INTERNATIONAL
Agrophysics
www.international-agrophysics.org

Int. Agrophys., 2019, 33, 1-10
doi: 10.31545/intagr/103748

Solutions for improvement of saccharification and fermentation of high gravity rye mashes**

Katarzyna Pielech-Przybylska, Maria Balcerek, Piotr Patelski, and Urszula Dziekońska-Kubczak*

Department of Spirit and Yeast Technology, Institute of Fermentation Technology and Microbiology, Faculty of Biotechnology and Food Sciences, Łódź University of Technology, Wólczańska 171/173, 90-924 Łódź, Poland

Received March 2, 2018; accepted October 18, 2018

A b s t r a c t. The aim of the study was to evaluate the effects of ultrasound pretreatment, pullulanase digestion and hop α-acids preparation activity on the efficiency of simultaneous saccharification and fermentation of high gravity mashes prepared from rye starch. As a result of the ultrasonic pretreatment alone, or such treatment combined with pullulanase digestion, a decrease in viscosity of 60-69 and 85%, respectively, was observed. Also, the higher concentrations of reducing sugars were determined in these mashes ($p < 0.05$). The pretreatment of rye starch with ultrasound for 10 min resulted in a higher (by over 21%) fermentation efficiency as compared to the control mash ($p < 0.05$). Pullulanase digestion preceded by the use of ultrasound pretreatment and the antimicrobial action of hop α-acid preparation resulted in a further increase in fermentation efficiency (by *ca*. 30%), in comparison to the control sample ($p < 0.05$).

K e y w o r d s: fermentation of high-gravity mash, hop α-acids, rye starch, pullulanase, ultrasound

INTRODUCTION

High pressure/thermal pretreatment of starch raw materials for ethanol production is energy-demanding. Pressureless liberation of starch (PLS) is an alternative technology that significantly reduces thermal energy and water consumption, enabling the preparation of high-gravity cereal mashes (Srichuwong *et al.*, 2009). Particular attention must be paid to the viscosity of mashes prior to fermentation, which rises in parallel with dry matter content. High viscosity is a result of the presence of non-starch polysaccharides (NSP), including mainly pentosans and β glucans, which at elevated temperatures lead to hi gh viscosity solutions (Kłosowski *et al.*, 2010). Therefore, efforts should be made to achieve simultaneous saccharification and fermentation of starch with enzymes capable of its degradation without gelatinization (Szymanowska and Grajek, 2009).

Liquefaction and saccharification of starch during mashes preparation is carried out by means of amylolytic enzyme preparations. Since amyloglucosidase degrades α-1,6-linkages much slower than α-1,4-linkages (Roy and Gupta, 2004), substantial inhibition of enzymatic degradation can be observed (Kłosowski *et al.*, 2010). This leads to the production of limit dextrins which are unfermentable by yeast.

In order to solve these problems, supportive enzymes can be applied, including pullulanase which converts starch into linear dextrins and oligosaccharides that are easily digested into glucose by glucoamylase (Hii *et al.*, 2012) and enzymes hydrolyzing NSP. In our previous paper (Balcerek and Pielech-Przybylska, 2012) the effect of supportive enzymes (xylanase and pullulanase) on rye mashes viscosity decrease and ethanol yield efficiency increase was demonstrated.

An interesting solution is also to use ultrasound pretreatment of starchy raw materials, which brings several benefits. During ultrasound treatment, the processes of formation, growth and rapid collapse of microbubbles are observed (Li *et al.*, 2009; Manchun *et al.*, 2012). The collapse microbubbles phenomenon is accompanied by an increase in both pressure and temperature which affects the depolymerisation of polysaccharides by the mechanical breakage of macromolecular C-C bonds and chemical degradation of these polymers, as a result of its reaction with hydroxyl radicals produced during the cavitation process (Jambrak *et al.*, 2010). It was confirmed that ultrasonic

*Corresponding author e-mail:
katarzyna.pielech-przybylska@p.lodz.pl

**This work was partially supported by the Polish National Centre for Research and Development under grant PBS2/B8/9/2013.

pretreatment of starch granules reduces the molecular weight of amylose and amylopectin (Jambrak *et al.*, 2010), as well as improves starch solubility (Li *et al.*, 2009). Some authors (Pejin *et al.*, 2012; Pietrzak and Kawa-Rygielska, 2014) use sonication to improve effectiveness of starch hydrolysis and fermentation. Pejin *et al*. (2012) and Nikolić *et al*. (2010) studied ultrasound application in bioethanol production from triticale and corn, respectively. Ultrasonic pretreatment caused an increase in the fermentable sugars content (glucose and maltose) in the obtained triticale mashes and, as a result, higher ethanol concentration was observed. Similar conclusions were given in Nikolić *et al*. (2010) work, where 5 min sonication at temperature of 60°C was deemed to constitute optimal conditions to obtain higher (by 6.82 %) concentration of glucose in the liquefied corn-based mash, along with higher (by 11.15%) ethanol content in the fermented mash, in comparison with the untreated sample.

Ultrasonic pretreatment also induces changes in the structure of the lignin-hemicellulose complex (Ebringerová and Hromádková, 2002; Ivetić *et al.*, 2017). Extraction of polysaccharides, including cellulose and arabinoxylans, caused by the phenomenon of cavitation, can increase the efficiency of their further hydrolysis with the use of enzyme preparations. Moreover, during ultrasonic pretreatment their partially hydrolysis can occur. Ultrasound application to sugar beet shreds treatment in research carried by Ivetić *et al.* (2017) affected the loss in substrate weight which was caused by partial degradation and solubilisation of some components. Results showed, among others, lower (by 25%) xylan content in the obtained medium.

The results obtained by Sun *et al.* (2004) showed that ultrasonic treatment of bagasse increased accessibility of the hemicelluloses caused by the cleaved linkages between lignin and hemicelluloses. Li *et al*. (2016) investigated the effect of ultrasounds on arabinoxylans properties and concluded that molecular weight of arabinoxylans decreased after using this treatment process.

Another advantage of using ultrasound is the fact that it is known to destruct living cells, among others, bacteria. Moreover, their effect can be enhanced by the combined action with bactericides (Mason *et al*., 1996).

The objective of this study was to determine the effect of the time of ultrasound pretreatment, pullulanase digestion and hop α-acids activity on the course and efficiency of simultaneous saccharification and fermentation of rye starch. An estimation of the microbial contamination of the rye mashes during fermentation was also performed.

MATERIALS AND METHODS

The Dańkowskie Diament rye grain variety (Danko Plant Breeding Ltd., Poland) was used. Average starch content 62.02±1.18% was measured with the polarimetric Evers method (BS EN ISO 10520:1998).

Fermentations were carried out using the Ethanol Red dry distillery yeast (*S. cerevisiae*, Fermentis-Division of S.I. Lesaffre, France). Prior to fermentation, the yeast was hydrated and acid-washed (15 min incubation of cells suspended in water with an addition of the sulfuric acid solution 25% w/w, pH 2.5, at room temperature). The yeast cream was added to the mashes in the amount of 0.3 g d.m. l^{-1} of mash.

For starch and non-starch polysaccharides hydrolysis the following enzyme preparations were used: GC 626 – acid-stable α-amylase (DuPont™ Genencor® Science, USA), Optimash™VR – blend of xylanase and cellulase (DuPont™ Genencor® Science, USA), STARGEN 002™ – blend of α-amylase and glucoamylase (DuPont™ Genencor® Science, USA), and Promozyme 200L – pullulanase (Novozymes, Denmark).

Before fermentation an aqueous solution of $(NH_4)_2HPO_4$ at a dose of 0.2 g l^{-1} mash was added as a mineral nutrient for yeast.

In order to prevent bacteria growth in the prepared mashes, antimicrobial hop α-acids preparation IsoStab® (BetaTec GmbH, Germany) was used at a dose of 80 ppm.

Experiments included fermentation trials using six variants of the mashing process:

I. Control sample
II. Ultrasound pretreatment (5 min)
III. Ultrasound pretreatment (10 min)
IV. Ultrasound pretreatment (15 min)
V. Ultrasound pretreatment (10 min) and digestion with pullulanase
VI. Ultrasound pretreatment (10 min) and digestion with pullulanase, and the IsoStab preparation addition.

For variants II-VI, mashes preparation was carried as follows: milled rye grains (1.5 kg) were mixed with warm (*ca*. 35°C) water (2.8 l 1 kg^{-1}); the obtained mixture was divided into three portions; each portion (1.5 l) was subjected to ultrasonic pretreatment using an UP400S ultrasonic processor (Hielscher, Germany) at an amplitude of 100% (ultrasound power 400 W, 24 kHz), at room temperature; the samples were then subjected to the mashing process, carried out in a cylindrical vessel placed in a water bath and equipped with a double blade impeller and a thermometer. The mashing process was carried out as presented in Fig. 1. A control sample (I) was prepared in a similar manner, but without the ultrasound treatment. Total solids content in the prepared mashes was approx. 28.5 ± 0.3% (w/w).

Prior to fermentation, mashes were supplemented with $(NH_4)_2HPO_4$ and inoculated with yeast. Finally, they were mixed thoroughly. The IsoStab® hop acid preparation was added to the selected rye mashes as an inhibitor to microbial infections. Fermentation was conducted for 3 days at 35-38°C in a 2 l glass bottles containing 1.3 l of mash, and closed with fermentation locks containing paraffin oil. The course of the process was controlled gravimetrically. During fermentation, mashes samples were collected for the analysis of ethanol, reducing sugars, dextrins and

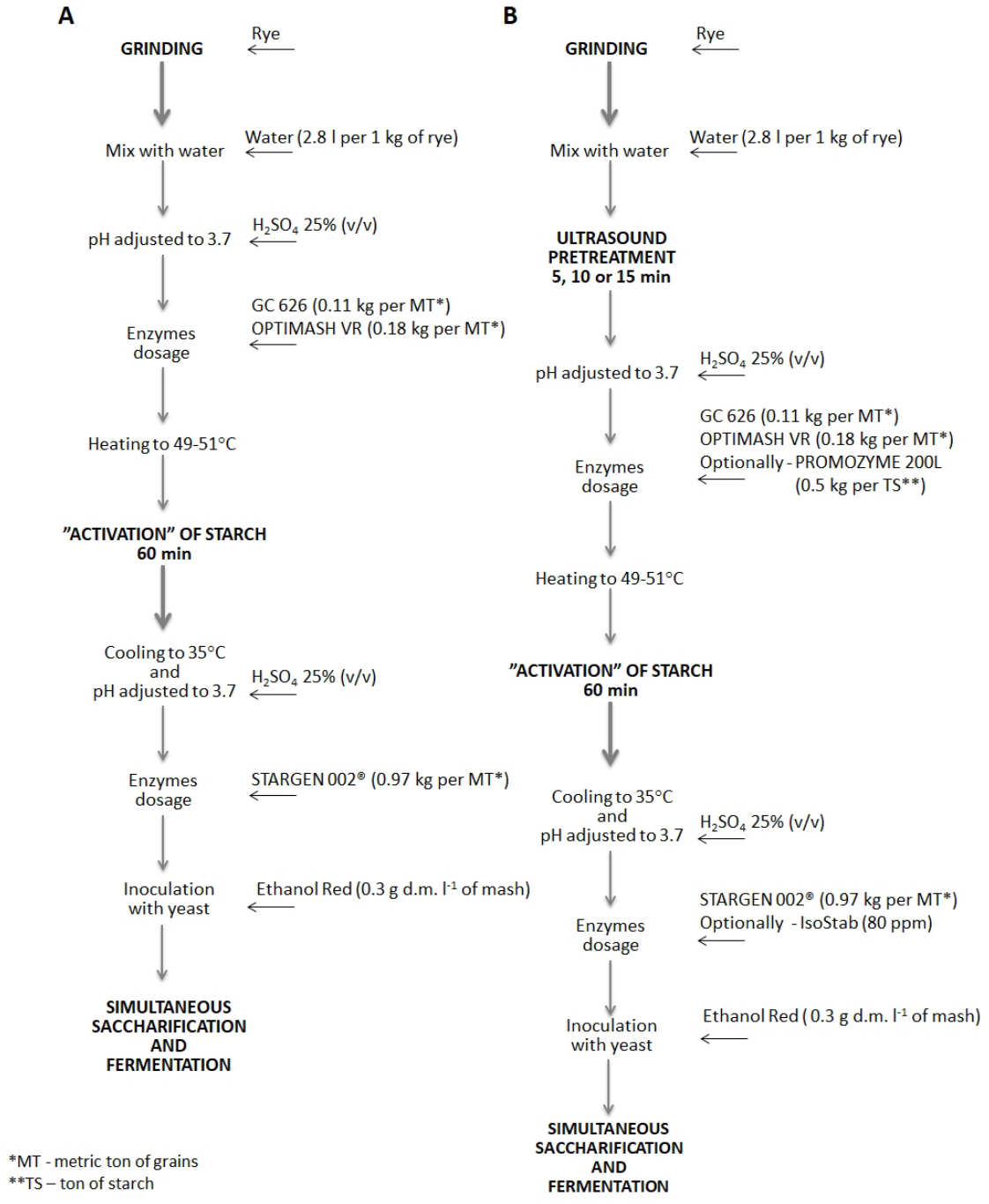

Fig. 1. Scheme of high gravity rye mashes preparation: A – control sample without ultrasound pretreatment; B – samples with ultrasound treatment.

dissolved solids content. The microbial contamination of mashes (before, during and on completion of the process) was also evaluated.

Rye mashes were analyzed for sugars and dissolved solids, both before and after fermentation. Reducing and total sugars were determined using the DNS reagent (Miller, 1959) and expressed in g glucose l^{-1} mash. Dextrins content was calculated as a difference between total sugars and reducing sugars, using a conversion coefficient of 0.9, and finally expressed in g l^{-1} mash (Balcerek and Pielech-Przybylska, 2012). The content of dissolved solids in prepared mashes was measured with a Brix hydrometer (AOAC, 2006) and results were expressed in % (w/w). On completion of the fermentation process, the concentration of dissolved solids in mashes was determined after ethanol distillation. The viscosity of mashes was determined using a falling ball viscometer (Visco Ball Höppler, Fungilab) (Sapińska *et al.*, 2013).

During fermentation and on completion of the process, mashes were analysed for ethanol concentration after distillation in a digital distilling unit Super Dee (Gibertini, Italy). Ethanol concentration in distillates was measured using a hydrometer with the scale expressed in % (v/v) of ethanol.

For microbial analysis, the samples of distillery mashes were prepared in accordance with ISO 6887 (ISO 6887-1:1999). The total mesophilic count (TMC) was determined on plate count agar (PCA, Merck), following incubation at 30°C for 96 h. Lactic acid bacteria (LAB) were determined on de Man, Rogosa, Sharpe (MRS) agar (Merck), following incubation at 30°C for 72 h under anaerobic conditions (Gas-Pack System, BBL, Becton Dickinson, Franklin Lakes, NJ USA) (ISO 4833:2004). Yeast count was determined on Dichloran Rose Bengal Chloramphenicol (DRBC) agar (Oxoid, Wesel, Germany), following incubation at 25°C for 96 h (ISO 21527-1:2008).

Fermentation energy was calculated as the ratio of the amount (g) of carbon dioxide liberated in successive hours of the fermentation process to the total carbon dioxide decrement (g), and expressed in %. Fermentation productivity was expressed as the amount of absolute ethanol (A_{100}) produced in one litre of mash within 1 h (ml A_{100} l^{-1} h^{-1}). Fermentation yield was calculated in relation to total sugars (according to the stoichiometric Gay-Lussac's 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^{-1} rye). The total sugar intake was calculated as the ratio of sugars used up in the fermentation process to their content in the mash prior to this process, and expressed in %.

All samples were prepared and analyzed in triplicate. The results were tested by means of the analysis of variance (ANOVA) at a significance level of $p < 0.05$ using Origin 7.5 software.

RESULTS AND DISCUSSION

Apart from starch, cereal grains contain various amounts of non-starch polysaccharides (NSPs), which are composed predominantly of arabinoxylans (pentosans), β-glucans, and cellulose (Căpriţă *et al.*, 2010). Arabinoxylans (AX) are the predominant NSP in rye (8.9%) and wheat (6-8%), while β-glucans are the predominant NSP in barley (7.6%) (Schweizer and Würsch, 1979; Wang and Zhang, 2010). These polymers are present in plant cell walls and form viscous colloidal solutions in water. The viscosity of cereal mashes is an important factor in distilleries, because it may cause problems during mashing, fermentation and distillation processes (Balcerek and Pielech-Przybylska, 2009). The treatment of cereal mashes with supportive enzyme preparations containing hydrolases of non-starch polysaccharides, *i.e.* xylanase, cellulase and endo-beta-1:3,1:4-glucanase, may decrease the viscosity of sweet mashes (especially very high gravity (VHG) mashes) (Wang *et al.*, 1999), while improving the efficiency of the technological process. When xylanase and α-amylase act in concert, both pentosans and starch undergo degradation. Besides, the depolymerisation of xylan attached to starch granules renders the latter more accessible to amylolytic enzymes (Scheffler and Bamforth, 2005).

The obtained results presented in Table 1 demonstrate that the viscosity of mashes decreased by 21.4% ($p < 0.05$) upon extending sonication time from 5 to 10 min. In comparison with the control sample, a sonication for 10 min resulted in the reduced viscosity by 68.6% ($p < 0.05$). However, its further extension did not lead to a significant decrease in mash viscosity ($p > 0.05$). As a consequence, 10 min was selected as the optimum pretreatment duration.

T a b l e 1. Viscosity of rye mashes (before fermentation)

Fermentation trial	Viscosity (Pa s)
Control sample (without ultrasound treatment)	5.39±0.21a
Ultrasound pretreatment (5 min)	2.14±0.09b
Ultrasound pretreatment (10 min)	1.68±0.07c
Ultrasound pretreatment (15 min)	1.74±0.07c
Ultrasound pretreatment (10 min) and digestion with pullulanase	0.79±0.03d

Different letters (a-d) indicate significant differences ($p < 0.05$) between means (ANOVA) at a significance level of 0.05.

Huang *et al.* (2007) reported that the degree of hydrolysis of corn starch increased sharply when the sonication time was prolonged from 3 to 9 min while a further extension of the time of this treatment did not improve the degree of starch hydrolysis any more. This is due to the fact that ultrasound action is effective towards amorphous regions in starch granules.

Digestion of distillery mashes with supportive enzymes, pretreated with ultrasounds, may significantly reduce their viscosity. It is connected with the fact that arabinoxylan molecules tend to form macromolecular aggregates in the dilute polysaccharide solutions. Ultrasonication helps to reduce the aggregates of polysaccharides effectively (Iida *et al.*, 2008) and, as a consequence, to lower viscosity.

The application of pullulanase, preceded by10 min ultrasound action, resulted in a decrease in mash viscosity of 55% as compared to the mash obtained under the same treatment conditions, but without pullulanase addition, and of 85% as compared to the controls ($p < 0.05$), (Table 1). Balcerek and Pielech-Przybylska (2009) reported that the use of this enzyme during the mashing process lowered the viscosity of rye mashes by 50-73% relative to the controls.

The results of the microbial analysis of rye mashes are summarized in Table 2. The yeast count introduced to all tested rye mashes was similar ($p > 0.05$). A significant increase in yeast count was observed up to 24 h of fermentation (the pre-fermentation phase).

T a b l e 2. Microbial analysis of rye mashes during fermentation

Fermentation trial	Time of fermentation (h)	Yeast count	Bacteria count	
			lactic acid	total
		log (cfu g^{-1})		
Control sample (without ultrasound pretreatment)	0	6.85±0.25bc	2.00±0.15d	2.70±0.17d
	16	7.34±0.40c	5.40±0.35h	7.65±0.55ij
	24	8.40±0.55d	8.53±0.75ij	8.68±0.65ij
	36	8.34±0.50d	8.70±0.75j	8.83±0.72ij
	48	7.08±0.42b	7.74±0.65ij	8.85±0.75ij
	60	6.40±0.35ab	7.65±0.60ij	8.76±0.55ij
	72	6.26±0.22a	7.34±0.60i	7.52±0.45i
Ultrasound pretreatment (5 min)	0	6.65±0.25b	1.30±0.05b	2.08±0.15c
	16	7.40±0.41c	2.18±0.15de	3.40±0.25e
	24	8.53±0.55d	3.15±0.25f	4.48±0.35f
	36	8.34±0.45d	3.36±0.28f	5.75±0.35g
	48	7.77±0.45c	4.43±0.35g	6.79±0.50h
	60	7.51±0.40c	4.41±0.35g	6.81±0.55h
	72	7.36±0.42c	3.40±0.25f	6.80±0.55h
Ultrasound pretreatment (10 min)	0	6.66±0.55bc	1.00±0.05a	1.18±0.05a
	16	7.40±0.65c	1.70±0.15c	3.08±0.55de
	24	8.59±0.75d	2.40±0.18e	3.32±0.15e
	36	8.51±0.65d	3.11±0.25f	3.48±0.22e
	48	8.45±0.62d	3.48±0.25f	5.66±0.45g
	60	7.54±0.55c	3.40±0.25f	5.72±0.55g
	72	7.48±0.50c	3.30±0.22f	5.78±0.55g
Ultrasound pretreatment (15 min)	0	6.84±0.35b	1.00±0.05a	1.00±0.15a
	16	7.40±0.55c	1.78±0.15c	1.90±0.15c
	24	8.60±0.70d	2.18±0.18de	2.49±0.20d
	36	8.65±0.75d	3.18±0.25f	3.40±0.25e
	48	8.32±0.55d	3.18±0.25f	3.40±0.25e
	60	7.93±0.45c	3.18±0.25f	3.48±0.25e
	72	7.92±0.45c	3.11±0.22f	3.48±0.25e
Ultrasound pretreatment (10 min) and digestion with pullulanase	0	6.75±0.37b	1.00±0.05a	1.18±0.05a
	16	7.38±0.44b	1.70±0.15c	3.08±0.20e
	24	8.56±0.65d	2.40±0.15e	3.32±0.25e
	36	8.51±0.65d	3.11±0.25f	3.48±0.25e
	48	8.40±0.66d	3.48±0.25f	4.66±0.35f
	60	7.54±0.55c	3.45±0.25f	4.74±0.35f
	72	7.48±0.50c	3.40±0.25f	5.72±0.35g
Ultrasound pretreatment, 10 min and digestion with pullulanase and IsoStab® hop α-acids preparation addition	0	6.80±0.45b	1.00±0.03a	1.18±0.05a
	16	7.40±0.55c	1.18±0.12ab	1.30±0.05b
	24	8.59±0.70d	1.00±0.03a	1.18±0.03a
	36	8.51±0.65d	1.00±0.03a	1.18±0.05a
	48	8.45±0.75d	1.00±0.02a	1.18±0.05a
	60	8.40±0.75d	1.00±0.05a	1.18±0.05a
	72	7.54±0.65c	1.00±0.05a	1.18±0.05a

Different letters (a-j) in columns designate statistically significant differences ($p < 0.05$).

The number of the total mesophilic bacteria in the control sample at the beginning of the fermentation process amounted to 2.70±0.17 log (cfu ml^{-1}) while the lactic acid bacteria count indicated the level of 2.00±0.15 log (cfu ml^{-1}). Unfortunately, the lack of antimicrobial protection resulted in the successive increase in the number of bacteria. The highest level of bacteria was observed in the control mash after 36 h of fermentation.

It was found that 10 min of ultrasound pretreatment is sufficient to reduce the initial level of microbial contaminants in mashes by *ca*. 50% as compared to the control sample. Prolongation of the sonication time to 15 min did not reduce the number of bacteria ($p > 0.05$). The lethal effect of high power ultrasound is due to the cavitation phenomena (Kentish and Feng, 2014)

The ultrasound pretreatment applied in our study did not ensure the complete elimination of microbes which resulted in the subsequent increase in the number of bacteria during fermentation. However, it remains valid that the number of bacteria in all fermented mashes treated with ultrasound before mashing was significantly lower than in the control sample ($p < 0.05$).

The growing use of the pressureless methods of starchy raw materials processing in the distilling industry encourages us to look for efficient and safe methods of microbial protection. The latest solution recommends the use of hop α-acids preparations as natural inhibitors of microbial infections (Rückle and Senn, 2006).

The use of the IsoStab hop α-acids preparation during fermentation of high gravity rye mashes (preceded by ultrasound pretreatment and pullulanase digestion) resulted in the inhibition of microbial infections and number of bacteria was low and similar during the whole fermentation process. The obtained results stay in agreement with our previous findings (Balcerek and Pielech-Przybylska, 2012; Pielech-Przybylska *et al.*, 2017).

Samples of rye mashes before and during fermentation were collected for reducing sugars, dextrins and dissolved solids content determination. Moreover, fermentation energy, ethanol concentration, its productivity and efficiency, the intake of sugar and ethanol yield from the unit (100 kg) of raw material were established. The obtained results are presented in Tables 3-4 and in Figs 2-3.

The application of ultrasound pretreatment (10 min) of rye starch resulted in higher ($p < 0.05$) contents of reducing sugars, in comparison with the control sample. Consequently, the concentration of dextrins was lower ($p < 0.05$). However, it was observed that an increase in the time of ultrasound action from 10 to 15 min did not significantly improve the degree of starch saccharification. On the other hand, as shown in Table 3, the application of a pullulanase preparation enhanced the initial starch hydrolysis. The concentration of reducing sugars rose by over 25% ($p < 0.05$) with a corresponding decline in dextrin content, in comparison with the sample treated by ultrasound only (10 min). This means that the subsequent action of ultrasounds and pullulanase constitutes an advantage of this variant over other studied variants.

The increase in ethanol concentration in the control mash was slower than in all the other trials. Lower availability of fermentable sugars resulted in the extension of fermentation phases. It was observed in the changes of fermentation energy (Fig. 2). Application of ultrasound pretreatment (10 min) before mashing and digestion with pullulanase resulted in a significant improvement of fermentation energy. The process proved to be the most dynamic when the mash was supplemented before fermentation with the IsoStab hop α-acids preparation. In this mash, the liberation of carbon dioxide stopped after 60 h while in the remaining trials the fermentation process was completed after 72 h (Fig. 2).

Simultaneously, it should be noted that the intake of total sugars in this variant reached 94.58±0.9% and did not statistically differ from the analogous variant containing no hop α-acids preparation (Table 3).

The obtained results indicate that the lack of microbial infections during the fermentation of mashes supplemented with the hop α-acids preparation contributed to improving yeast activity towards ethanol production. When the undesirable microorganisms compete with yeasts for nutritional substances, the fermentation process is impeded and runs more slowly (Broda and Leja, 2010).

For all mashes, the maximum ethanol productivity was observed between 24 and 36 h of the process (Table 4). In the samples collected at later fermentation hours, alcohol productivity successively decreased. It was observed that the sonication for 10 min caused the most intense ethanol production as compared to the controls ($p < 0.05$).

Pejin *et al.* (2012) examined the feasibility of ultrasound pretreatment to enhance the release of fermentable particles and increase bioethanol yield in the simultaneous saccharification and fermentation (SSF) of triticale meal. The results of their study showed that ultrasound-pretreatment at 60°C (5 min, a frequency of 40 kHz) was helpful in obtaining the maximum ethanol content of 9.55% (w/v), ethanol yield of 0.43 g g^{-1} starch, and percentage of the theoretical ethanol yield of 84.56%. On the other hand, Pietrzak and Kawa-Rygielska (2014) who studied several pretreatment methods to improve the course of fermentation of waste wheat-rye bread (after shelf-life, returns from shops), using the granular starch hydrolyzing enzyme, stated that if the sonication (5 min, the power of the bath of 250 W and ultrasonic frequency of 40 kHz) was applied, the ethanol yield reached 82.81 ± 4.45% of the theoretical (366.78 ± 19.70 g absolute ethanol kg^{-1} raw material, whereas the fermentation of untreated waste bread ended with 80% ethanol yield that corresponded to 354.36 g of absolute ethanol kg^{-1} of raw material).

Table 3. Changes of reducing sugars, dextrins, and dissolved solids during fermentation

Hours of fermentation (h)	Parameters	Fermentation trial					
			Ultrasound pretreatment				
		Control sample (without ultrasound pretreatment)	5 min	10 min	15 min	10 min and digestion with pullulanase	10 min digestion and with pullulanase and hop α-acid preparation addition
0	Reducing sugars (g glucose 100 ml^{-1})	6.02±0.15a	7.50±0.20b	9.10±0.26c	9.44±0.1c	11.44±0.31d	11.50±0.31d
	Dextrins (g 100 ml^{-1})	12.44±0.75c	11.60±0.79c	9.55±0.67b	9.18±0.92b	7.15±0.37a	7.25±0.35a
	Dissolved solids % (w/w)	24.21±1.02a	24.10±1.10a	24.21±1.08a	24.24±0.95a	24.17±0.99a	24.21±0.90a
16	Reducing sugars (g glucose 100 ml^{-1})	5.68±0.11c	5.56±0.10c	4.81±0.12b	4.83±0.09b	2.60±0.12a	2.55±0.12a
	Dextrins (g 100 ml^{-1})	3.41±0.07c	3.04±0.06b	2.31±0.05a	3.05±0.06b	4.93±0.10d	4.93±0.10d
	Dissolved solids % (w/w)	13.84±0.28d	11.60±0.23c	12.76±0.26b	12.84±0.26b	10.58±0.21a	10.48±0.20a
24	Reducing sugars (g glucose 100 ml^{-1})	2.42±0.05d	1.54±0.03b	2.29±0.05c	1.56±0.03b	0.53±0.05a	0.45±0.05a
	Dextrins (g 100 ml^{-1})	1.55±0.03a	2.26±0.05b	1.63±0.03a	2.33±0.05b	2.66±0.15c	2.46±0.08bc
	Dissolved solids % (w/w)	9.36±0.19ab	9.76±0.20b	8.90±0.18ad	9.32±0.19ab	8.54±0.27cd	8.24±0.17c
36	Reducing sugars (g glucose 100 ml^{-1})	0.50±0.01c	0.54±0.01d	0.40±0.01b	0.50±0.01c	0.24±0.01a	0.24±0.01a
	Dextrins (g 100 ml^{-1})	1.53±0.03b	1.30±0.03a	1.21±0.02a	1.84±0.04c	1.31±0.13a	1.11±0.10a
	Dissolved solids % (w/w)	7.44±0.15bc	7.14±0.14b	6.74±0.13a	7.62±0.15c	6.62±0.13a	6.52±0.12a
48	Reducing sugars (g glucose 100 ml^{-1})	0.47±0.01c	0.41±0.01ac	0.39±0.01a	0.38±0.01a	0.24±0.05b	0.20±0.01b
	Dextrins (g 100 ml^{-1})	1.42±0.03a	1.24±0.02e	1.17±0.02d	1.46±0.03a	0.92±0.02c	0.82±0.02b
	Dissolved solids % (w/w)	6.94±0.14ab	7.00±0.14ab	6.64±0.13a	7.22±0.14b	6.20±0.12c	5.95±0.12c
60	Reducing sugars (g glucose 100 ml^{-1})	0.41±0.01a	0.32±0.01d	0.39±0.01a	0.38±0.01a	0.18±0.01b	0.25±0.02c
	Dextrins (g 100 ml^{-1})	1.34±0.03b	1.18±0.02a	1.12±0.02a	1.39±0.03b	0.88±0.08d	0.65±0.05c
	Dissolved solids % (w/w)	6.74±0.13b	5.98±0.12a	6.02±0.12a	6.74±0.13b	6.14±0.12a	3.36±0.09c
72	Reducing sugars (g glucose 100 ml^{-1})	0.56±0.01d	0.45±0.05cd	0.32±0.02ab	0.39±0.09bc	0.25±0.02a	0.24±0.02a
	Dextrins (g 100 ml^{-1})	3.77±0.11b	2.98±0.72b	1.43±0.02a	1.46±0.24a	0.72±0.08a	0.65±0.07a
	Dissolved solids % (w/w)	6.72±0.17d	6.08±0.07c	4.29±0.09b	4.51±0.11b	3.46±0.09a	3.34±0.08a
	Intake of total sugars (% of theoretical)	76.06±2.04c	81.11±1.91d	90.31±2.21ab	89.67±2.17a	94.58±0.9ab	94.99±1.2b

Different letters (a-e) in lines indicate significant differences ($p < 0.05$) between means (ANOVA at a significance level of 0.05).

Table 4. Results of the simultaneous saccharification and fermentation of high gravity rye mashe

Hours of fermentation (h)	Parameters	Fermentation trial					
			Ultrasound pretreatment				
		Control sample (without ultrasound pretreatment)	5 min	10 min	15 min	10 min and digestion with pullulanase	10 min and digestion with pullulanase and hop α-acid preparation addition
16	Ethanol concentration (%, v/v)	2.06±0.02b	2.57±0.02c	3.48±0.03a	3.53±0.04a	4.30±0.04d	5.30±0.04e
	Fermentation productivity (ml A_{100} l^{-1} h^{-1})	1.29±0.01b	1.61±0.01c	2.18±0.02a	2.21±0.03a	2.69±0.03d	3.31±0.03e
24	Ethanol concentration (%, v/v)	4.08±0.04a	4.35±0.04b	6.40±0.05c	6.42±0.05c	7.93±0.05d	9.68±0.05e
	Fermentation productivity (ml A_{100} l^{-1} h^{-1})	1.70±0.02a	1.81±0.02b	2.67±0.02c	2.67±0.02c	3.30±0.02d	4.03±0.02e
36	Ethanol concentration (%, v/v)	6.70±0.04a	6.90±0.05b	7.75±0.05c	7.68±0.05c	9.67±0.05d	11.50±0.05e
	Fermentation productivity (ml A_{100} l^{-1} h^{-1})	1.86±0.01a	1.92±0.01c	2.15±0.01b	2.13±0.01b	2.69±0.01d	2.96±0.01e
48	Ethanol concentration (%, v/v)	7.92±0.04a	8.50±0.05b	9.20±0.06c	9.45±0.06d	11.05±0.05e	11.37±0.05f
	Fermentation productivity (ml A_{100} l^{-1} h^{-1})	1.65±0.01a	1.77±0.01b	1.92±0.01c	1.97±0.01d	2.30±0.01e	2.37±0.01f
60	Ethanol concentration (%, v/v)	8.15±0.04a	9.20±0.06b	10.25±0.06c	10.50±0.06d	11.28±0.06e	11.70±0.06f
	Fermentation productivity (ml A_{100} l^{-1} h^{-1})	1.36±0.01a	1.53±0.01b	1.71±0.01c	1.75±0.01c	1.88±0.01c	1.95±0.01d
72	Ethanol concentration (%, v/v)	9.18±0.22a	9.66±0.46a	11.10±0.07bc	10.91±0.15b	11.67±0.12d	11.71±0.07d
	Fermentation productivity (ml A_{100} l^{-1} h^{-1})	1.27±0.01a	1.34±0.01b	1.57±0.01d	1.51±0.01c	1.62±0.01e	1.96±0.01f
	Fermentation efficiency (% of theoretical)	71.43±1.86a	74.94±1.75a	86.94±1.38b	86.55±1.12b	92.96±1.93c	93.00±1.85c

Different letters (a-f) in lines indicate significant differences ($p < 0.05$) between means (ANOVA at a significance level of 0.05).

The ethanol concentration in mashes additionally digested with pullulanase was 27% higher ($p < 0.05$) relative to the non-sonicated controls, and 7% higher ($p < 0.05$) relative to the samples subjected to sonication only (10 min). The obtained results are in line with the ones presented by Sapińska *et al.* (2013).

In our study, the highest ethanol concentration and its productivity were determined in the rye mash initially treated with ultrasounds, then digested with pullulanase and supplemented with the hop α-acids preparation. As regards the efficiency of the process, fermentation of this mash resulted in the efficiency comparable to the similar trial but without the IsoStab preparation. After completion of the process, ethanol concentration it the reference mash reached 11.70±0.06% (v/v).

The utilisation of sugars during fermentation was associated with their availability for yeast. The lowest value of this parameter was observed in the control mash which contained the highest content of dextrins. The ultrasound pretreatment (10 min), digestion with pullulanase and supplementation with the IsoStab preparation were shown as the best solution to significantly improve the degree of

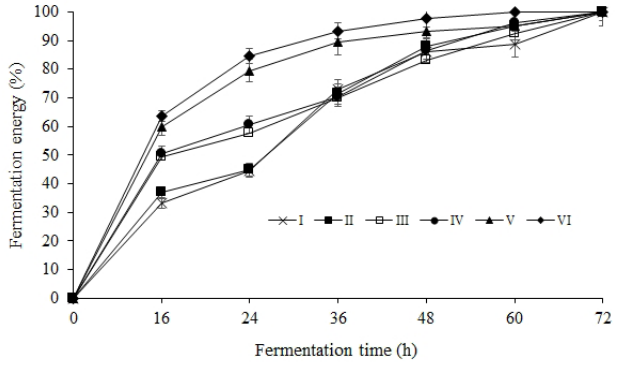

Fig. 2. Changes in the fermentation energy of high gravity rye mashes Fermentation trials: I – control sample, II – ultrasound pretreatment (5 min), III – ultrasound pretreatment (10 min), IV – ultrasound pretreatment (15 min), V – ultrasound pretreatment (10 min) and digestion with pullulanase, VI – ultrasound pretreatment (10 min) and digestion with pullulanase, and the IsoStab preparation addition.

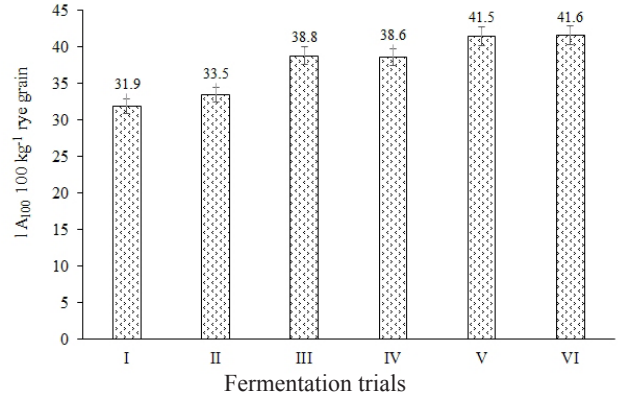

Fig. 3. Comparison of ethanol yield from 100 kg of rye grain. Designation of fermentation trials as on Fig. 2.

starch saccharification, and to maintain the microbiological safety of process, consequently leading to an increase in the sugars intake which reached 94.99±1.2%. Also, the highest ethanol yield was calculated for this fermentation trial (Table 4).

Under the favourable conditions found in these experiments, 41.6±0.3 l of absolute ethanol (A_{100}) could be obtained from 100 kg of rye grain (Fig. 3).

CONCLUSIONS

1. The use of ultrasound pretreatment is an attractive method of decreasing the viscosity of high-gravity distillery mashes. In our study, the optimum sonication time was 10 min, at amplitude of 100%. Moreover, by supporting the mashing process with pullulanase digestion, the degree of starch hydrolysis was increased and the highest ethanol yield was obtained (more than 92% of the theoretical yield).

2. The application of the hop α-acids preparation was found to be beneficial and considerably improved the microbial status of mashes during fermentation, as well as resulted in very dynamic and efficient fermentation.

3. The applied treatments during simultaneous saccharification and fermentation of high gravity rye mashes can be viewed as increasing the efficiency of starch hydrolysis and ethanol yield.

Conflict of interest: The Authors do not declare any conflict of interest.

REFERENCES

AOAC, **2006.** Official Method 932.14. Solids in Syrups. Official Methods of Analysis of AOAC International, 18th Ed. AOAC International, Gaithersburg, MD, USA.

Balcerek M. and Pielech-Przybylska K., 2009. Effect of supportive enzymes on chemical composition and viscosity of rye mashes obtained by the pressureless liberation of starch method and efficiency of their fermentation. European Food Res. Technol., 229, 141-151.

Balcerek M. and Pielech-Przybylska K., 2012. Effect of simultaneous saccharification and fermentation conditions of native triticale starch on the dynamics and efficiency of process and composition of the distillates obtained. J. Chemical Technol. Biotechnol., 8, 615-622.

Broda M. and Leja K., 2010. The microbiological situation of distilleries in Poland. Polish J. Environ. Studies, 19, 901-906.

BS EN ISO 10520:**1998**. Native starch. Determination of starch content. Ewers polarimetric method.

Căpriţă R., Căpriţă A., and Julean C., 2010. Biochemical aspects of non-starch polysaccharides. Animal Sci. Biotechnol., 43, 366-375.

Ebringerová A. and Hromádková Z., 2002. Effect of ultrasound on the extractibility of corn bran hemicelluloses. Ultrasonics Sonochemistry, 9, 225-229.

Hii S.L., Tan J.S., Ling T.C., and Ariff A.B., 2012. Pullulanase: role in starch hydrolysis and potential industrial applications. Enzyme Research, doi:10.1155/2012/921362.

Huang Q., Li L., and Fu X., 2007. Ultrasound effects on the structure and chemical reactivity of corn starch granules. Starch, 59, 371-378.

Iida Y., Tuziuti T., Yasui K., Towata A., and Kozuka T., 2008. Control of viscosity in starch and polysaccharide solutions with ultrasound after gelatinization. Innovative Food Sci. Emerging Technol., 9, 140-146.

ISO 21527-1:**2008**. Microbiology of food and animal feeding stuffs. Horizontal method for the enumeration of yeasts and moulds. Part 1: Colony count technique in products with water activity greater than 0.95.

ISO 4833:**2004**. Microbiology of food and animal feeding stuffs. Horizontal method for the enumeration of microorganisms. Colony-count technique at 30 degrees C.

ISO 6887-1:**1999**. Microbiology of food and animal feeding stuffs. Preparation of test samples, initial suspension and decimal dilutions for microbiological examination.

Ivetić D.Z., Omorjan R.P., Tatjana R., Dord T.R., and Antov M.G., 2017. The impact of ultrasound pretreatment on the enzymatic hydrolysis of cellulose from sugar beet shreds: modeling of the experimental results. Environ. Progress Sustainable Energy, 361164-1172.

Jambrak A.R., Herceg Z., Šubarić D., Babić J., Brnčić M., Brnčić S.R., Bosiljkov T., Čvek D., Tripalo B., and Gelo J., 2010. Ultrasound effect on physical properties of corn starch. Carbohydrate Polymers, 79, 91-100.

Kentish S. and Feng H., 2014. Applications of power ultrasound in food processing. Annual Review of Food Sci. Technol., 5, 263-284.

Kłosowski G., Mikulski D., Czupryński B., and Kotarska K., 2010. Characterisation of fermentation of high-gravity maize mashes with the application of pullulanase, proteolytic enzymes and enzymes degrading non-starch polysaccharides. J. Biosci. Bioengineering, 109, 466-471.

Li L., Ma S., Fan L., Zhang C., Pu X., Zheng X., and Wang X., 2016. The influence of ultrasonic modification on arabinoxylans properties obtained from wheat bran. Int. J. Food Sci. Technol., 51, 2338-2344.

Li Q., Ma Y., Mao C., and Wu C., 2009. Grafting modification and structural degradation of multi-walled carbon nanotubes under the effect of ultrasonics sonochemistry. Ultrasonics Sonochemistry, 16, 752-757.

Manchun S., Nunthanid J., Limmatvapirat S., and Sriamornsak P., 2012. Effect of ultrasonic treatment on physical properties of tapioca starch. Advanced Materials Res., 506, 294-297.

Mason T.J., Paniwnyk L., and Lorimer J.P. 1996. The uses of ultrasound in food technology. Ultrasonics Sonochemistry, 3, S253-S260.

Miller G.L., 1959. Use of dinitrosalicylic acid reagent for determination of reducing sugar. Analytical Chemistry, 31, 426-428.

Nikolić S., Mojović L., Rakin M., Pejin D., and Pejin J., 2010. Ultrasound-assisted production of bioethanol by simultaneous saccharification and fermentation of corn meal. Food Chemistry, 122, 216-222.

Pejin D.J., Mojović L.V., Pejin J.D., Grujić O.S., Markov S.L., Nikolić S.B., and Marković M.N., 2012. Increase in bioethanol production yield from triticale by simultaneous saccharification and fermentation with application of ultrasound. J. Chemical Technol. Biotechnol., 87, 170-176.

Pielech-Przybylska K., Balcerek M., Nowak A., Wojtczak M., Czyżowska A., Dziekońska-Kubczak U., and Patelski P., 2017. The effect of different starch liberation and saccharification methods on the microbial contaminations of distillery mashes, fermentation efficiency, and spirits quality. Molecules, 22, 1647, doi:10.3390/molecules22101647

Pietrzak W. and Kawa-Rygielska J., 2014. Ethanol fermentation of waste bread using granular starch hydrolyzing enzyme: Effect of raw material pretreatment. Fuel, 134, 250-256.

Roy I. and Gupta M.N., 2004. Hydrolysis of starch by a mixture of glucoamylase and pullulanase entrapped individually in calcium alginate beads. Enzyme and Microbial Technol., 34, 26-32.

Rückle L. and Senn T., 2006. Hop acids as natural antibacterials in ethanol fermentation. Int. Sugar J., 108, 139-147.

Sapińska E., Balcerek M., and Pielech-Przybylska K., 2013. Alcoholic fermentation of high-gravity corn mashes with the addition of supportive enzymes. J. Chemical Technol. Biotechnol., 88, 2152-2158.

Scheffler A. and Bamforth C.W., 2005. Exogenous β-glucanases and pentosanases and their impact on mashing. Enzyme Microbial Technol., 36, 813-817.

Schweizer T.F. and Würsch P., 1979. Analysis of dietary fibre. J. Sci. Food Agric., 30, 613-619.

Srichuwong S., Fujiwara M., Wang X., Seyama T., Shiroma R., Arakane M., Mukojima N., and Tokuyasu K., 2009. Simultaneous saccharification and fermentation (SSF) of very high gravity (VHG) potato mash for the production of ethanol. Biomass and Bioenergy, 33, 890-898.

Sun J.X., Sun R.C., Sun X.F., and Su Y.Q., 2004. Fractional and physico-chemical characterization of hemicelluloses from ultrasonic irradiated sugarcane bagasse. Carbohydrate Res., 339, 291-300.

Szymanowska D. and Grajek W., 2009. Fed-batch simultaneous saccharification and ethanol fermentation of native corn starch. Acta Scientiarum Polonorum. Technologia Alimentaria, 8, 5-16.

Wang J.M. and Zhang G.P., 2010. β-glucans and arabinoxylans. In: Genetics and Improvement of Barley Malt Quality (Eds Zhang, Guoping, Li, Chengdao). Springer, New York, ISBN 978-3-642-01279-2

Wang S., Thomas K.C., Sosulski K., Ingledew W.M., and Sosulski F.W., 1999. Grain pearling and very high gravity (VHG) fermentation technologies for fuel alcohol production from rye and triticale. Process Biochemistry, 34, 421-428.