Potential influence of compounds released in degradation of phytates on the course of alcoholic fermentation of high gravity mashes – simulation with analogs of these compounds

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Aim of the study was to evaluate the effect of supplementation of high gravity media with mineral compounds and myo-inositol, at concentration which would be obtained as a result of degradation of phytates present in raw material during alcoholic fermentation. The process of alcoholic fermentation was conducted under laboratory conditions in a 72 h system at 37°C with the use of *S. cerevisiae* D-2 strain. Calcium chloride proved to be the most effective of all supplements tested. Final ethanol concentration increased by 1.2% v v^{-1} and the yield of process increased by ca. 7 dm^{-3} ethanol 100 kg^{-1} of starch in comparison with control. Selective supplementation with KH_2PO_4, $ZnSO_4$ and $MgSO_4$ also increased the ethanol concentration, but the effect was accompanied by a deterioration in composition of volatile products. The hydrolysis of phytate complexes with microbial phytases can be an alternative solution to supplementation of HG mashes presented in this work.

Keywords: high gravity alcoholic fermentation, mineral compounds, inositol.

INTRODUCTION

The process of alcoholic fermentation using high gravity (HG) mashes exhibits a number of desirable properties, mainly in the economic aspect. Due to the higher productivity of fermentation vats and lower consumption of energy per unit volume of produced ethanol, overall costs are reduced. On the other hand, the possibility of ethanol concentration reaching lethal levels as well as a high osmotic pressure associated with a shortage of minerals and other nutrients essential for yeast, can make this technology difficult to use. *Saccharomyces cerevisiae* yeast cells efficiently use the available sugar substrates in the media if they are accompanied by appropriate amounts of essential minerals and biologically active substances. In studies conducted under laboratory conditions the fermentation media are often supplemented with tryptone and yeast extract. However, this method is too expensive to be economically viable on an industrial scale[1, 2].

Phytic acid is the principal storage form of phosphorus and inositol in plant seeds and cereals and therefore it is present in plant raw materials. The chelating effect of the phosphate groups causes phytic acid to bind easily to divalent cations such as calcium, magnesium, zinc forming insoluble salts, phytates[3–5]. Because distillery yeast cells do not produce phytase, the enzyme hydrolyzing phytic acid, they cannot utilize phytates. In this situation, a careful selection of suitable compounds that could remedy the existing deficiencies when used as supplements, is of utmost importance. The alternative solution is an effective hydrolysis of phytates to liberate ions (divalent cations, orthophosphates) and compounds (inositol, proteins) from phytates.

When using raw materials rich in phytic acid complexes, it is important to determine whether biogenic compounds released during phytate hydrolysis can positively affect the fermentation activity of *S. cerevisiae*. The answer to this question can help to establish whether there is a need to conduct further research on phytate degradation leading to an increase in the bioavailability of nutrients. So far, it has been shown that supplementation of culture media with inositol affects the viability of yeast cells and determines a higher activity of membrane ATPase[6]. Transmembrane transport in yeast cells largely depends on this enzyme. Inhibition of ATPase in the presence of ethanol impedes the transport of sugar substrates and biogenic compounds through the cell membrane, which has a negative effect on the cell biomass growth rate and the fermentation activity[7]. Myo-inositol plays also an important role in many physiological and biochemical processes, e.g. in synthesis of membrane phospholipids and in nucleic processes[8]. Phosphorus, present in the cells mainly in the form of orthophosphates, accounts for about 3–5% of the dry mass of the yeast cells[9]. Orthophosphates, which are components of nucleic acids and phospholipids, are accumulated primarily in the vacuoles reaching 110-fold higher concentration than in the cytoplasm. Studies have shown that an exogenous source of phosphorus in the culture medium during alcoholic fermentation increases the yield and the productivity of the process[9, 10]. The micronutrient zinc also plays a vital role in the fermentation process. As a trace element, it is important for cell growth and metabolism. It is a functional and structural component of proteins and nucleic acids, and a cofactor of many enzymes[11]. Being a cofactor of alcohol dehydrogenase, it plays an important role mainly at the final stage of alcoholic fermentation. In must used for wine production, the concentration of zinc is sufficient, therefore supplementation is not required. On the other hand, in the process of brewing beer, the wort sometimes requires zinc supplementation for optimal bioavailability of the element. Too low concentrations of this element may slow down the fermentjon process and adversely affect the quality of the resulting product. Supplementation of fermentation media with zinc is also beneficial for the production of ethanol. A protective effect of this element on cells under toxic stress induced by high ethanol concentration levels during the fermentation process has also reported[11, 12].

When supplementing the fermentation media, it is important to ensure the proper proportions of the introduced substances, especially in the case of calcium and magnesium ions because of ion antagonism. A higher ratio of Mg^{2+} to Ca^{2+} increases the initial fermentation rate and yield, and improves cell viability[2]. On the other hand, increased ratio of Ca^{2+} to Mg^{2+} slows down the first phase of the fermentation and reduces the ethanol production. The magnesium to calcium ratio in beer wort varies from 2:1 to 6:1. Magnesium accounts for ca. 0.3% of the dry mass of yeast cells. This intracellular cation activates more than 300 different enzymes including all phosphatases, kinases and synthetases[2]. The mineral ion is involved in the stabilization of cell membranes, and in the synthesis of proteins, nucleic acids, structural and storage polysaccharides, and ribosomes. It plays an important role in the regulation of cell growth and division. It also regulates the metabolism of pyruvate. Calcium ions also play a role in enhancing the stability of the cell membrane and are also required for the activity of α-amylase. Too high level of Ca^{2+} may inhibit the activity of yeast cells due to the existing magnesium/calcium ion antagonism[2, 9].

Limited availability of nutrients during fermentation of high gravity mashes, and binding process of essential divalent cations to phytic acid adversely affects the fermentation output. As shown above, all of these substances play an important role in the cell functioning. Therefore, it is important to check how the supplementation with selected mineral compounds affects the course and parameters of alcoholic fermentation of high gravity mashes. The aim of the study was to evaluate the effect of supplementation of high gravity media with mineral compounds and myo-inositol on the fermentation activity of yeast *S. cerevisiae* D-2 strain and the production of volatile fermentation by-products.

MATERIALS AND METHOD

Raw material

Laboratory analysis was carried out using ground corn grain (*Zea mays*) obtained from Rolnas Ltd, Kotomierz, Poland and characterized by the following parameters: dry matter (DM) 91.59 ±0.17%; starch concentration 49.43 ±0.70%; ethanol yield 35.50 ±0.50 dm^3 EtOH 100 kg^{-1} of raw material; phytic acid concentration 2.56 ±0.09 mg g^{-1} DM; total phosphorus concentration 3.30 ±0.03 mg g^{-1} DM. The concentration of minerals in

the raw material was as follows: calcium 0.43 mg g^{-1}; magnesium 1.13 mg g^{-1}; zinc 0.01681 mg g^{-1}.

Yeast

In this study we used a preparation of active dry yeast *S. cerevisiae*, strain D-2. The distillery yeast strain used is thermophilic (34–38°C), osmophilic and resistant to increased ethanol concentration (above 12% v v^{-1}). This strain comes from the collection of pure cultures of the Institute of Biotechnology and Food Industry in Warsaw, Poland, and is commonly used by industry distillers. The active dry yeast preparation was rehydrated (5 g of the preparation was mixed for 15 min with 50 cm^3 of water at 30°C). Yeast cream (1.05 ±0.07 x 10^9 CFU cm^{-3}) was added to the fermentation medium in the amount of 3.3 cm^3 dm^{-3} of mash (the total volume of HG mash was 7 dm^3).

Enzymatic preparations

The enzymatic hydrolysis of starch during the preparation of HG mashes was carried out with the use of enzymatic preparations by Novozymes® (Denmark, Bagsvaerd). The following basic preparations were applied. For starch liquefaction Termamyl S.C. D.S. preparation (activity of 240 KNU g^{-1}) containing heat-stable α-amylase *Bacillus licheniformis* (genetically modified) with a lower demand for Ca^{2+} was used; one Kilo Novo alpha-amylase Unit (KNU) hydrolyses 5.26 g of starch per hour under standard conditions (in the Novozymes standard method for the determination of alpha-amylase); 70 cm^3 of the preparation per one ton of starch was applied.

For starch saccharification Saczyme preparation (activity of 750 AGU g^{-1}) containing glucoamylase *Aspergillus niger* (genetically modified) was used; Amylo Glucosidase Unit (AGU) catalyzes the conversion of 1 μmol of maltose per minute under standard conditions; the preparation does not exhibit the activity of transglucosidase, which eliminates the so called reversion, i.e., secondary creation of non-fermenting sugars (isomaltose, panose, nigerose); 300 cm^3 of the preparation per one ton of starch was applied.

Experimental variants

Laboratory studies included 10 experimental variants of the fermentation process was presented in Table 1.

The applied doses of supplements were calculated based on the concentrations of respective substances in the raw material and corresponded to the maximal theoretical concentrations obtained by the degradation of phytic acid complexes.

Table 1. Characteristics of research variants

Research variant	Concentration of supplements of fermentation media [g dm^{-3} of mash]				
	inositol	KH$_2$PO$_4$	ZnSO$_4$	MgSO$_4$	CaCl$_2$
Control variant	–	–	–	–	–
Variant I	0.27	–	–	–	–
Variant II	0.54	–	–	–	–
Variant III	–	1.00	–	–	–
Variant IV	–	2.00	–	–	–
Variant V	0.27	1.00	–	–	–
Variant VI	–	–	0.03	–	–
Variant VII	–	–	–	2.16	–
Variant VIII	–	–	–	–	0.91
Variant IX	0.27	1.00	0.03	2.16	0.91

Preparation of HG mashes

Maize mashes were prepared with the use of pressureless liberation of starch technology. Laboratory conditions reflected those applied in an industrial scale. The ground corn was mixed with water to obtain the concentrations about 18.834 ±0.174 g 100 cm^{-3}. The mash was pasteurized for 60 min at 90°C. At the same time starch liquefaction was performed with the use of α-amylase (Termamyl SC DS preparation). After pH was adjusted to 5.5 with 25% H_2SO_4, starch saccharification was performed using glucoamylase (in the form of Saczyme preparation). The real extract content in the obtained fermentation media was 23.1 ±0.5° Brix. Experiments were carried out in sterile 0.5 dm^3 glass flasks (filled to 0.3 dm^3) closed with fermentation bungs containing glycerine. In order to control the fermentation process and to evaluate the correctness of its course, three series of pitches were prepared for each variant of the experimental fermentation. For by-products determination each pitch contained additionally 2 flasks of 3.0 dm^3 (filled to 1.7 dm^3) intended for distillation after the fermentation had been completed (after 72 h)[14]. The total concentration of starch in each fermentation variant was identical. The mashes after inoculation with yeast cream were subjected to fermentation for 72 hours at 37°C.

ANALYTICAL METHODS

Characteristics of raw material

The dry matter (DM) content (%) in the ground corn grain was determined using a moisture analyzer WPS-30S by Radwag. The ethanol yield in the fermentation process was determined after enzymatic hydrolysis of starch. The results are shown as dm^3 of EtOH obtained from 100 kg of the raw material. The concentration of starch was determined by Ewers polarimetric method in accordance with BS EN ISO 10520:1998[15]. The concentration of phytic acid was measured colorimetrically using WADE reagent[16]. The total phosphorus content was determined with vanadate-ammonium molybdate reagent[17]. Colorimetric analyzes were performed with spectrophotometer UV-Vis Pharo 300 by Merck®. The content of magnesium and zinc was measured after mineralization with atomic absorption spectrometer (at the wavelengths λ = 285.2 nm and λ = 213.9 nm, respectively). The concentration of calcium was measured after mineralization with a flame emission spectrometer. All analysis of mineral compounds were carried out at the Regional Chemical Station in Bydgoszcz, Poland.

Analysis of the alcoholic fermentation process

Parameters of the alcoholic fermentation were defined as follows[14]. Fermentation productivity: the amount of absolute ethanol produced in one liter of mash within 1 h (cm^3 EtOH dm^{-3} h^{-1}); fermentation yield: the amount of absolute ethanol obtained from 100 kg of starch (dm^3 EtOH 100 kg^{-1} of starch). Ethanol concentration (% v v^{-1}) was measured after distillation of 100 cm^3 of filtrated mash using immersion refractometer (Carl-Zeiss Jena) and alcoholometric tables. The real extract was determined with Abbe refractometer calibrated in Brix degrees (°Brix), after filtration. The content of reducing sugars (mg cm^{-3}) in mashes (before and after acid hydrolysis) was determined by Lane–Eynon's method[18]. Measurements were done after 16, 24, 40, 48, 64, and 72 hours.

Determining the physiological state of yeast

Yeast cells were counted (CFU cm^{-3} of mash) using the plate method[19]. Viability of the yeast cells (%) was determined after staining with methylene blue. Yeast cells were counted using a Thoma cell counting chamber[20]. The phosphorus content in the yeast cells (% of DM) was determined after dry mineralization colorimetrically with molybdate reagent[21]. Measurements were performed after 24, 48 and 72 hours of the process initialization.

Analysis of volatile by-products of the alcoholic fermentation

Distillate samples with the ethanol concentration of 89.0 ±0.5% v v^{-1} were subjected to analysis of volatile by-products of the alcoholic fermentation. The content of volatile by-products in the samples was determined by capillary gas chromatography method using HP 6890 chromatograph equipped with FID detector. The column used was 50-m long Varian CP WAX 57 CB column with the internal diameter of 0.32 mm. The conditions of the chromatographic analysis were described by Kłosowski and Mikulski[22].

Statistical analysis

The results of the experiments were subjected to statistical analysis (determination of SD, analysis of variance) carried out with the use of STATISTICA software, version 12. Tukey's range test and ANOVA analysis were performed at the 5% significance level. The data were from three independent experiments.

RESULTS AND DISCUSSION

The influence of supplementation of HG fermentation media on the parameters of the alcoholic fermentation process

We analyzed the effect of supplementation of HG fermentation media on basic parameters characterizing the process of alcoholic fermentation, i.e. the ethanol concentration, productivity and yield, as well as the ratio of the actual yield to the theoretical one (Table 2, Fig. 1).

It was observed that the experimental variant VIII with the Ca^{2+} supplementation had the highest ethanol concentration of all the variants tested. At the 72 hour of the process the ethanol concentration amounted to 10.8 % v v^{-1} and was significantly higher (by 1.2% v v^{-1}) than in the control variant (Table 2). It was also observed that in the experimental variant VIII the concentration of reducing sugars between the 48 and 72 hour of the process was the lowest (Table 3). The lowest concentration of reducing sugars at the beginning of the fermentation was reported for the experimental variant III in which the fermentation media were supplemented with the basic dose of phosphorus ions (Table 3). The addition of calcium chloride to the medium resulted in the highest fermentation productivity and yield throughout the process (Table 2). The experimental variant VIII had also the highest ratio of the actual yield to the theoretical one. The increase was ca. 10%, as compared to the control

Table 2. The effect of supplementation of HG media on selected parameters of the alcoholic fermentation

Research variant	Hours of fermentation								
	24 h			48 h			72 h		
	Ethanol concentration [% v v^{-1}]	Fermentation productivity [cm^3 EtOH dm^{-3} h^{-1}]	Fermentation yield [dm^3 EtOH 100 kg^{-1} of starch]	Ethanol concentration [% v v^{-1}]	Fermentation productivity [cm^3 EtOH dm^{-3} h^{-1}]	Fermentation yield [dm^3 EtOH 100 kg^{-1} of starch]	Ethanol concentration [% v v^{-1}]	Fermentation productivity [cm^3 EtOH dm^{-3} h^{-1}]	Fermentation yield [dm^3 EtOH 100 kg^{-1} of starch]
Control variant	4.3ab ±0.2	1.78ab ±0.06	22.55ab ±0.81	7.8ab ±0.1	1.63ab ±0.03	41.23ab ±0.53	9.6a ±0.0	1.33a ±0.00	50.75a ±0.00
Variant I	4.1bd ±0.1	1.69bd ±0.02	21.41bc ±0.27	7.7a ±0.1	1.59a ±0.01	40.44a ±0.27	10.1b ±0.2	1.41b ±0.02	53.57bc ±0.81
Variant II	3.9d ±0.1	1.63d ±0.05	20.92c ±0.54	7.5a ±0.2	1.57a ±0.03	40.62a ±0.83	9.4a ±0.1	1.31a ±0.02	50.43a ±0.54
Variant III	4.6ac ±0.1	1.90ac ±0.02	24.41de ±0.27	8.5c ±0.1	1.76c ±0.01	45.33c ±0.27	10.3b ±0.2	1.43b ±0.03	55.08c ±1.12
Variant IV	4.8ce ±0.2	2.01ce ±0.06	25.93ef ±0.82	8.3c ±0.1	1.73c ±0.02	44.5cd ±0.54	10.1b ±0.2	1.40b ±0.02	54.00bc ±0.82
Variant V	3.9d ±0.1	1.63d ±0.05	20.30c ±0.30	7.8ad ±0.2	1.63ad ±0.05	40.94ae ±1.05	10.1b ±0.1	1.41b ±0.02	53.19b ±0.61
Variant VI	4.5a ±0.2	1.88a ±0.08	23.79ad ±0.92	8.3c ±0.1	1.73c ±0.02	43.87cf ±0.53	10.2b ±0.0	1.42b ±0.00	53.92bc ±0.00
Variant VII	4.5ac ±0.1	1.89ac ±0.02	23.97ad ±0.31	8.3c ±0.1	1.73c ±0.02	43.87cf ±0.53	10.2b ±0.1	1.41b ±0.01	53.65bc ±0.27
Variant VIII	5.1e ±0.1	2.11e ±0.03	27.28f ±0.31	9.3e ±0.2	1.94e ±0.04	49.89h ±1.07	10.8c ±0.1	1.50c ±0.01	57.76d ±0.31
Variant IX	4.4a ±0.1	1.83a ±0.05	23.10ad ±0.53	8.1bcd ±0.1	1.70bcd ±0.01	42.69bdef ±0.30	10.1b ±0.1	1.41b ±0.02	53.19b ±0.61

The mean values given in columns with different letter index are significantly different (α < 0.05).
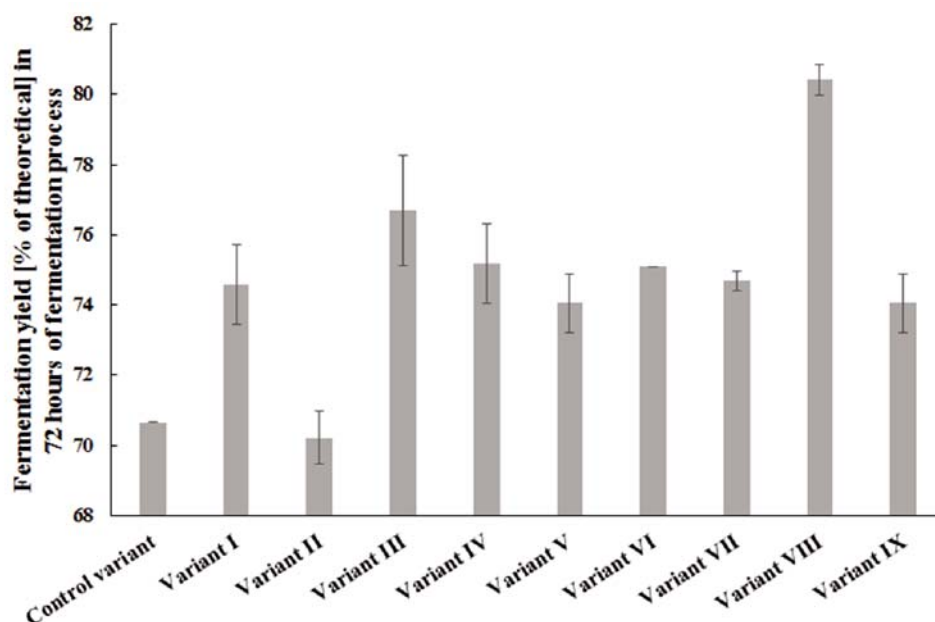
Figure 1. The ratio of the actual yield of the alcoholic fermentation to the theoretical one for all mashes analyzed at the 72nd hour of the process

variant (Fig. 1). Our results obtained with the HG corn media confirm the tendency shown in the studies by Nabais et al., who observed that the addition of calcium ions in the form of CaCl$_2$ to the model media positively affected the fermentation activity of *S. bayanus*[23]. Studies conducted by Zeng et al. also showed a significant effect of calcium ions added to the model fermentation media on both the amount of ethanol produced and the glucose assimilation rate by *Zymomonas mobilis*[24].

The analysis of the key parameters of the alcoholic fermentation process showed that the alcohol concentration, fermentation productivity and yield were reduced in the experimental variant II with the double inositol dose as compared to the experimental variant I with basic dose of 0.27 g dm^{-3}. This trend continued throughout the whole alcoholic fermentation process. Similar results were observed when the fermentation media were supplemented with orthophosphate. In the experimental variant IV with a double dose of this compound the fermentation parameters were lower than those observed

for the experiment variant III (Table 2). All variants but the variant II, where the double inositol dose (ca. 0.5 g dm^{-3}) was applied, had a higher ethanol concentration, productivity, yield, and a higher ratio of actual yield to the theoretical one in the last 24 hours of the fermentation process, as compared to the control (Table 2, Fig. 1). A positive effect of the supplementation of the model fermentation media and sake mashes with inositol on the yeast viability was reported by many authors, but the effect of this compound on the fermentation activity of the yeast, and the amount of obtained ethanol was strongly dependent on the concentration of the used supplement[25, 26]. In the studies of Ishmayan et al., a negative impact of inositol on the concentration of ethanol in fermentation media was reported. In comparison to the results of the present paper, in these studies a lower concentration of glucose (15%) was applied[25]. However, the application of a higher carbohydrate concentration (up to 20%) along with inositol supplementation resulted in a higher ethanol concentration, as compared to the

Table 3. The effect of supplementation of HG media on the concentration of reducing sugars during the alcoholic fermentation process

Research variant	Hours of fermentation							
	0 h		24 h		48 h		72 h	
	concentration of reducing sugars [mg cm^{-3}]		concentration of reducing sugars [mg cm^{-3}]		concentration of reducing sugars [mg cm^{-3}]		concentration of reducing sugars [mg cm^{-3}]	
	−	+	−	+	−	+	−	+
Control variant	12.87abc ±0.11	14.61a ±0.50	8.47ac ±0.21	9.74ab ±0.08	6.39a ±0.48	6.80ab ±0.30	5.12a ±0.38	5.68a ±0.15
Variant I	12.62abc ±0.06	15.85bcd ±0.04	8.50ac ±0.17	9.33bd ±0.10	5.41b ±0.19	6.11bc ±0.03	4.42bd ±0.04	4.76bd ±0.04
Variant II	13.02ac ±0.03	16.09b ±0.04	10.05b ±0.02	10.93c ±0.27	6.55a ±0.20	7.20a ±0.01	5.10a ±0.17	5.56ac ±0.26
Variant III	12.74abc ±0.07	15.11ac ±0.30	7.90c ±0.33	8.96d ±0.14	5.02b ±0.15	5.59c ±0.33	4.00cd ±0.01	4.25d ±0.04
Variant IV	12.77abc ±0.22	15.08ac ±0.22	8.79a ±0.53	10.17a ±0.01	6.37a ±0.11	6.91ab ±0.37	4.68ab ±0.12	4.98be ±0.25
Variant V	12.42abc ±0.03	15.22ac ±0.08	8.92a ±0.13	10.12a ±0.13	6.87a ±0.08	7.25a ±0.02	4.47bd ±0.19	5.39af ±0.29
Variant VI	12.09ab ±0.81	14.59a ±0.33	8.43ac ±0.23	9.29bd ±0.49	6.36a ±0.10	6.75ab ±0.27	4.40bd ±0.25	5.19ae ±0.17
Variant VII	12.02b ±0.39	15.04ae ±0.23	8.88a ±0.14	9.84ab ±0.10	6.41a ±0.25	7.52a ±0.04	4.51bd ±0.20	5.38ag ±0.13
Variant VIII	12.50abc ±0.35	15.07ad ±0.34	8.46ac ±0.04	8.99d ±0.12	4.27c ±0.31	4.64d ±0.44	3.44c ±0.02	3.68h ±0.03
Variant IX	13.21c ±0.25	15.57bcde ±0.31	8.96a ±0.39	10.04a ±0.23	6.20a ±0.22	6.85a ±0.14	4.55abd ±0.28	5.08bcefg ±0.13

The mean values given in columns with different letter index are significantly different (α<0.05).
− analysis before acid hydrolysis of starch residues in mashes,
+ analysis after acid hydrolysis of starch residues in mashes.

experimental variant with no inositol added[26]. As shown by Furukawa et al., the supplementation of cereal media used for sake production with 10 μM inositol increased the ethanol productivity, but increasing inositol concentration to 90 μM resulted in a decrease of productivity, despite its positive effect on the yeast viability[27].

The influence of HG media supplementation on the physiological state of yeast

Selected parameters characterizing the physiological state of yeast during the alcoholic fermentation in all experimental variants are displayed in Figure 2. Supplementation with inositol (experimental variants I and II) and phosphorus (experimental variants III and IV) resulted in a significant increase in the number of yeast cells at the 24 and 48 hour of fermentation. The number of colony forming units gradually decreased with increasing alcohol concentration at the subsequent hours of fermentation in all experimental variants (Fig. 2A). Increasing concentration of ethanol also affected the cell viability, which decreased in subsequent days of fermentation. At the 24 hour of fermentation yeast
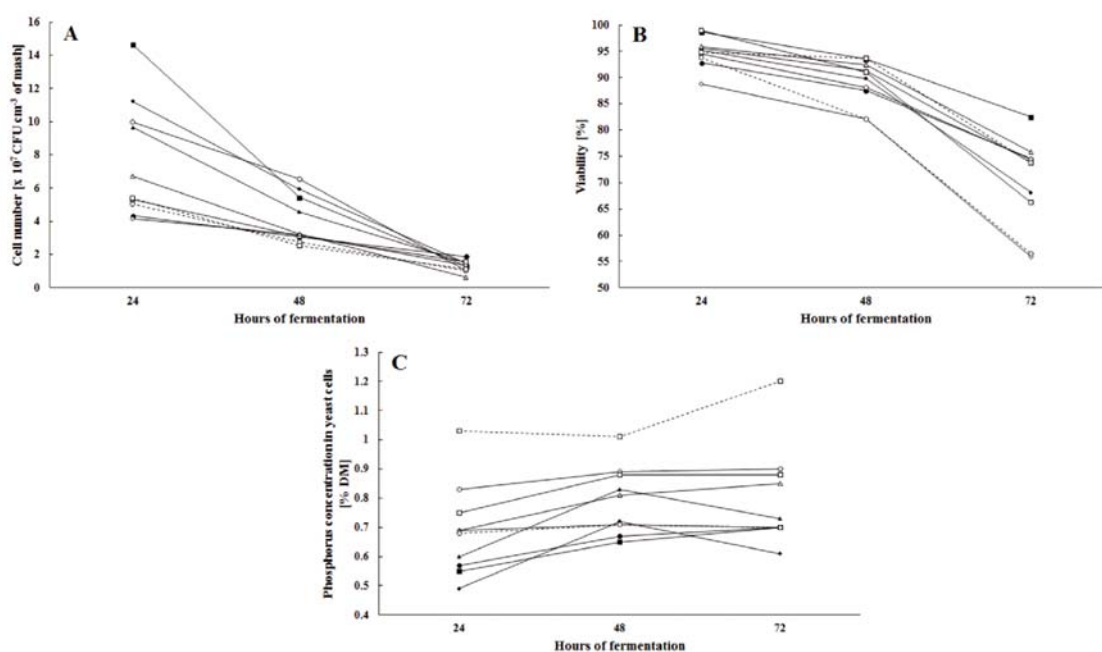

Figure 2. The evaluation of selected indicators of physiological state of yeast during the alcoholic fermentation process; A – cell number; B – viability; C – phosphorus concentration in yeast cells (Control variant – closed circle, solid line; Variant I – closed squares, solid line; Variant II – closed diamonds, solid line; Variant III – closed triangles, solid line; Variant IV – open circles, solid line; Variant V – open squares, solid line; Variant VI – open diamonds, solid line; Variant VII – open triangles, solid line; Variant VIII – open circle, dotted line; Variant IX – open squares, dotted line)

viability remained stable at a level of about 95%, and finally at the 72 hour of the process it decreased by about 25% as compared to the first day of the process (Fig. 2B). It is worth noting that the highest viability in the last day of the experiment was reported for the experimental variant I (supplementation with the basic dose of inositol). Chi et al. also observed that the viability of *S. cerevisiae* cells decreased at a slower rate when the availability of this compound in the medium was higher[26]. Similarly, the studies of Furukawa et al. showed that the supplementation of sake media with inositol increased the viability of yeast cells, despite a high concentration of ethanol[6]. However, it must be noted that our studies showed a decreased viability of yeast after the dose of inositol was doubled to about 0.5 g dm^{-3}. This confirms the need for an appropriate composition of supplements in the fermentation media. At the 72 hour of the alcoholic fermentation the viability in the experimental variant II decreased in comparison with that in the experimental variant I and in the control by ca. 14% and 6.5%, respectively (Fig. 2B).

The concentration of phosphorus in the yeast cells throughout the whole process was the highest in the experimental variant IX, where the medium was supplemented simultaneously with inositol, phosphorus, zinc, magnesium and calcium; the difference was statistically significant. In the last 24 hours of the alcoholic fermentation the concentration of phosphorus was 1.20% of dry mass and was higher by 0.5% than in the control (Fig. 2C).

The influence of HG media supplementation on the composition of volatile by-products of the fermentation process

The composition of volatile by-products in the spirits obtained by distillation of the mashes is shown in Table 4. The concentration of acetaldehyde varied from ca. 100.0 mg dm^{-3} EtOH (experimental variant IX) to ca. 230.0 mg dm^{-3} EtOH (experimental variant VII). An elevated acetaldehyde concentration may result from using corn mash with a higher extract, which was confirmed by Roustan and Sablayrolles[27]. In this study the authors observed that a high concentration of acetaldehyde in wine musts depended on the high initial concentration of sugars[28]. It is worth noting that in the experimental variant VIII, where supplementation with Ca^{2+} was applied, no significant differences in the concentration of acetaldehyde as compared to the control variant was observed despite the highest ethanol yield and concentration.

The observed effect probably results from a stabilizing influence of membrane cell under the ethanol stress conditions as well as from an increase in the activity of H$^+$-ATPase induced by Ca^{2+}. H$^+$-ATPase stabilizes the intracellular pH at a level suitable for the acetaldehyde reduction to ethanol by ADH[29]. Similar relationships were observed for the total concentration of higher alcohols in the obtained distillates. In the distillate obtained in the variant VIII (mash with calcium ions added) the total concentration of higher alcohols was similar to control (ca. 1460 mg dm^{-3} EtOH). The analysis of all the distillates for the total concentration of aldehydes indicates that the maximum levels (exceeding 300 mg dm^{-3} EtOH)

were found in the spirits derived from substrates containing phosphorus and inositol (experimental variant V), because of an increased concentration of acetaldehyde and propionaldehyde.

A high total concentration of aldehydes (at the level of 290.5 mg dm^{-3} EtOH, Table 4) was also observed in the distillate obtained by distillation of mash enriched with MgSO$_4$ (2.16 g dm^{-3} of mash; experimental variant VII). Elevated levels of aldehyde concentration were also reported for the media supplemented with magnesium sulfate (2.0 g dm^{-3} of rye mash) in the studies by Kotarska et al.[13]. A high concentration of acetaldehyde in the fermentation media with additional magnesium ions can result from increased activity of pyruvate decarboxylase, which uses Mg^{2+} as a cofactor[28]. It should be noted that the supplementation with inositol, phosphorus, zinc, magnesium and calcium (as in the experimental variant IX) reduced the total aldehyde concentration to about 100 mg dm^{-3} EtOH which was the lowest concentration in all the analyzed distillates and more than three times lower than in variant V (Table 4). The lowest acetaldehyde concentration in the raw spirits obtained by the distillation media supplemented with magnesium and zinc ions may result from a synergistic effects of these ions on the activity of both pyruvate decarboxylase and alcohol dehydrogenase that are the key enzymes involved in the conversion of pyruvate to acetaldehyde[28, 30].

Our study also revealed that the addition of all supplements except for calcium ions (experimental variant VIII) caused an increase in the total concentration of higher alcohols as compared to the control, which was mainly due to increased concentrations of iso-butanol and 2-methyl-1-butanol (Table 4). The variant with calcium supplementation was the only one for which no statistically significant difference in the concentration of n-propanol, isobutanol, n-butanol and 2-methyl--1-butanol relative to the control variant was reported (Table 4). Different results were obtained in the study by Kotarska et al., which showed that supplementation with magnesium and zinc compounds lowered the total concentration of higher alcohols. These discrepancies can result from different raw materials used in both studies. Kotarska et al. used rye grain and a lighter extract (ca. 19.5° Blg) and reported much higher total concentration of higher alcohols in the control (about 4.25 g dm^{-3} EtOH)[13].

CONCLUSIONS

The supplementation of HG media with biogenic compounds analogous to those released in the phytate hydrolysis improved the parameters of alcoholic fermentation. The enrichment of the media with selected compounds of limited bioavailability associated with the complexing action of phytic acid, seems to be particularly important in large scale applications employing HG technology. Increased availability of divalent cations, required for the proper metabolism of yeast cells, had a positive effect on their physiological state. It should be noted, however, that the use of excessively high doses of some supplements had a negative impact on the process of alcoholic fermentation. The comparison of the obtained results in the experimental variants I and II, as well as

Table 4. The effect of supplementation of HG media on the composition of volatile by-products of the alcoholic fermentation

Research variant	Aldehydes [mg dm^{-3}]			Higher alcohols [mg dm^{-3}]					Esters [mg dm^{-3}]			
	acetaldehyde	propionaldehyde	Σ aldehydes	n–propanol	isobutanol	n–butanol	2-methyl-1-butanol	Σ higher alcohols	ethyl acetate	isoamyl acetate	ethyl heptanoate	Σ esters
Control variant	109.4a ±1.4	14.7a ±0.4	124.2a ±1.2	187.8aef ±9.4	652.2ab ±22.0	6.6ae ±0.1	615.2abef ±11.3	1461.9a ±20.0	9.3a ±0.8	0.0a ±0.0	0.0a ±0.0	9.3a ±0.8
Variant I	196.7b ±18.6	42.5bc ±10.2	239.4bc ±28.2	241.0bcf ±41.6	942.1bce ±132.7	6.3ae ±1.0	775.4befg ±67.1	1964.8b ±236.1	21.9b ±7.0	3.9bc ±1.0	0.9bd ±0.1	26.7b ±8.1
Variant II	201.7bc ±3.0	52.6ce ±1.5	254.6cdh ±1.2	341.4cd ±45.6	1156.8cde ±161.1	12.0b ±1.7	1291.4cd ±101.7	2801.5c ±310.0	20.1b ±0.3	4.5b ±0.8	1.6c ±0.1	26.1b ±1.2
Variant III	224.7bc ±2.9	52.1ce ±2.0	276.9df ±1.0	346.4d ±28.7	1325.2d ±115.5	10.8bc ±1.0	1388.5d ±144.7	3071.0c ±290.0	13.5a ±0.6	5.7b ±0.2	1.2cd ±0.1	20.9b ±1.0
Variant IV	167.4d ±0.9	34.1b ±2.7	201.6e ±3.6	225.9abe ±7.8	890.4ae ±23.1	7.0ae ±0.2	840.0efg ±11.4	1949.8b ±11.6	11.3a ±1.3	0.0a ±0.0	0.6be ±0.0	11.9ab ±1.3
Variant V	221.6bc ±5.5	81.1d ±8.3	303.1f ±2.8	229.4abe ±5.5	907.9abc ±37.2	5.5ae ±0.4	681.0fi ±46.3	1823.7a ±89.3	34.9b ±12.1	5.5b ±0.0	0.8bdf ±0.2	41.1c ±11.9
Variant VI	210.0bc ±7.2	55.8ce ±1.3	265.9bdg ±5.9	326.3bcd ±50.1	1168.3cde ±192.0	10.9bd ±2.2	986.6gh ±84.5	2630.9bc ±407.3	16.8a ±0.9	4.9b ±0.9	1.1bc ±0.3	22.8b ±2.2
Variant VII	229.1c ±22.9	61.2e ±7.4	290.5fgh ±27.6	313.2bcd ±58.0	1246.1cd ±138.3	11.0bd ±1.9	1150.0ch ±66.3	2720.8c ±238.1	20.0b ±5.5	5.2b ±0.7	1.1bc ±0.3	26.3b ±5.8
Variant VIII	114.3a ±0.6	15.8a ±3.7	130.1a ±3.2	186.3aef ±38.0	750.6ab ±152.3	4.0e ±0.9	546.8bi ±102.3	1487.9a ±293.7	14.3a ±3.0	2.4cd ±0.7	0.7bdf ±0.1	17.4ab ±3.8
Variant IX	99.7a ±5.1	1.8a ±1.8	101.5a ±3.3	200.7aef ±13.1	766.3ab ±50.7	8.3acd ±0.4	981.7gh ±78.6	1957.3b ±142.7	9.8a ±0.4	0.7ad ±0.7	0.4aef ±0.4	10.8a ±0.1

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

III and IV, indicates that applying double doses of both phosphorus and inositol may inhibit the fermentation activity of the yeast, which is manifested by a lower ethanol concentration, productivity, and yield. This means that appropriate concentrations of these supplements should be used, which is also extremely important from the economic point of view. It is noteworthy that the addition of CaCl$_2$ improved the fermentation rate which was manifested in a statistically significant increase of all analyzed fermentation parameters. The hydrolysis of phytate complexes with exogenic microbial phytases can be a solution alternative to the supplementation of HG mashes presented in this work, with the advantage of eliminating the costs of supplementation.

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