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Changes in the Concentration of Carbonyl Compounds during the Alcoholic Fermentation Process Carried out with *Saccharomyces cerevisiae* Yeast

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Abstract

The aim of the study was to determine the influence of the source material and the applied *S. cerevisiae* strain on the concentrations of carbonyl fractions in raw spirits. Acetaldehyde was the most common aldehyde found, as it accounted for 88–92% of the total amount of aldehydes. The concentration of acetaldehyde in maize, rye and amaranth mashes was highly correlated with fermentation productivity at a given phase of the process, and reached its highest value of 193.5 mg/l EtOH in the first hours of the fermentation, regardless of the yeast strain applied. The acetaldehyde concentration decreased over the time with the decreasing productivity, reaching its lowest value at the 72nd hour of the process. The final concentration of acetaldehyde depended on the raw material used (*ca* 28.0 mg/l EtOH for maize mashes, 40.3 mg/l EtOH for rye mashes, and 74.4 mg/l EtOH for amaranth mashes). The effect of the used yeast strain was negligible. The overall concentration of the analyzed aldehydes was only slightly higher: *ca* 30.3 mg/l EtOH for maize mashes, 47.8 mg/l EtOH for rye mashes, and 83.1 mg/l EtOH for amaranth mashes.

Key words: *Saccharomyces cerevisiae*, alcoholic beverages, fermentation technology, yeast fermentation

Introduction

Controlling the concentration of carbonyl compounds in raw spirits and other fermented alcoholic beverages is very important, because these compounds affect organoleptic features of the products and can be harmful to human health. The application of different raw materials and yeast strains not only influences the course and yield of the fermentation process, but it also affects the composition of volatile by-products. These compounds determine the sensory quality of alcoholic beverages. The accepted levels of volatile fractions in spirits and alcoholic beverages are regulated by law in many countries. Therefore the identification of all factors that can modify the composition of this group of compounds is of utmost importance. Carbonyl compounds, *i.e*. aldehydes and ketones, constitute one of the fractions that affect the sensory quality of spirits and alcoholic beverages (Biernacka and Wardencki, 2012; Cachot *et al*., 1991; Longo *et al*., 1992; Plutowska *et al*., 2010). Carbonyl compounds have various fruity or floral flavours that resemble the scent of apples, lemons or nuts. They have different detection thresholds and differ in the relative influence on the organoleptic features of alcoholic beverages (Moreno-Arribas and Polo, 2009; Ribéreau-Gayon *et al*., 2006a). Acetaldehyde can account for up to 90% of the total amount of carbonyl fraction in the spirits. Short-chain and branched aldehydes are produced by the yeast during the alcoholic fermentation process from sugars, fatty acids and amino acids (Lambrechts and Pretorius, 2000; Longo *et al*., 1992; Moreno-Arribas and Polo, 2009).

A disturbance in the reaction of decarboxylation or reduction, caused either by a decreased availability of thiamine pyrophosphate, magnesium or zinc ions, or by the redox potential that favors rather oxidation than reduction processes, can result in an elevated concentration of acetaldehyde in the fermentation medium (Cheraiti *et al*., 2010; Moreno-Arribas and Polo, 2009). An increased concentration of acetaldehyde can also result from an alteration in fermentation procedure parameters such as oxygenation of the medium, pH, the concentration of fermenting sugars, yeast strain, inoculum size, temperature at which the process is conducted,

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and the distillate storing conditions (Cachot *et al*., 1991; Cheraiti *et al*., 2010; Li and Orduña, 2011; Liu and Pilone, 2000). It has to be stressed that the presence of inhibitors of enzymatic reactions (especially those catalyzed by dehydrogenases) can result in the production of small amounts of higher aldehydes that are produced along metabolic pathways leading also to higher alcohols (Moreno-Arribas and Polo, 2009). The inhibition effect is brought about by many heavy metals and chemical compounds, including mycotoxins (Kłosowski and Mikulski, 2010; Ueno and Matsumoto, 1975).

The presence of aldehydes in the spirits and alcoholic beverages produced by the fermentation industry is determined by many of the aforementioned factors. The composition of the fermentation media, that depends on the raw material used, is crucial in this context. The production of aldehydes during the fermentation process, including acetaldehyde, is an individual feature of each yeast species and strain (Longo *et al*., 1992; Li and Orduña, 2011; Liu and Pilone, 2000). Various species and strains of wine yeast can produce from 4 to 490 mg of aldehydes per one liter of must (Lambrechts and Pretorius, 2000; Liu and Pilone, 2000; Longo *et al*., 1992). The application of different raw materials during the media preparation as well as changes in the concentration of fermenting sugars can increase the concentration of aldehydes in the medium (Biernacka and Wardencki, 2012; Cachot *et al*., 1991; Li and Orduña, 2011). The elevated glucose concentration in the wine must, from 10 g/l to 240 g/l, increased the concentration of acetaldehyde by more than 100 mg/l (Li and Orduña, 2011). The application of high gravity (HG) mashes increased the acetaldehyde concentration by more than 480 mg/l of raw distillate (Mikulski *et al*., 2014).

The aim of the study was to examine the differences in both the kinetics of carbonyl compounds production and the composition of carbonyl fraction in the distillates obtained by fermentation of various starchy materials, *i.e.* maize, rye and amaranth grain, with the use of two *S. cerevisiae* strains: D-2 and As-4. The authors also made an attempt at establishing the correlation between some fermentation parameters, such as the stage of the process and the application of various raw materials and yeast strains, and the concentration of selected aldehydes. The type of raw material used can influence the fermentation kinetics and the aldehyde concentration. Being aware of these relationships can be useful in the industrial practice, because the level of carbonyl compounds in liquors is an important quality parameter (subjected to law and industry regulations in many countries) that affects the sales price. Moreover, the assessment of the concentration of aldehyde contaminants, including acetaldehyde, in the spirits and alcoholic beverages is of utmost importance, because aldehyde contaminants are listed by the World Health Organization (WHO) among potentially carcinogenic compounds (Nascimento *et al*., 1997).

Experimental

Materials and Methods

Raw materials. The fermentation media were prepared with the use of maize grain (Anna variety), rye grain (Danikowskie Złote variety) and amaranth seeds (*Amaranthus cruentus* L.). Rye and maize grain were purchased from Rolnas Ltd, Kotomierz, Poland. Amaranth grain was delivered by APC Kuma Ltd., Bydgoszcz, Poland. The starch concentration in the samples of maize, rye and amaranth grain, determined by Evers' polarimetric method (BS EN ISO 10520:1998), was 69.1%, 50.3% and 52.5%, respectively.

Microorganisms. Fermentation media were obtained by mixing ground grain with water (1:3.7; real extract value: 16.5° Brix) and inoculated with two distillers yeast strains: *S. cerevisiae* D-2 or As-4. These strains, originating from a collection of pure cultures of the Institute of Agricultural and Food Biotechnology, (Warsaw, Poland) are commonly used in the Polish distilling industry. Dry yeast were rehydrated by stirring in of distilled water and then added to the mashes in the amount of 1 ml/l of mash ($1.05 \pm 0.07 \times 10^9$ CFU/ml), according to the vendor recommendations.

Enzymes preparations. The enzymatic hydrolysis of starch and non-starch polysaccharides was carried out with Novozymes® (Bagsvaerd, Denmark) preparations. All mashes were prepared with the same set of enzymes applied at doses recommended by the producer. Starch liquefaction was carried out with thermostable α-amylase (Termamyl 120L), the dose was 150 ml/ton of starch. For starch saccharification an *Aspergillus* preparation SAN Super 240 l was used (1000 ml/ ton of starch). For hydrolysis of non-starch polysaccharides in the mashes, Viscozyme 120 l preparation was applied. The preparation exhibits the activity of arabanase, cellulase, β-glucanase, hemicellulase, and xylanase. The recommended dosage was 200 ml per 1 ton of the raw material. All enzymatic preparations were applied at doses recommended by the producer.

Mash preparation. All fermentation media were prepared under laboratory conditions with the application of pressureless liberation of starch (PLS) technology. After the hydrolysis of polysaccharides, the mashes were cooled to 30°C, then inoculated with yeast cream and subjected to fermentation for 72 h at 37°C. The total amount of starch was identical in each variant of fermentation (12.9 g 100 ml of mash). The initial concentrations of starch hydrolysis products and fructose in maize, rye and amaranth mashes are presented in Fig. 1.

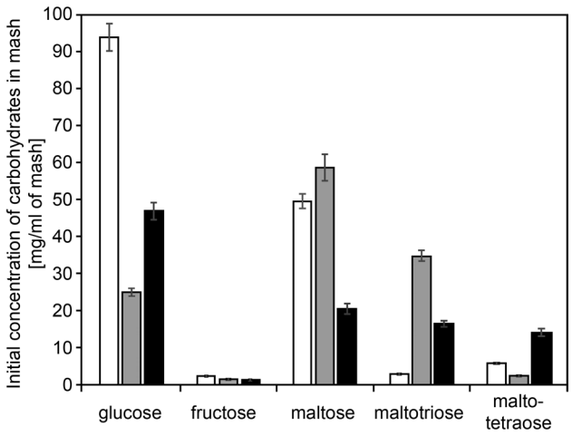

Fig. 1. The initial concentration of sugars in the fermentation media (white bars – maize mashes, grey bars – rye mashes, black bars – amaranth mashes).

Analytical methods

Analysis of the alcoholic fermentation process. Fermentation factors were defined as follows (Kłosowski *et al.*, 2010). Fermentation productivity: the amount of absolute ethanol produced in one liter of mash within 1 h (ml EtOH/l/h); fermentation yield: the amount of absolute ethanol obtained from 100 kg of starch (l/EtOH 100 kg of starch). The ethanol concentration was measured with a chromatograph by Agilent Technologies, model 1260, equipped with a refractometric detector. Chromatographic separation was carried out using a Hi-Plex H column (Agilent technologies) operating at 60°C under isocratic conditions with 5 mM H_2SO_4 as the eluent, with the flow rate of 0.6 ml/min. The ethanol concentration was determined with the use of external standards (ESTD) and an appropriate calibration curve. Integration and quantification were performed with Chem-Station LC by Agilent Technologies. The separation parameters were in agreement with the manufacturer's recommendations for examining ethanol concentration in fermentation media.

Determination of the alcoholic fermentation volatile by-products. Analysis of the aldehyde fraction was performed after 16, 24, 36, 48, 65 and 72 hours of the process. Distillate samples were obtained with a glass distillation apparatus equipped with 25 bubble-cap plates. The ethanol concentration in such obtained raw spirits was 91.0 ± 0.5% v/v. The content of aldehydes in the distillates was determined by capillary gas chromatography method using the Agilent Technologies 7890 chromatograph with FID detector on the 50-m long Agilent Technologies CP WAX 57 CB column with the internal diameter of 0.32 mm. Chromatographic separation conditions were described in Kłosowski and Mikulski (2010).

Analysis of the products of enzymatic degradation of polysaccharides. The products of enzymatic hydrolysis of polysaccharides were analyzed by high-performance liquid chromatography (HPLC) directly after enzymatic hydrolysis. Before the analysis of sugars, the fermentation media samples were diluted and filtered through a 0.44 µm filter. Chromatographic separation was carried out using a chromatograph by Agilent Technologies, model 1260, equipped with a refractometric detector, under conditions described in 2.5.1. The concentration of sugars was determined with the use of external standards (ESTD) and appropriate calibration curves. Integration and quantification were performed with Chem-Station LC by Agilent Technologies. The separation parameters followed the manufacturer's recommendations for examining the concentration of carbohydrates in fermentation media.

Statistical analysis. Statistical analysis (analysis of variance, determination of SD), was carried out using Statistica software, version 10. ANOVA and RIR Tukey's tests were applied at the significance level of $\alpha < 0.05$. The data were from three independent experiments.

Results and Discussion

Concentration of selected aldehydes in the distillates at subsequent hours of the fermentation process. The concentration of acetaldehyde, isobutyraldehyde, valeraldehyde, isovaleraldehyde, propionaldehyde in the obtained distillates at the subsequent hours of the fermentation of maize, rye and amaranth mashes was analyzed (Fig. 2, Fig. 3). Acetaldehyde was the main component of the aldehyde fraction, both at the

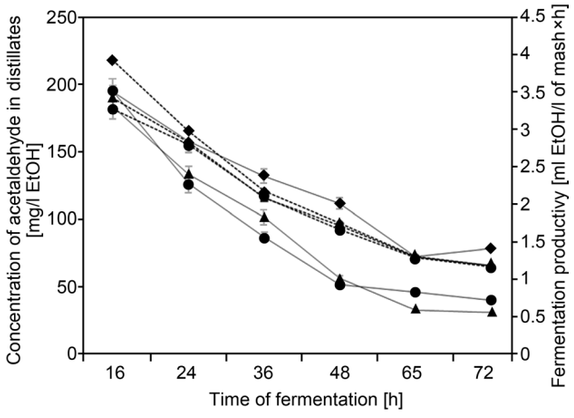

Fig. 2. Fermentation productivity (dotted line) and the concentration of acetaldehyde (solid line) in the mashes *vs* fermentation time (closed triangles – maize mashes with D-2 strain, open triangles – maize mashes with As-4 strain, closed circles – rye mashes with D-2 strain, open circles – rye mashes with As-4 strain, closed diamonds – amaranth mashes with D-2 strain, open diamonds – amaranth mashes with As-4 strain).

beginning of the process and after 72 hours of fermentation. At the 16th hour of the process, the compound accounted for almost 88% of the total amount of aldehydes. Its concentration at the first hour of the process was *ca* 193.5 mg/l EtOH in all distillates, with no statistically significant differences across the analyzed samples (Fig. 2). At the next hours of the process a decrease in the acetaldehyde concentration was observed, due to the activity of alcohol dehydrogenase (ADH) that reduced the aldehyde to ethanol. Similar changes in the concentration of acetaldehyde were also observed by other researchers (Cachot *et al*., 1991; Li and Orduña, 2011), who indicated that the acetaldehyde reduction at the initial phase of the fermentation process is relatively less efficient in comparison to the pyruvate decarboxylation process. At the subsequent hours of the process, the accumulated acetaldehyde, a hydrogen acceptor, is gradually reduced to ethanol which results in a decrease of its concentration in the fermentation medium.

During the first 24 hours of the fermentation, the reduction of the accumulated acetaldehyde was the fastest in maize and rye media, irrespective of the yeast strain used (D-2 or As-4). After 24 hours, the acetaldehyde concentration in these media decreased by as much as 63 mg/L EtOH as compared to the concentration of 192 mg/l EtOH recorded at the 16th hour of the process (Fig. 2). This downward trend lasted till the end of the fermentation. It was also observed that the concentration of acetaldehyde depended on the yeast strain used. The differences found were statistically significant. The lowest final acetaldehyde concentration, *ca* 26.1 ± 0.4 mg/l EtOH, was reported for maize spirits obtained with As-4 yeast strain (Fig. 2). The final acetaldehyde concentration in rye spirits reached the level of *ca* 40.3 mg/l EtOH, with no statistically significant differences across the rye mashes analyzed. The highest final acetaldehyde concentration, more than 70 mg/l, was in amaranth spirits, irrespective of the yeast strain used (Fig. 2). The results suggest that the yeast metabolism, especially the kinetics of acetaldehyde concentration, can be modified by the available medium components. Such relationships for rye distillates were shown by other authors. It was demonstrated that the concentration of particular volatile by-products can be significantly changed even by the rye variety used (Pietruszka and Szopa, 2014).

Similar changes in the acetaldehyde concentration were reported by Cachot *et al*. (1991), who observed the highest acetaldehyde concentration (exceeding 340 mg/l EtOH) during the first 8 hours of alcoholic fermentation of cane molasses. The concentration decreased to *ca* 20 mg/l EtOH at the 24th hour of the process. Li and Orduña (2011) also observed a downward trend in the acetaldehyde concentration during the subsequent hours of the alcoholic fermentation. These authors also reported significant differences in the acetaldehyde concentration in the media fermented with various *S. erevisiae* strains. The ongoing acetaldehyde reduction was also observed during the alcoholic fermentation in the wine making procedure, but because of a lower temperature of the process, the reduction reaction was slower and could take more than ten days (Pan *et al*., 2011).

The analysis of distillates at subsequent hours of the fermentation process revealed that the concentration of isobutyraldehyde, propionaldehyde, valeraldehyde and isovaleraldehyde in the spirits was decreasing over time. This phenomenon can be explained by the ongoing reduction of the aldehydes to their alcohol counterparts, *e.g.* propionaldehyde to propanol, isobutyraldehyde to isobutanol and isovaleraldehyde to isoamyl alcohol (Nykänen and Suomalainen, 1983). In the examined samples of spirits obtained from maize media the concentration of isobutyraldehyde decreased from *ca* 8 mg/l EtOH at the 16th hour of the fermentation to 1 mg/l EtOH at the 72nd hour of the process (Fig. 3A), regardless of the yeast strain applied. In spirits obtained from rye mashes, the isobutyraldehyde concentration was *ca* 4 mg/l EtOH and did not change much during the whole fermentation process, regardless of the yeast strain used (D-2 or As-4). In the distillates obtained from the amaranth media, isobutyraldehyde concentration decreased during the fermentation by *ca* 2.5 mg/l EtOH in comparison with that measured at the16th hour of the process, but then it remained at a higher level of *ca* 5 mg/l EtOH (Fig. 3A). The analysis of isovaleraldehyde concentrations at the 16th hour of fermentation revealed significant differences between distillates from media prepared with different raw materials. An elevated concentration of the aldehyde was reported for maize and rye spirits. The highest concentration of isovaleraldehyde, *ca* 15 mg/l EtOH, was observed at the 16th hour of the fermentation in the maize spirits, slightly lower, *ca* 12.5 mg/l EtOH, was reported for rye spirits (Fig. 3B), irrespective of the yeast strain applied. However, in both maize and rye spirits the isovaleraldehyde concentration decreased during the subsequent hours, and at the 72nd hour of the process the aldehyde was fully reduced (Fig. 3B). In amaranth distillates neither valeraldehyde nor isovaleraldehyde was detected (Fig. 3B, C). The presence of both aldehydes in maize and rye distillates is associated with the metabolism of leucine that is present in the fermentation media. This amino acid is deaminated *via* Ehrlich pathway to a corresponding α-keto acid, then decarboxylated to isovaleraldeyde, and reduced to 3-methyl-1-butanol (Ribéreau-Gayon *et al*., 2006b).

Spirits obtained from rye mashes had the highest initial valeraldehyde concentration, *ca* 19 mg/l EtOH, irrespective of the yeast strain used (Fig. 3C). Similarly

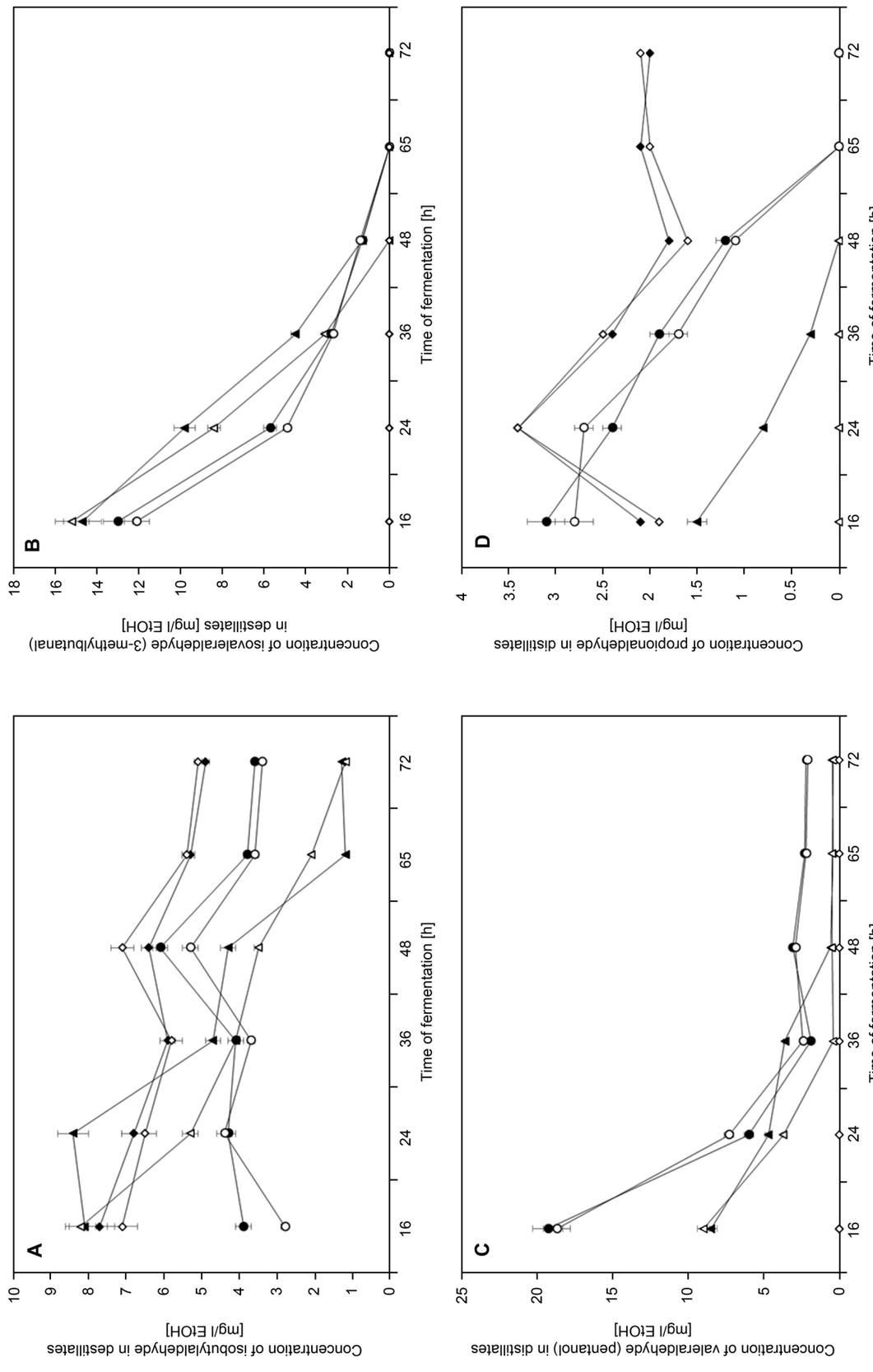

Fig. 3. The concentration of selected aldehydes in the obtained distillates at the subsequent hour of the alcoholic fermentation: **A** – isobutyraldehyde, **B** – isovaleraldehyde, **C** – valeraldehyde, **D** – propionaldehyde (closed triangles – maize mashes with D-2 strain, open triangles – maize mashes with As-4 strain, closed circles – rye mashes with D-2 strain, open circles – rye mashes with As-4 strain, closed diamonds – amaranth mashes with D-2 strain, open diamonds – amaranth mashes with As-4 strain).

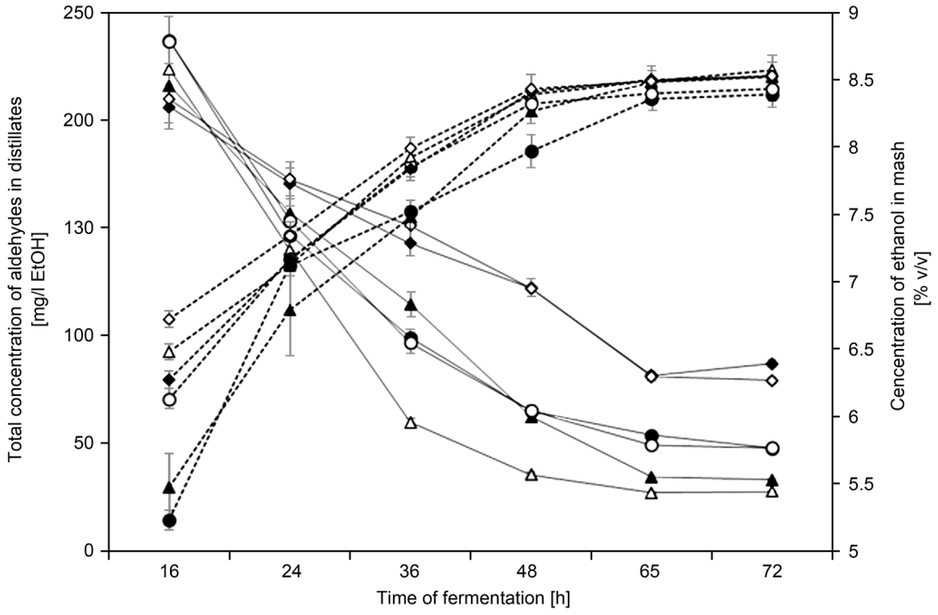

Fig. 4. The overall concentration of aldehydes in the spirits (solid line) and the ethanol concentration in the mashes (dotted line) during the alcoholic fermentation process (closed triangles – maize mashes with D-2 strain, open triangles – maize mashes with As-4 strain, closed circles – rye mashes with D-2 strain, open circles – rye mashes with As-4 strain, closed diamonds – amaranth mashes with D-2 strain, open diamonds – amaranth mashes with As-4 strain).

to the isovaleraldehyde, the concentration of valeraldehyde in rye and maize spirits was decreasing over the fermentation time, but its concentration at the 72nd hour of the process was 2 mg/l EtOH, irrespective of the yeast strain applied, so the aldehyde was not fully reduced at the end of the process (Fig. 3C).

All analyzed spirit samples had a low concentration of propionaldehyde (Fig. 3D). In the distillate obtained from a maize mash, propionaldehyde was not found at all (in samples inoculated with As-2 strain), or it was detected only at the 16th hour of the fermentation process at the concentration of 1.5 mg/l EtOH (in samples inoculated with D-2 strain) (Fig. 3D). The concentration of propionaldehyde in rye distillates decreased from 3 mg/l EtOH at 16th hour of the fermentation to zero at 72nd hour, regardless of the yeast strain applied (Fig. 3D). In amaranth distillates the propionaldehyde concentration was *ca* 2 mg/l EtOH and constant over the time, regardless of the yeast strain used.

The effect of alcohol fermentation kinetics on the total concentration of aldehydes in the obtained distillates. A correlation between the fermentation productivity and the overall concentration of aldehydes was observed, including acetaldehyde as the main component of the aldehyde fraction. The highest ethanol concentration, fermentation productivity and yield at the initial phase of the process were associated with the elevated concentration of aldehydes, especially acetaldehyde. It must be stressed, however, that statistically significant differences in productivity between different yeast strains were observed at the beginning of the fermentation process and were not accompanied by significant differences in the acetaldehyde concentration. At this fermentation stage the productivity of As-4 yeast strain was statistically higher than that of D-2 strain, regardless of the raw material used, but no significant differences in the acetaldehyde concentration were found (Fig. 2, Fig. 4, Table I). At the 16th hour of the fermentation, in maize and rye mashes inoculated with As-4 the yield was higher by ca. 7.4 l EtOH/100 kg of starch in comparison to that of the mashes inoculated with D-2 strain (Table I). In the following hours of the fermentation process the fermentation activity of D-2 was rising so that at the 65th hour of the process no statistically significant differences in the yield between the two strains were observed. At this stage of the process no differences in the yield between different raw materials were found (Fig. 4, Table I). At the 72nd hour of the alcoholic fermentation the ethanol concentration, the fermentation yield and the ratio of the actual yield to the theoretical one were similar in all media analyzed, reaching 8.49% v/v, 65.8 l EtOH/100 kg of starch, and 91.6%, respectively (Fig. 4, Fig. 5, Table I).

During the subsequent hours of the process, the concentration of the analyzed aldehydes decreased with decreasing fermentation yield, as an effect of the ongoing reduction of aldehydes to their corresponding alcohols (Nykänen and Suomalainen, 1983). Although no statistically significant differences in the parameters of the fermentation process were observed at

Table I
Fermentation yield at subsequent hours of the alcoholic fermentation process.

Raw material	Yeast strain	Fermentation yield [l EtOH/100kg of starch] at subsequent hours of the alcoholic fermentation process					
		16	24	36	48	65	72
Maize grain	D-2	42.35a ± 1.94	52.57ac ± 2.63	57.91a ± 0.62	64.03abc ± 0.70	65.66a ± 0.62	65.96a ± 0.85
	As-4	50.17bd ± 0.46	55.13abc ± 0.62	61.32b ± 0.85	64.96b ± 1.01	65.73a ± 0.62	66.35a ± 0.85
Rye grain	D-2	40.50a ± 0.54	52.03c ± 0.62	58.23a ± 0.62	61.71c ± 0.93	64.73a ± 0.70	64.96a ± 0.77
	As-4	47.46c ± 0.54	55.52ad ± 0.77	60.78b ± 0.54	64.42ab ± 0.77	65.04a ± 0.85	65.27a ± 0.70
Amaranth grain	D-2	48.56bc ± 0.46	55.45ae ± 0.46	60.72b ± 0.70	65.13ab ± 1.01	65.83a ± 0.77	65.99a ± 0.70
	As-4	52.04d ± 0.46	56.85bde ± 0.77	61.88b ± 0.62	65.29ab ± 0.77	65.75a ± 0.70	66.06a ± 0.70

The mean values given in columns with different letter index are significantly different ($\alpha < 0.05$).

the 72nd hour of the process, the concentration of all analyzed aldehydes differed significantly across the source materials used (Fig. 4). The lowest final concentration of aldehydes was found in the distillates obtained from maize media, regardless of the yeast strain used (D-2 or As-4). The aldehyde concentration in the maize media was *ca* 30.3 mg/l EtOH and it was lower than that observed in rye and amaranth distillates by *ca* 17.5 mg/l EtOH and 52.8 mg/l EtOH, respectively. The results justify the conclusion that the application of different yeast strains does not considerably influence the final acetaldehyde concentration. The most pronounced differences in the acetaldehyde concentration was observed between samples taken at different process stages. The final acetaldehyde concentration was associated with the type of source material used. This effect can be caused by different availability of minerals that are enzyme cofactors, such as zinc and magnesium, in the source material. The active site of alcohol dehydrogenase contains a bound zinc atom. Magnesium and thiamine pyrophosphate are cofactors of pyruvate decarboxylase. Different concentrations of Zn and Mg cations in the maize, rye and amaranth grain can affect the activity of ADH and pyruvate decarboxylase and thus influence the acetaldehyde concentration in the distillates (Lorenz and Wright, 1984; Mikulski and Kłosowski, 2015; Moreno-Arribas and Polo, 2009; Ribéreau-Gayon *et al*., 2006b).

Conclusions

A general relationship was observed: there was a correlation between the ethanol productivity and the acetaldehyde concentration at a given fermentation stage. A high productivity at the initial phase of the process is accompanied with an elevated acetaldehyde concentration. The drop in the productivity at the final phase of the fermentation is correlated with a decrease in the concentration of acetaldehyde. Therefore, should any disturbing factors prematurely terminate or

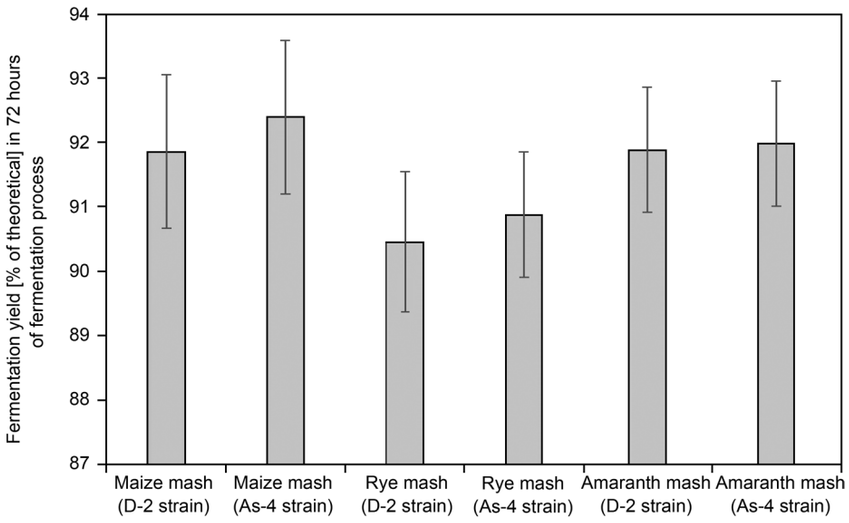

Fig. 5. The ratio of the actual yield of the alcoholic fermentation to the theoretical one for all mashes analyzed at the 72nd hour of the process.

considerably shorten the technological process of the alcoholic fermentation, an elevated concentration of carbonyl compounds would have to be expected. The final concentration of acetaldehyde depends significantly on the type of source material used, without any noticeable influence of the applied yeast strain.

Similar relationships for the other aldehydes (propionaldehyde, valeraldehyde, isovaleraldehyde) are much more difficult to find. These compounds are also products of yeast metabolism, but they are not directly associated with the main alcoholic fermentation pathway. However, a certain trend can be observed: the concentration of these aldehydes in the distillate samples taken at subsequent fermentation stages gradually decreases over the time.

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