-L-Ara*f*-(1→3)]-β-D-Xyl*p*-(1→4)}-{[α-L-Ara*f*-(1→2)]-[α-L-Ara*f*-(1→3)]-β-D-Xyl*p*-(1→4)}-β-D-Xyl*p*-(1→4)-D-Xyl; ( ) XA^{3}A^{3}X = β-D-Xyl*p*-(1→4)-{[α-L-Ara*f*-(1→3)]-β-D-Xyl*p*-(1→4)}-{[α-L-Ara*f*-(1→3)]-β-D-Xyl*p*-(1→4)}-D-Xyl; ( ) A^{3}A^{2+3}XX = {[α-L-Ara*f*-(1→3)]-β-D-Xyl*p*-(1→4)}-{[α-L-Ara*f*-(1→2)]-[α-L-Ara*f*-(1→3)]-β-D-Xyl*p*-(1→4)}-β-D-Xyl*p*-(1→4)-D-Xyl (arabinoxyloologosaccharides abbreviations are according to Fauré et al. [27]).

noxylans then to xylose. In addition to the pentoses, a high increase in glucose was obtained during the enzymatic hydrolysis of cell wall materials. This suggests that a significant fraction of cellulose or β-glucans was solubilized by the action of other glycoside hydrolases presented in commercial enzyme preparations.

In this study, in addition to monosaccharides and linear xylooligosaccharides, short chain mono- and di-substitited arabinoxylooligosaccharides with degree of polymerization DP2-DP5 were found in cereal cell wall materials after enzymatic treatment with tested enzyme preparations. The structures of identified arabinoxylooligosaccharides are indicated in Fig. 3.

The presence of different side glycoside hydrolases next to endoxylanase in the enzyme preparation could be the reason for different promotion for hydrolysis. Tenkanen [25] purified two β-xylanases, one β-xylosidase, one α-glucuronidase and two acetyl esterases produced by *T. reesei*. Blomstedt et al. [26] reported endoglucanase and cellulase activity in enzyme preparation Ecopulp from *T. reesei*. Additional enzyme activities could synergize xylanase to convert cereal arabinoxylans to xylose.

The results of this analysis showed that the type of cereal has also effect on the release of monosaccharides. The tested enzymes preparations acted more effective on rye, and released more monomers. The differences in the amount and composition of rye and wheat cell wall materials might be the reason for higher yields of monosacharides from rye. This difference has already been noticed in our previous study of the composition of rye and wheat cell wall material (Table 1).

3.2. Inhibitory activity against microbial β-xylanases in rye grains

The enzymes differed significantly in their substrate specificities. Table 4 presents the kinetic parameters of the commercial xylanases from *T. reesei* and *Th. lanuginosus* determined under reaction conditions: pH 4.5; 40 °C; 60 min. The results showed that the rate of hydrolysis of the soluble wheat arabinoxylan (AX) was on an average 3 times higher than of the birchwood xylan (X) by using both xylanases. Kinetic studies show the higher specificity of *Th. lanuginosus* xylanase to both substrates: K_m values of *Th. lanuginosus* xylanase determined were lower by 30% and 38%, respectively, than *T. reesei*. The results indicated that the *Th. lanuginosus* xylanase can be characterized as to have a 1.6 times higher catalytic activity than *T. reesei*.

Table 4
Kinetic parameters of wheat arabinoxylan (AX) and birchwood xylan (X) hydrolysis by β-xylanases from *Th. lanuginosus* and *T. reesei*.

Parameter	*T. reesei* xylanase		*Th. lanuginosus* xylanase	
	AX	X	AX	X
V_{max} (mg ml^{-1} min^{-1})	0.21 ± 0.02	0.06 ± 0.01	0.15 ± 0.01	0.05 ± 0.01
$V_{maxi,}$ (mg ml^{-1} min^{-1})	0.21 ± 0.02	0.06 ± 0.01	0.15 ± 0.01	0.05 ± 0.01
K_m (mg ml^{-1})	3.17 ± 0.36	0.29 ± 0.04	2.21 ± 0.22	0.19 ± 0.02
K_i (mg ml^{-1})	4.23 ± 0.28	0.39 ± 0.05	3.46 ± 0.38	0.32 ± 0.03

V_{maxi}, max reaction velocity with presence of inhibitor.

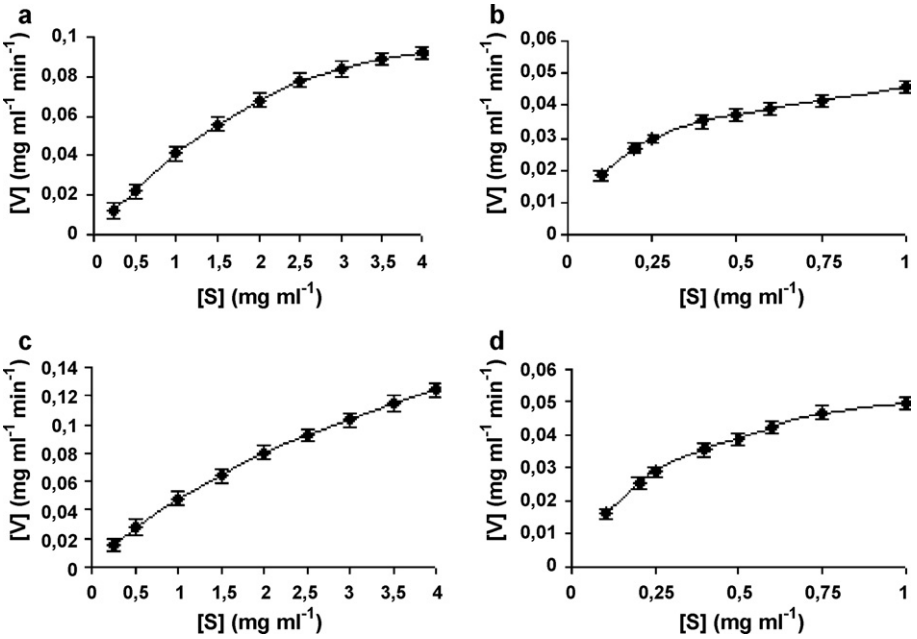

Fig. 4. Activity of the *Th. lanuginosus* (a and b) and the *T. reesei* (c and d) xylanases towards wheat arabinoxylan (a and c) and birchwood xylan (b and d).

However, the efficiency and functionality of microbial xylanases in biotechnological applications involving cereal processing may be influenced to different degrees by the relative quantities of proteinaceous xylanase inhibitors presented in cereals, and by the sensitivity of the endoxylanase to the inhibition [28]. Therefore, the rye extract was tested for its inhibitory activity against chosen xylanases. Results indicate that xylanases from *Th. lanuginosus* and *T. reesei* were inhibited, the former more than the latter. The presence of rye extract in reaction mixture decreased the activity of *Th. lanuginosus* xylanase by 30–41% and *T. reesei* xylanase by 18–27%. This data indicated that rye contain xylanase inhibitors which have a strong impact on the functionality of the xylanases. The inhibitors could influence these fermentation processes either by reducing the level of activity of the xylanases or by altering certain properties of these target enzymes. Finally, the inhibitors themselves could have an impact on the biotechnological application through their action on arabinoxilan population [29].

The kinetic studies with both of xylanases and different substrates in the presence of proteins with inhibitory activity indicated that the V_{max} did not change for both of the β-xylanases, but the K_m for *T. reesei* and *Th. lanuginosus* increased to 4.23 and 3.46 mg xylose ml^{-1} min^{-1} (wheat AX) and to 0.39 and 0.32 mg xylose ml^{-1} min^{-1} (birchwood xylan), respectively (Fig. 4). This indicates competitive inhibition, suggesting that the inhibitors bind at or are close to the xylanase active site.

In a previous study the structurally different endoxylanase inhibitors: TAXI-type with molecular weight of 11, 30.1, 29.8 and 39.9 kDa and p*I* values between 8.65 and 9.3, and TL-XI-type with molecular mass of 18.4 kDa and p*I* values of 9.3, have been partially purified from Lithuanian rye varieties [30]. All known TAXI-type xylanase inhibitors are high-p*I* proteins and occur in two molecular forms (form A, with a molecular weight of approximately 40 kDa, and form B, made up of two subunits of approximately 30 and 10 kDa) and p*I* values of at least 8.9 [31]. In wheat TAXI-I and TAXI-II were isolated, differing from one another in p*I* (8.8 and 9.3, respectively) and showing different activities towards endoxylanases. Four isoforms of TAXI-type xylanase inhibitors purified from rye have similar structure and specificity with TAXI-I from wheat [32]. A TL-XI has been identified in wheat as a basic (p*I* > 9.3) protein with a molecular weight of approximately 18 kDa which occurs as multiple isoforms [33]. All these proteins can specifically inhibit the activity of fungal and bacterial xylanases belonging to families 10 and 11 of glycoside hydrolases [34]. The inhibition is highly dependent on the nature of the enzyme used as well as specificity of inhibitor towards different β-xylanases [35,36]. Although both tested xylanases belong to the 11 family, the results of this study showed that *Th. lanuginosus* β-xylanase was more sensitive to rye xylanase inhibitors than *T. reesei*.

3.3. Enzymatic hydrolysis of cereal residues

Based on the results presented above, *T. reesei* xylanase was selected as organic catalysts for further trials.

An increment of *T. reesei* xylanase activity per assay was 50 XU at selected range (Table 1). The assays contained constant activities of amylolytic enzyme preparations, selected with reference to previous investigations [37,38] and producer recommendations (1.5 KNU g^{-1} $starch^{-1}$ and 4 AGU g^{-1} $starch^{-1}$, respectively).

In order to determine the impact of a complex of amylolytic enzymes with the xylanase preparation on the hydrolysis process, the content of SDM, FC in the wort and NFC after fermentation was measured. Results of analysis are shown in Table 5.

During the enzymatic hydrolysis with complex of a amylolytic enzymes and xylanase, the SDM content in the wort increased by 30% for both kinds of bran, with the increase of xylanase activity until 4 XU g^{-1} $bran^{-1}$. Addition of xylanase also resulted in 35% higher FC concentration in the wort in comparison with the reference sample without xylanase. Acidity of the wort and fermented broth after 72 h was measured. Rye bran as well as wheat bran turned out to be having almost the same wort acidity $0.24 \pm 0.01^{\circ}$. The obtained acidity values after fermentation were $0.40 \pm 0.02^{\circ}$. An increase of $0.06 \pm 0.02^{\circ}$ was found in comparison with the reference sample without xylanase preparation. Regular baker's yeast is most active in the medium acidity range of 0.3–0.5° [39]. The normal acidity of fermented broth shows the purity of the fermentation. It is possible to infer that the addition of xylanase resulted in higher acidity of the fermented broth and could play a positive role on the fermentation process.

Table 5
Effect of added enzyme complex on yields (g 100 g^{-1}) of soluble dry matter (SDM), fermentable carbohydrates (FC), non-fermented carbohydrates (NFC) and ethanol (Y_{Et}).

Raw material	SDM				FC		NFC		Y_{Et}[a]
	Wort		Fermented broth		Reference	Enzyme complex	Reference	Enzyme complex	
	Reference	Enzyme complex	Reference	Enzyme complex					
Wheat bran	8.4 ± 0.1	11.9 ± 0.1	0.44 ± 0.02	0.45 ± 0.02	6.7 ± 0.2	10.5 ± 0.2	0.40 ± 0.02	0.36 ± 0.02	0.441
Rye bran	8.6 ± 0.2	12.8 ± 0.2	0.46 ± 0.02	0.51 ± 0.02	7.3 ± 0.2	11.2 ± 0.2	0.40 ± 0.02	0.38 ± 0.02	0.457

Data are the mean ± SD of three analyses.
[a] Ethanol yield in g alcohol per g of FC.

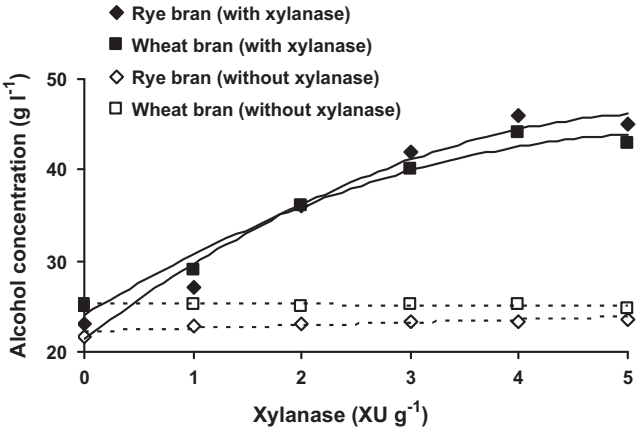

Fig. 5. Effect of *T. reesei* xylanase on the ethanol concentration in the distillate from rye and wheat bran.

3.4. Fermentability tests

Firstly, the influence of *T. reesei* xylanase preparation (Ecopulp) with complex of amylolytic enzymes on ethanol yield was investigated. The ethanol concentration increased with the increase of xylanase activity until 4 XU g^{-1} bran^{-1}. The maximum ethanol concentrations achieved after hydrolysis and fermentation were 44 g l^{-1} for both kinds of bran. The presented results showed that by application of xylanase, it is possible to increase the ethanol concentration on average by 24% and 45% for grain and bran, respectively, in comparison with the reference sample without xylanase preparation (Fig. 5). The composition of optimal amounts of amylase, glucoamylase and xylanase preparations resulted in the ethanol yields of 0.40–0.46 g g^{-1} FC^{-1} (or approximately 78–90% of the theoretical value), which were comparable with data obtained in the literature varying between 0.1 and 0.4 [40,41].

Xylanases catalyze the hydrolysis of xylans, the major component of NSP, by formation of pentoses, which in general are not fermentable by *S. cerevisiae*. However, pentoses could increase the synthesis of the yeast biomass, thereby influencing the ethanol production efficiency. Furthermore, the increase of the ethanol concentration could be influenced by synergetic action of the xylanase/cellulase/glucanase complex of the enzyme preparation Ecopulp, which renders the solubilization and depolymerization of NSP to their monomeric constituent sugars and the higher concentration of hexoses in the medium.

The alcohol concentration (C_{Et}) was plotted as a function of the amount of xylanase by using a non-linear (polynomial) regression curve fit. The regression coefficients and the slopes of the linear part of the regression curves, which are shown in Fig. 5, are presented in Table 6. Ethanol yield increased with increasing of the xylanase activity until 4 XU g^{-1} bran^{-1}. Results indicate that there is a high correlation between the added amount of *T. reesei* xylanase preparation and concentration of the end-product.

Concentrations of SDM and FC were plotted as a function of time using a linear equation. Also a high correlation between xylanase activity per assay and concentrations of fermentable carbohydrates and solid dry matter was found. Regression coefficients and the slopes of the linear part of the curves are presented in Table 6.

3.5. The qualitative determination of bioethanol

The composition of bioethanol produced from cereal residues were determined by gas chromatography (GC) with flame ionisation detection. The impact of the complex of amylolytic enzyme with xylanase on the quality of bioethanol has been evaluated. Results are shown in Fig. 6. Apart from ethanol, also methanol, propanol, isobutanol, isoamyl and amyl alcohol, acetaldehyde, ethyl acetate and methyl acetate were found in the distillate.

Application of a complex system of amylase, glucoamylase and xylanase to cereal bran enables to decrease the content of higher alcohols (fusel oil fraction) by 34% and significantly decreases the methanol concentration in the distillate, in comparison with the reference sample. Acetaldehyde and ethyl acetate were obtained at low concentrations in all samples, and it seems that xylanase preparation has no significant influence. Acetaldehyde is the intermediate product of glycolysis, which is transformed enzymatically to ethanol. Some of minor compounds (esters, acetones) are produced as by products of a secondary metabolism of yeast, especially by involving the interconversion of amino acids [42]. The results showed that the xylanase/cellulase/glucanase complex of *T. reesei* preparation Ecopulp may influence the enrichment of the yeast growing medium. A higher activity of yeast enzymes and biomass formation are obtained, herewith fostering the carbohydrate metabolism to ethanol and carbon dioxide, the two major products of glycolysis. The formation of methyl acetate during the fermentation of rye bran indicated, that rye bran was a pure grow-

Table 6
Results of regression analysis.

Raw material	C_{Et}[a]		SDM[b]		FC[c]	
	Slope K	R^2	Slope K	R^2	Slope K	R^2
Wheat bran	0.096	0.993	0.056	0.994	0.052	0.998
Rye bran	0.120	0.989	0.067	0.992	0.063	0.992

[a] Ethanol concentration.
[b] Soluble dry matter.
[c] Fermentable carbohydrates.

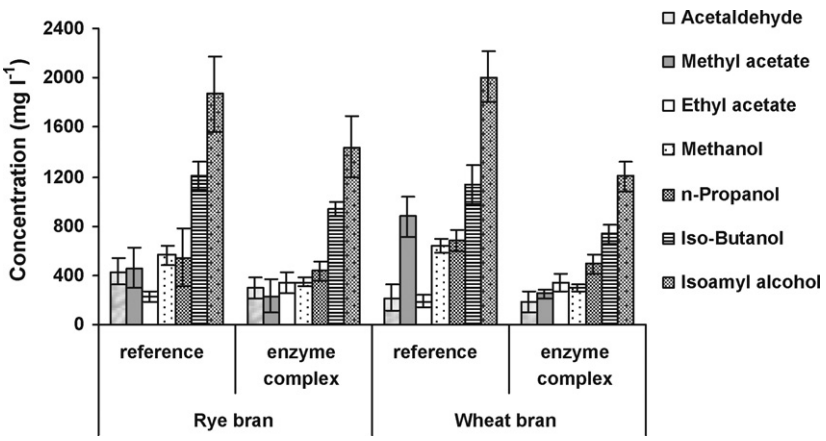

Fig. 6. Effect of *T. reesei* xylanase on the concentrations of fusel oils and methanol in the distillates from rye and wheat bran.

ing medium for yeasts. Also the different enzyme systems and lower activity of endogenous enzymes in comparison with wheat bran is shown. A high decrease in methyl acetate, obtained after enzymatic hydrolysis of rye bran using *T. reesei* xylanase preparation, sustains the advisable use of hemicellulase/cellulase complex.

4. Conclusions

To increase the efficiency of biocatalysis for second generation of bioethanol production inhibitors activity in cereal residues has been taken in consideration. Special attention has been paid for local agricultural waste from rye and decomposition specificity of the material into fermentable sugars (hydrolysis) and transformation of the sugars into bioethanol (fermentation).

Kinetic studies of different origin xylanases from *Th. lanuginosus* and *T. reesei* in the presence of proteins with inhibitory activity indicated that *Th. lanuginosus* xylanase was found more sensitive to proteinaceous xylanase inhibitors presented in rye than *T. reesei.* Furthermore *T. reesei* xylanase demonstrated affinity to hydrolysis of standard substrates of the soluble wheat arabinoxylan or birchwood xylan.

Enzymatic treatment of rye and wheat cell wall materials confirmed that the level of hydrolysis depends from origin of xylanase preparations. The highest yield of xylose and arabinose was achieved by adding *T. reesei* xylanase to these substrates, while *Th. lanuginosus* xylanase converted arabinoxylans only into xylooligosaccharides, and monosaccharides were not released. Based on the enzymatic hydrolysis of rye as well as wheat bran the optimal activity of *T. reesei* xylanase in composition with amylase and glucoamylase was selected to achieve a higher ethanol yield in the distillate.

The addition of *T. reesei* xylanase improved the quality of bioethanol by decreasing the concentrations of propanol, isobutanol, isoamyl and amyl alcohols and the methanol concentration. Furthermore the content of ethanol after enzymatic treatment with selected type of xylanase increased.

The study showed that the efficiency of fermentation of cereal residues could be increased by selecting the right type of xylanase based on substrate studies as well as on evaluation of the inhibitors in the raw material. Future research involves the study of the variability of inhibitors at the genetic and phenotypic level. The elucidation of their physiological roles in local plants will most likely reveal additional applications in biotechnology.

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