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Fermentation Efficiency of High-Gravity Rye Mashes using Pressureless Starch Liberation Methods

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Abstract

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The efficiency of ethanol production during simultaneous saccharification and fermentation (SSF) of gelatinised starch or in its native form, in high-gravity rye mashes (approx. 21% and approx. 25% dry matter) prepared by pressureless methods of starch release were compared. The obtained fermentation efficiency expressed in % of the theoretical yield was 72.98 ± 1.46% for gelatinised starch and 84.27 ± 1.68% for native starch in the mashes with 21% dry matter, while the use of mashes with 25% dry matter content resulted in 71.22 ± 1.42% and 77.36 ± 1.54% of the theoretical yield, respectively. However, the presence of residual dextrins (1.99 ± 0.82 to 3.04 ± 0.39 g/100 ml) in the fermented mashes suggests the need of further research on the improvement of the process.

Keywords: native starch; gelatinised starch; SSF; high-gravity mash

Starchy raw materials such as cereal grains or potatoes are commonly used in Polish distilleries. In the process of ethanol production, apart from costs of raw material which currently account for 50–60% of the ethanol price (Kawa-Rygielska *et al*. 2007), significant part of costs is associated with the liberation of starch from raw material (especially by pressure cooking) and its liquefaction (Sapińska *et al*. 2013).

Starch is a polysaccharide of glucose, and is not directly fermented by yeasts, thus it is necessary to subject it to some technological processes in order to obtain fermentable sugars (Kłosowski *et al*. 2010). In Poland, the most commonly used method of pre-treatment of starchy raw materials is a pressure-thermal method with the use of Henze steamer (at 140–150°C, 0.3–0.4 MPa) (Jarosz & Jarociński 1994). Energy consumption associated with the steaming of raw materials can be effectively reduced by replacing it with mechanical treatment by means of appropriate mills (Roehr 2001). This is called pressureless liberation of starch (PLS) (Kłosowski *et al*. 2006), and its total energy gain, in comparison with the pressure-thermal method, stands at 19–34% (Balcerek & Pielech-Przybylska 2012). In the PLS method, the raw material is milled at first, then mixed with water and, after the addition of appropriate enzyme preparation, the whole mixture is heated only to the temperature of 80–90°C, which considerably reduces the energy consumption (Strąk & Balcerek 2015). Moreover, the PLS method enables to work with high-gravity (HG) mashes (Balcerek & Pielech-Przybylska 2012), which results in lower water consumption (Srichuwong *et al*. 2009), but on the other hand, it exposes yeasts to osmotic stress and to the presence of inhibitors.

With the latest developments in the field of biotechnology it is possible to modernise the methods of starch hydrolysis. Using the enzymatic preparations containing enzymes that have their optimal activities below the temperature of starch gelatinisation it can reduce the cost of production of distillates of agricultural origin (Szymanowska & Grajek 2009).

Poland is the fourth largest producer of vodka in the world, behind Russia, Ukraine, and the United

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States. Polish vodka is strictly regulated and protected thanks to its standing as a geographical indication under the EU law (European Commission 2008). The term Polish Vodka may be applied exclusively in relation to spirits made of wheat, barley, potatoes, oat, or rye, and the entire production process ought to take place in Poland (Polish Journal of Statutes 2006). The selection of raw materials covered by the above-mentioned indication is a reflection of the diversity and distinctive character of Polish Vodka. To maintain its high position in the global market, research needs to be focused on the production of the best quality distillate of agricultural origin with the use of energy-saving and environmentally-friendly technologies.

The objective of this study was to compare the efficiency of ethanol production in the process of simultaneous saccharification and fermentation of gelatinised or native rye starch in the mashes with two concentrations of dry matter – DM (21 and 21% w/w).

MATERIAL AND METHODS

Rye cultivar. The Dańkowskie Amber rye cultivar purchased from Danko Plant Breeding Ltd. (Poland) was used as a raw material in all experiments. The raw material was analysed for moisture, protein, and starch content according to the methods recommended in the food industry (AOAC 1995; BS EN ISO 10520:1998).

Enzymes. Gelatinised starch was hydrolysed using the following enzyme preparations purchased from Novozymes (Denmark):

Termamyl SC – thermostable α-amylase from *Bacillus licheniformis* (pH 5.0–6.0, T = 85–110°C);

San Extra L – glucoamylase from *Aspergillus niger* (pH 4.0–5.0, T = 65–70°C).

Native starch was hydrolysed using the following enzyme preparations purchased from DuPont™ Genencor® Science (USA):

GC 626 – acid-stable α-amylase from genetically modified *Trichoderma reesei* (substantial activity at pH less than 5.0; peak activity at 62–70°C but for the 'activation' of native rye starch the temperature should be in the range of 48–51°C);

STARGEN 002™ – a blend of *Aspergillus kawachi* alpha-amylase expressed in *Trichoderma reesei* and glucoamylase from *Aspergillus niger* (pH 3.3–4.5; the recommended minimum temperature is 48°C and is specific of the type of raw material).

Yeast. In the experiments, a commercial preparation of dry distillery *Saccharomyces cerevisiae* yeast Ethanol Red (Fermentis – Division of S.I. Lesaffre, France) was used in an amount of 0.3 g/l mash.

Mineral nutrient. A mineral nutrient was an aqueous solution of $(NH_4)_2HPO_4$ in an amount of 0.2 g/l sweet mash.

Sweet mash preparation. Mashes were prepared by PLS methods as described below. The raw material was ground with the use of a FidiBus XL laboratory disc mill (KoMo, Germany) prior to experiments (particle size below 1.5 mm diameter). The process was carried out in a vessel placed in a water bath and equipped with a laboratory stirrer and thermometer. Ground grain was mixed with water at the ratio of: 3.5 l of water per 1 kg of grain (to obtain approx. 21% DM); 2.8 l of water per 1 kg of grain (to obtain approx. 25% DM).

Preparation of mashes from gelatinised starch. Milled rye grains were mixed with tap water (at a temperature of 28°C) at an appropriate ratio and heated to approx. 90 ± 2°C (stirring continuously), then the Termamyl SC preparation was added at a dose of 0.15 ml/kg of starch (pH 5.0). These conditions were maintained for 60 min, so the liquefaction (dextrinisation) of starch occurred. Subsequent stages of the process were conducted according to the principles of the SSF (Simultaneous Saccharification and Fermentation) process, i.e., the mash was cooled to approximately 60°C, supplemented with saccharifying glucoamylase (SAN Extra preparation at a dose of 0.6 ml/kg of starch; pH 5.0) and immediately cooled to the temperature at which fermentation was started (30°C). Before inoculation with yeast, the mash was acidified with sulphuric acid solution (25% w/w) to obtain the pH value of 4.0.

Preparation of mashes from native starch. Milled rye grains were mixed with tap water (at a temperature of 28°C) at an appropriate ratio and acidified to pH 4.0 with the use of 25% (w/w) sulphuric acid solution. Subsequently, the GC 626 enzymatic preparation was added in an amount of 0.3 ml/kg of the raw material. In the next step, the mixture was heated to 48°C. At that temperature, pH was controlled again, and if necessary, it was adjusted to 4.0. Next, the STARGEN 002 enzymatic preparation (in the amount of 1.2 ml per kg of raw material) was added, and the mixture was kept in these conditions for 60 min (pH 4.0, T = 48°C). The next steps of the mash preparation for fermentation were the same as in the case of the method using the gelatinised

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starch, described above. During the experiments, the following variants were conducted:

Variant I – gelatinised starch-based mashes with approx. 21% DM; variant II – gelatinised starch-based mashes with approx. 25% DM; variant III – native starch-based mashes with approx. 21% DM; variant IV – native starch-based mashes with approx. 25% DM content.

Fermentation. The fermentation was performed in 6 l flat-bottomed flasks, each containing 3.5 l of the inoculated mash supplemented with mineral nutrient. Yeasts were previously rehydrated, the yeast slurry was acidified with 25% (w/w) sulphuric acid solution to pH approx. 2.5 and allowed to stand under these conditions for 10 minutes. This procedure is designed to eliminate the weaker yeast cells, cleansing their cell membranes and destroying the undesirable bacterial microflora. The yeast slurry was added to the mashes at a ratio of 0.3 g DM/l mash. The inoculated mashes were thoroughly mixed prior to fermentation. The fermentation was conducted at 30 ± 1°C for 72 hours.

Analytical methods. Sweet mashes were analysed for total extract (using a hydrometer graduated in degrees Balling – °Blg; it refers to the concentration of dissolved solids, mostly sugar, as the weight percentage of sucrose or maltose), reducing sugars and total sugars after hydrolysis, both expressed in g glucose/100 ml of mash. Dextrins were calculated as the difference between total sugars and reducing sugars taking into consideration the conversion coefficient into dextrins –0.9 and therefore expressed in g/100 ml of mash. Fermented mashes were analysed in terms of pH, apparent extract (in the presence of alcohol), and real extract, both expressed in °Blg. All analyses were performed according to the methods recommended in the distilling industry (AOAC 1995).

The sugar profile of sweet and fermented mashes as well as ethanol concentration were determined by the HPLC method using Agilent 1260 Infinity (Agilent Technologies, USA) with a Hi-Plex H column (7.7 × 300 mm, 8 µm) (Agilent Technologies, USA), equipped with a refractive index detector (RID) at 55°C. Column temperature was maintained at 60°C and 5 mM H_2SO_4 was used as a mobile phase at a flow rate of 0.7 ml/min with the sample volume of 20 µl. Preparation of liquid samples was performed by filtration through a 0.45 µm PES (polyethersulphone) membrane prior to analysis.

Calculation of fermentation indicators. The saccharification degree (SC) was calculated as the ratio of reducing sugars released by the enzymatic hydrolysis to total sugars (after acid hydrolysis) in the sweet mash, and expressed in %.

$$SC = \frac{G_{(\text{reducing sugars released by the enzymatic hydrolysis})}}{G_{(\text{total sugars in sweet mash})}} \times 100$$

where: $G_{(\text{reducing sugars released by the enzymatic hydrolysis})}$ – g of glucose/100 ml mash; $G_{(\text{total sugars in sweet mash})}$ – g of glucose/100 ml mash

Fermentation yield was calculated in relation to total sugars (according to Gay-Lussac's stoichiometric equation) and expressed as % of the theoretical yield. Ethanol yield was expressed as the amount of absolute ethanol (A_{100}) obtained from 100 kg of rye – (l A_{100}/100 kg rye) (Kłosowski *et al*. 2010). The intake of sugars was calculated as the ratio of total sugars used up during fermentation to their content in the mash prior to this process, and expressed in %.

Statistical analysis. The samples were prepared and analysed in duplicate. The results were tested by analysis of variance at a significance level of $P < 0.05$ using the Origin 7.5 software.

RESULTS AND DISCUSSION

Chemical characterisation of raw material. The Dańkowskie Amber rye cultivar was characterised by the moisture content of 4.47 ± 0.22% (Table 1). The water content in the raw material affects its possible storage (Geissler 2009). The proteins contained in the raw material are the natural nitrogen source, essential in the metabolism of the yeast. However, a too high amount of proteins can adversely affect the course of the fermentation due to the excessive foaming of mashes (Roehr 2001). The tested rye grain was characterised by protein content (11.92 ± 0.51% DM) similar to the literature data (11.3%) (Hansen *et al*. 2004). Rye grain used in our research contained 68.50 ± 2.55% of starch, which was also

Table 1. Chemical composition of raw material

Cultivar	Moisture (%)	Protein (% DM)	Reducing sugars (g/100 g)	Starch (%)
Dańkowskie Amber	4.47 ± 0.22	11.92 ± 0.51	1.83 ± 0.25	68.5 ± 2.55

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Table 2. Chemical composition of sweet mashes

	Reducing sugars (g glucose/100 ml mash)	Dextrins (g/100 ml mash)	Total sugars (g glucose/100 ml mash)	Saccharification degree (%)
Variant I	5.01 ± 0.25	11.90 ± 0.14	18.23 ± 0.50	27.48 ± 1.50
Variant II	4.26 ± 0.85	16.85 ± 0.11	22.98 ± 0.94	18.54 ± 1.13
Variant III	8.66 ± 0.33	10.88 ± 0.85	20.75 ± 0.35	41.73 ± 1.45
Variant IV	6.54 ± 0.62	15.15 ± 0.77	23.37 ± 0.69	27.98 ± 1.09

Variant I – gelatinised starch-based mashes with approx. 21% DM; variant II – gelatinised starch-based mashes with approx. 25% DM.; variant III – native starch-based mashes with approx. 21% DM; variant IV – native starch-based mashes with approx. 25% DM

similar to the literature data (Pietruszka & Szopa 2014), and reflects the high technological usefulness of this cultivar of rye grain.

The results of the chemical analyses of sweet mashes are presented in Table 2. Samples of rye mashes before and after fermentation were analysed for reducing sugars, dextrins, and total sugar content.

The results of the chemical analyses of mashes after fermentation (Table 3) showed a lower concentration of total sugars (2.98 ± 0.41 g glucose/l00 ml – variant III, 3.19 ± 0.22 g glucose/l00 ml – variant IV) in mashes based on native starch. The mashes prepared from native starch contained almost twice less reducing sugars (0.52 ± 0.13 g/l00 ml – variant III, 0.98 ± 0.37 g/l00 ml – variant IV) compared to those prepared from gelatinised starch (1.64 ± 0.62 g/l00 ml – variant I, 1.55 ± 0.53 g/l00 ml – variant II).

Particular attention should be given to the differences in dextrins contents (Table 3). Mashes prepared from native rye starch were distinguished by a lower content of dextrins than those prepared from liquefied starch. The analysis of the results indicates that starch gelatinisation is not necessary for the proper course of saccharification and fermentation of starch. The use of the efficient STARGEN 002 enzymatic preparation, which contains a complex of amylolytic enzymes able to conduct relatively efficient saccharification of native starch without its pre-treatment, allows reaching the high efficiency of process (Balcerek & Pielech-Przybylska 2012) in comparison with the traditional PLS method. However, the dextrin content which remained in the mashes was still high, which indicates the insufficient saccharification of starch. This suggests the need of further research to improve the saccharification rate. The addition of the pullulanase preparation, catalysing the hydrolysis of α-1,6-glycosidic linkages in amylopectin, is recommended. This is consistent with the results obtained by Balcerek and Pielech-Przybylska (2009) on the impact of supportive enzymes on the course and results of fermentation of distillery mashes. The authors observed that the residues of unhydrolysed dextrins in mashes without pullulanase treatment were higher than in mashes containing pullulanase, which led to differences in the alcohol yield. During fermentation of corn mashes with the extract content of 20–21°Blg, Kłosowski *et al*. (2010) also observed that the treatment of mashes with pullulanase resulted in the acceleration of the starch hydrolysis degree and lower amounts of unhydrolysed dextrins, and as a consequence higher ethanol yield.

Chromatographic analysis of reducing sugars and ethanol content in mashes. During the fermentation process, samples were taken every 24 h and the chromatographic analysis of mashes was performed. The results of glucose, maltose, and maltotriose content are shown in Figure 1.

Table 3. Chemical composition of fermented mashes

	Apparent extract (°Blg)	Real extract (°Blg)	Reducing sugars (g glucose/100 ml mash)	Dextrins (g 100 ml mash)	Total sugars (g glucose/100 ml mash)
Variant I	1.88 ± 0.75	3.02 ± 0.45	1.64 ± 0.62	2.38 ± 0.55	4.28 ± 0.37
Variant II	4.31 ± 0.49	5.88 ± 0.77	1.55 ± 0.53	3.04 ± 0.39	4.93 ± 0.79
Variant III	2.15 ± 0.88	3.26 ± 0.22	0.52 ± 0.13	2.21 ± 0.71	2.98 ± 0.41
Variant IV	3.79 ± 0.13	5.78 ± 0.61	0.98 ± 0.37	1.99 ± 0.82	3.19 ± 0.22

variant I – gelatinised starch, mashes with 21% DM; variant II – gelatinised starch, mashes with 23% DM; variant III – native starch, mashes with 21% DM; variant IV– native starch, mashes with 23% DM

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Noteworthy is the high initial glucose content in mashes prepared using the STARGEN 002 preparation (13.68 ± 0.85 g/100 ml mash – variant III and 11.09 ± 0.67 g/100 ml mash – variant IV). Moreover, in the above-mentioned samples, a higher reduction in the concentration of glucose during fermentation was observed (the initial concentration of glucose: 13.68 ± 0.68 g/100 ml mash – variant III and 11.09 ± 0.56 g/100 ml mash – variant IV), in comparison with the samples prepared using liquefied starch (1.64 ± 0.45 g/100 ml mash – variant I and 2.55 ± 0.58 g/100 ml mash – variant II). A similar relation was also observed in the case of maltose and maltotriose concentrations, but without such a high initial concentration. This proved the high activity of the enzymes contained in enzymatic preparations in the first hours of fermentation. Low sugar content in the mashes after the first 24 h of fermentation confirmed the occurrence of simultaneous saccharification and fermentation process. The intake of fermentable sugars by yeast was directly related with the increasing ethanol concentration in fermented mashes.

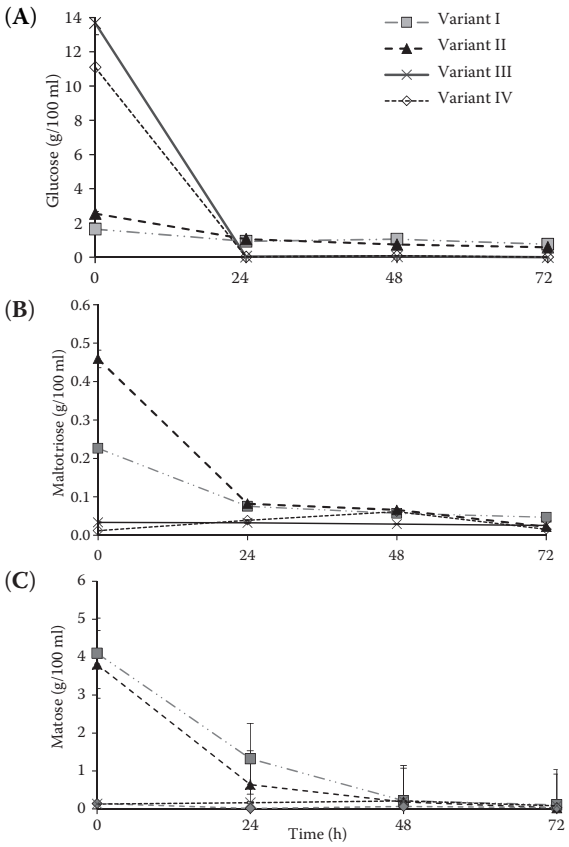

Figure 1. Changes in concentrations of (A) glucose, (B) maltose, and (C) maltotriose during the fermentation of rye mashes

variant I – gelatinised starch-based mashes with approx. 21% DM; variant II – gelatinised starch-based mashes with approx. 25% DM.; variant III – native starch-based mashes with approx. 21% DM; variant IV – native starch-based mashes with approx. 25% DM

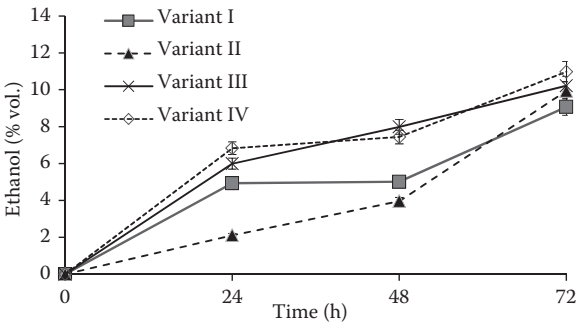

Figure 2. Changes in ethanol concentrations in rye mashes during fermentation (designation of fermentation variants – see Figure 1)

The changes in ethanol concentration in fermented mashes are shown in Figure 2. The highest final concentration of ethanol was observed in mashes based on native starch (10.98 ± 0.33% vol. for the mash with 25% DM and of 10.62 ± 0.59% vol. for the mash with 21% DM). In the case of mashes prepared from gelatinised starch, a higher ethanol concentration was observed for the mash containing 25% DM (9.94 ± 0.32% vol.), while the mash containing 21% DM reached the final concentration of ethanol at the level of 9.08 ± 0.37% vol. The rapid increase in ethanol concentration in the mashes indicates the proper course of fermentation, and additionally inhibits the growth of bacteria and wild yeasts (Puligundla *et al*. 2011).

Evaluation of fermentation indicators. Based on the obtained results, the use of sugars by yeasts during fermentation (Figure 3), fermentation yield expressed in % of the theoretical yield (Figure 4) and ethanol yield from 100 kg of raw material (Fig-

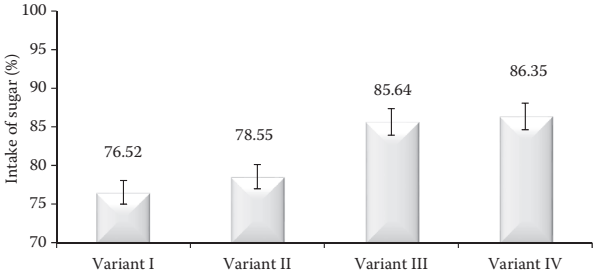

Figure 3. Intake of sugars by yeasts during the fermentation of rye mashes with various dry matter content (designation of fermentation variants – see Figure 1)

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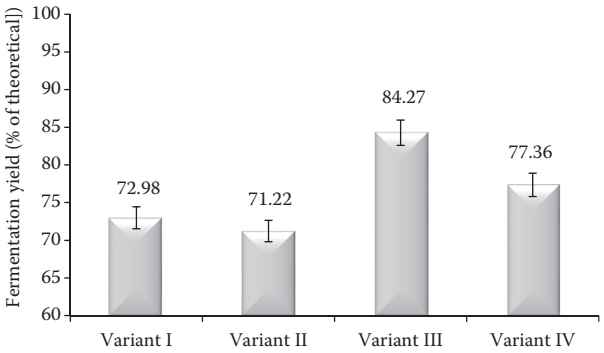

Figure 4. Fermentation yield of rye mashes (designation of fermentation variants – see Figure 1)

ure 5) were calculated. The results indicated that the higher intake of sugars was observed in simultaneous saccharification and fermentation of native starch (86.35 ± 0.85% – variant IV, 85.64 ± 0.92% – variant III) than in the mashes based on gelatinised starch (76.52 ± 0.78% – variant I, 78.55 ± 0.57% – variant II).

The highest fermentation yield (84.27 ± 1.68% of the theoretical yield) was observed in the mash with 21% DM, prepared with the STARGEN 002 preparation. However, the fermentation of samples with the same dry matter content, but prepared from gelatinised starch gave lower results (72.98 ± 1.46% – variant I). The fermentation of samples containing 25% DM resulted in higher yield when the mash was prepared from native starch compared to gelatinised starch; the yield of the process was 77.36 ± 1.54% and 71.22 ± 1.42% of the theoretical yield, respectively. This confirmed the high activity of enzymes intended to ethanol production from high-gravity mashes.

The factor which should be considered in order to evaluate the effectiveness of the process is the yield of ethanol from 100 kg of raw material (A_{100}). The highest ethanol yield was calculated for the fermentation of mashes prepared from native starch and

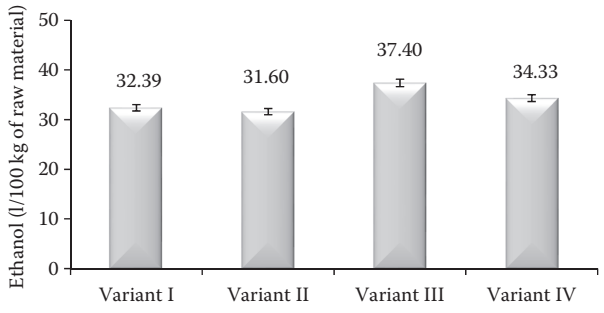

Figure 5. Ethanol yield from 100 kg of raw material (designation of fermentation variants – see Figure 1)

ranged between 34.33 ± 0.69 l A_{100} (variant IV) and 3.40 ± 0.75 l A_{100} (variant III). In the fermentation of liquefied starch-based mashes, the ethanol yield was significantly lower, i.e. by approx. 5 l for mashes with 21% DM and by approx. 2 l from 100 kg of rye grain when the fermentation was carried out by using mashes with 25% DM (Figure 5).

CONCLUSIONS

The results clearly indicate that enzymatic preparations designed for the hydrolysis of native starch have higher activity in the fermentation of mashes than enzymes used in the hydrolysis of liquefied starch. As a consequence, the efficiency of the simultaneous saccharification and fermentation of mashes with 21% dry matter content, prepared from native starch, was higher (84.27 ± 1.84% of the theoretical value) than when gelatinised starch was hydrolysed (72.98 ± 1.43% of the theoretical value). A similar pattern was observed for mashes with 25% dry matter content (77.36 ± 1.35% for native starch-based mash and 71.22 ± 1.73% for liquefied starch-based mash of theoretical value). It resulted in a significantly higher ethanol yield from mashes prepared from native starch, especially those with 21% dry matter content.

However, the presence of dextrins remaining after the fermentation of mashes indicates the need of further research on improving the efficiency of simultaneous saccharification and fermentation of mashes with high extract content, excluding the stage of gelatinisation.

Summing up, our results show that the fermentation of high-gravity, native starch-based mashes could be an alternative solution for the fermentation of starchy raw materials, eliminating the need of starch gelatinisation, and simultaneously providing the high efficiency of the process, which can improve the economic index in the alcohol-distilling industry. The combined savings in the expenses on energy and increases in the ethanol production rate would raise the alcohol plant efficiency and its competitiveness.

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