SELECTED TOPICS IN FOOD BIOTECHNOLOGY

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Józefa Chrzanowska
and **Aleksandra Zambrowicz**

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Authors:

Augšpole Ingrida, Balcerek Maria, Błażewicz Józef, Borowski Sebastian, Chrzanowska Józefa, Dąbrowska Anna, Drozdowska Krystyna, Dziekońska Urszula, Dziugan Piotr, Eckert Ewelina, Gawrysiak-Witulska Marzena, Karklina Daina, Kordialik-Bogacka Edyta, Kozłowska Katarzyna, Kruma Zanda, Księżopolska Mirosława, Liszewski Marek, Nogala-Kalucka Małgorzata, Nowak Adriana, Patelski Piotr, Pielech-Przybylska Katarzyna, Pokora Marta, Riekstina-Dolge Rita, Seglina Dalija, Siepka Ewelina, Siger Aleksander, Szołtysik Marek, Szwed Łukasz, Trziszka Tadeusz, Ungure Eva, Wawrzyniak Jolanta, Zambrowicz Aleksandra, Zembold-Guła Agnieszka,

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Redaktor Naczelny – prof. dr hab. Andrzej Kotecki
ul. Sopocka 23, 50–344 Wrocław, tel. 71 328 12 77
e-mail: wyd@up.wroc.pl

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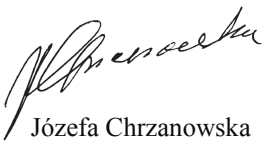
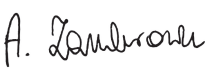
FOREWORD

Biotechnology has a long history of practice in food production. Since ancient times, man has applied different naturally occurring micro-organisms in manufacture of food products such as bread, beer, wine, vinegar or cheese. At present modern biotechnology, which uses a range of processes and techniques especially at the molecular level, offers new opportunities for the agriculture and food industries. In agro-biotechnology, the use of recombinant DNA technology has enabled the production of crops with increased yield and better resistance to diseases, pests and adverse environmental conditions. Thanks to biotechnology, it is also possible to obtain raw materials of plant and animal origin with quality parameters more suitable for processing and also with improved nutritional value. Biotechnology has achieved great successes in the modification of microorganisms for their ability to increase the biosynthesis of substances used as food additives or supplements. These modifications are made by both tissue engineering and genetic manipulation. In relation to food production, biotechnology makes it possible to improve the organoleptic characteristics of products and their nutritional value. It can also be used to ensure greater food safety by using advanced diagnostic techniques to detect pathogens and toxins in food. Enzymes are particularly important tools in this respect and their use in the modern food industry is increasing. It is expected that by 2015 the global market value of enzymes used in the food industry will grow to $1.3 billion, compared to $975 million in 2010 (i.e. annual growth of 5.1%). Enzymes enable the utilisation of less attractive raw materials and also to streamline technological processes and increase the effectiveness of production of certain products. Thanks to the use of enzymes, it is also possible to obtain new products with high pro-health value: prebiotics, structured polymers and biologically active compounds such as biopeptides. Last but not least, biotechnology is also extremely important in environmental protection: the search for new energy sources and management of waste and by-products of the agri-food industry.

This monograph focuses on selected issues in the area of food biotechnology. It discusses the content and stability of bioactive substances in raw materials (phenolic compounds, tocoferol, tocochromonols) and the efficiency of bioprocesses depending on the quality of raw material and technological factors. Some of the mentioned reports concern improvements of technological characteristics of industrial microorganisms. Other authors evaluate the possibility of using enzymes for the improvement of industrial processes and in the production of biopeptides which are of importance both for the food and pharmaceutical industries. It is also presented that sugar beet is an attractive raw material for bioethanol production.



Józefa Chrzanowska



Aleksandra Zambrowicz

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THE EFFECT OF NITROGEN FERTILIZATION OF BREWING BARLEY PLANTS ON THE MALTING PRODUCTIVITY

Introduction

Spring barley is an important cereal in the whole grain economy, which is caused by the universality of its application in feed, milling, groats and brewing industry.

Malting industry is located between agriculture and brewing industry. On the one hand, it faces the high variability of raw material, on the other hand the requirements of customers who demand a stable quality of malt.

Before World War II, Poland was a respected exporter of malting barley and brewing malt. The recipients were the countries of Western Europe [Gołębiewski 1998], and in 1939, Poland was one of the most important producers of malting barley. Export of this material was then 370 thou. tons and gave 4th place in the quantity of brewing barley production right after Germany, the Netherlands and Romania.

After the war export broadened on other countries as Japan, Brazil, Venezuela and many others. Those customers put up very high demands on the quality of malt [Gołębiewski, 1998, Baca et al. 2000, Kulinski 2001].

For many years in recent decades, breeders and producers of barley have worked primarily on increasing production of fodder cultivar, rich in protein. This gave include disastrous results. From the position of the exporter, Poland became an importer of large quantities of brewing barley and malt [Gołębiewski et al. 1997].

Polish participation in European harvest of barley in 2007 was 4.9% (8th place), while 3% in the world (11th place) [GUS 2010]. In 2008, 286.6 thou. tons of barley was imported from abroad, including 283.6 thou. tons from EU countries, and 3.0 thou. tons from Central and Eastern Europe [GUS 2009].

Production of malt is the most important industrial use of barley. Currently about 10% of worlds harvest is directed for malt production. The European Union is characterized by high yield of malt production, which accounts for about 60% of worlds brewing barley and nearly 50% of the world malt. A large part of the malt from the EU, however, is intended for export to other parts of the world. On individual approach, the largest malt production takes place in China (17%). While in Poland 5–6% of the annual production of barley are used for this purpose [Davies 2006, Pecio 2002, Zdeb 2001].

Particularly important in the brewing barley cultivation is nitrogen fertilization. Nitrogen is a nutrient that in highest degree affects not only the grain yield of barley, but also its protein content. Use of high dose nitrogen fertilization can result satisfactory yield, but adversely affects its quality, it also promotes lodging of plants. The method of nitrogen doses sharing carries the risk of an increased grain protein content, so the safest way is to use nitrogen entirely preplant.

Spring barley has among cereal the shortest period of vegetation. However, it is very sensitive to periodic, even short-term deficiency or excess of precipitation. Hot and dry weather shortens the growing season of barley [Gąsiorowski 1997]. Occurrence of drought periods have a significant impact on the uptake and transolcation of nitrogen in the plant. Results of research show that lowering supply of easily available water in the soil, in both phases before flowering, as well as during grain filling, leads to reduction of yield and grain plumpness, as well as increase of grain protein content, and thus deteriorating the brewing value [Kukuła et al. 1999, Pecio 2002, Pecio and Bichoński 2006, Qureshi and Neibling 2009, Thompson et al. 2004].

Despite cultivar and agrotechnical progress, temporal variability of meteorological conditions, which represent the main feature of Polish transition agroclimate, is a major limiting factor for production of high quality brewing barley [Błażewicz and Dawidowicz 2006, Pecio 2002]. Irrigation of plants, not yet widespread in Poland on a broad field production scale, can be effective treatment for counteracting negative effects of agricultural drought.

Barley is the grain most commonly used and subjected to malting, brewing, disstillery and food industry. In brewing industry quality and quantity of the extract obtained from the weight unit of purchased and malted barley is of vital importance. It is key economic indicator of barley and malt value. Hence it is important that grains should be rich in starch, well-filled and equalized. The aim of malthouse is not only production of good quality malt, but also satisfactory technological efficiency with minimal malting losses.

The purpose of this study was to determine the effect of growing season conditions, cultivar characteristic and the six variants of nitrogen fertilization on economic usefulness of brewing barley grain. Economic suitability of brewing barley was defined on the basis of plump grains yield, malting productivity and malt weight, which are obtainable from 1 hectare of cultivation in 2008–2010.

Materials and Methods

The experimental material was grain of spring brewing barley 'Mauritia' and 'Sebastian' cultivars. The grain originated from the vegetative seasons of 2008–2010, from a strict field experiment, that was conducted in Agricultural Research Station in Pawłowice near Wrocław, on brown soil, on different nitrogen fertilization levels (kg $N \cdot ha^{-1}$): 0, 20, 40, 60, 60 (40+20I), 60 (40+20II). Nitrogen fertilization was prosecuted preplant and top-dressing (divided doses) in two stages of growth: I – at the end of tillering ($BBCH_{29}$), II – in the stage of second node ($BBCH_{32}$).

Nitrogen has been applied as 34% ammonium nitrate water solution. Preplant, phosphorus fertilization in the dose of 40 kg $P_2O_5 \cdot ha^{-1}$ and potassium in the dose of 60 kg $K_2O \cdot ha^{-1}$ were also applied. During the growing season multicomponent foliar fertilizer was used. Forecrop for barley was winter wheat. Agricultural measures were made according to the intensive technology of spring barley cultivation, with full protection of plants against weeds, pests and diseases. The harvest of barley was carried out using the plot combine in the phase of full ripeness of barley. Final grain yield from each plot was determined.

To determine drought periods during the growing season of barley Sielianinov hydrothermal coefficient was used. It was calculated by dividing the sum of precipitation by the sum of the month temperatures reduced tenfold [Gałęzewska and Kapuściński 1978].

Technological evaluation of brewing barley grain and obtained from them malts was conducted in the Department of Food Storage and Technology of Wrocław University of Environmental and Life Sciences.

The grain was fractionated by means of Vogel screens, as well as deprived of contaminations and damaged corns. After a period of dormancy, grain with fractions of >2.5 mm thickness was used to produce 3-, 4-, 5- and 6-day Pilsner type malts under laboratory conditions. The steeping and malting of grain samples (200 g) were conducted in perforated foil bags in a climatic cabinet, with temperature kept at a level of 15–16°C and high air humidity. The steeping cycle spanned for 48 h. The grain was kept in water and air atmosphere according to the following scheme: 8 h – in water /w/, 11 h – in air atmosphere /a/, 5 h – w, 8 h – a, 11 h – w, and 5 h – a. The steeping allowed to obtain the assumed final moisture content of 43%. The time of malting was counted from the termination of the steeping cycle and lasted for 3, 4, 5 or 6 days. In the course of the malting process, the grain was weighed and agitated. The resultant malts were kilned in a laboratory wind dryer using the following temperature cycles: 10 h – 30°C, 5 h – 40°C, 3 h – 50°C, 3 h – 65°C, and 2 h – 82°C. After cooling of the kilned malts the rootlets were removed manually.

Grain of choice quality (well-filled fraction of barley), yield of choice quality grain and the level of natural losses of malting mass were calculated. Natural losses were used to calculate the weight of malt dry matter possible to obtain from one hectare field of brewing barley. The degree of utilization of substances, contained in the barley grain, was described as yield of malting process. This parameter is determined on the basis of barley grains dry matter yield obtained from one hectare compared/attributable to dry matter of produced from them malt.

Obtained results were subjected to one-way and two-way analysis of variance at a significance level of $\alpha = 0.05$. Homogenous groups, denoted in tables with subsequent letters of the alphabet, were determined with the method of multiple comparisons using a Duncan's test. All calculations were performed by means of *STATISTICA 8.0* package by StatSoft company.

Results and Discussion

Influence of weather conditions on yield of barley grain

Mean twenty-four hour temperatures of vegetation season, in researched years, were generally higher than mean long-term value for respective months (Tab. 1). Only in June of 2009 and May of 2010 mean twenty-four hour air temperatures were lower than long-term means. In May, June and July of 2008, as well as June and July of 2010 summed amount of rainfalls were lower than their respective long-term values. Sum of rainfalls in seasons of 2009 and 2010 significantly exceeded (by 162.6 and 90.4 mm) mean long-term sum of rainfalls (313.6 mm).

Table 1

Weather conditions in 2008–2009 (for the Agricultural Research Station Swojec near Wrocław)

Month	Temperature [°C]				Rainfalls [mm]				Sielianinov index [K]		
	2008	2009	2010	Mean 1976 –2005	2008	2009	2010	Mean 1976 –2005	2008	2009	2010
III	4.6	4.6	4.2	3.7	33.0	48.3	44.9	31.7	2.31	3.39	3.45
IV	8.9	12.0	9.3	8.3	87.1	30.9	45.4	30.5	3.27	0.86	1.62
V	14.3	14.2	12.7	14.1	37.3	67.6	140.7	51.3	0.84	1.53	3.57
VI	18.8	15.8	17.9	16.9	36.5	141.7	32.9	59.5	0.65	3.00	0.61
VII	19.8	19.5	21.4	18.7	65.6	134.2	78.6	78.9	1.06	3.24	1.19
VIII	18.8	19.3	18.9	17.9	94.0	53.5	61.5	61.7	1.61	0.89	1.86
Mean sum III–VIII	14.2	14.2	14.1	13.3	353.5	476.2	404.0	313.6	–	–	–

Cultivation habitat is compromised from all of the complex of soil and climatic factors, but circumscription of weather conditions to their individual components is insufficient. Important role in description is currently played by agronomic-climatic index. From agricultural meteorology point of view, atmospheric droughty is often cause to soil drought effecting in underdevelopment of plants and decreased yield of crops. Useful method of environment water ratio estimation is Sielianinov hydrothermal index, also known as factor of protection in water [Radomski 1987]. Sielianinov hydrothermal index is also used for estimation of duration and intensity of drought. Period of dry spell is assumed when K index is lower than 1.0, which means that plant is using greater amounts of water for vaporization than received with precipitation. As drought Sielianinov assumes period characterized by K index lower than 0.5, which equals, that amount of vaporized water is twice times bigger than their inlet In own research this index was used for example to estimate influence of weather conditions during barley vegetation on yield and quality of barley grain.

Weather conditions before sowing were favorable for satisfactory seedling emergence of barley.

Słaboński [1985] claims, that air temperature in tillering should not overcome 18°C, and rainfalls should be moderate. In all researched years weather conditions were favourable for good tillering of plants, but most intensive rainfalls occured in 2009.

Many authors [Liszewski and Błażewicz 2001, Słaboński 1985] claim, that weather conditions during shooting and earing phases are crucial, because of brewing barley greatest sensitivity on water shortages in mentioned periods. Optimal conditions allow to develop maximal assimilation leaf area, which grants potential of high yielding, with proper brewing parameters [Pecio 2002, Słaboński 1985]. Pecio [2002] published, that in stages of shooting and earing high temperature, from 17°C to 19°C, and moderate rainfalls are beneficial. In 2008 vegetation season shooting stage progressed in optimal air temperature, but with too low rainfalls. Unfavourable weather conditions in were confirmed by calculated Sielianinov indexes (V-0.84, VI -0.65) [Radomski 1987], which caused disorder in vegetative development. Many authors [Bertholdson 1999, Przulj and Momcilovic 2001] claim, that greater assimilation area, generated in period before blooming of barley, determines its

later size, that take part in photosynthesis after blooming. It is important for proper grain filling and obtainment of desired quality, especially in cultivation of barley for brewing usage. Optimal conditions during shooting took place in 2009 and 2010 seasons, when sufficient rainfalls sum allowed to obtain high grain yield.

According to Pecio [2002] in time after earing sunny and warm weather corresponds with proper conditions for intensive photosynthesis and good plumpness of grain. On the opposite side, insufficient rainfalls in this period disadvantageous influence quality of grain, causing increase in protein content [Pecio and Kubsik 2006, Rozbicki 1994].

In season of 2008 rainfalls were low, whereas in 2009 season, during grain filling and in the period of maturation weather was favorable for obtainment of grain with good quality parameters.

Influence of nitrogen fertilization
on yield of choice barley grain and malting productivity

Essential condition for obtainment of god malt is procession of homogenous mass, composed from grains of similar size, in which conversions may occur with specified intensity. Choice quality of brewing barley grain, which is described as percent share of grains of thickness >2.5 mm, should not be lower than 90% [Rozbicki 1994].

In own research, independently from dose of nitrogen used share of well-filled grain for both cultivars were high and reached values from 92.8 to 98.0%, relatively from season, cultivar and variant of nitrogen fertilization (Tab. 2). In 2010 season 'Mauritia' cultivar was characterized with share of grain of choice quality (fractions 2.5–2.8 and >2.8 mm) superior than 'Sebastian' cultivar. In 2008 determined shares of well-filled grains were significantly greater than in 2009 (by 3.9 pp) and 2010 (by 3.3 pp). After Liszewski and Błażewicz [2001], inferior share of choice quality of grain is caused by specific conditions, in which increase of plant's productive tillering causes necessity to nutrition greater amount of grains. This effects in decreased share of well-full fractions.

Application of nitrogen fertilization didn't contribute to substantial decrease of grain's choice quality in both tested cultivars. Previous research showed other dependencies [Błażewicz et al. 2008], in which it was claimed, that high doses of nitrogen don't favor development of yield with plumpness grain, but only allow better vegetation development of plants, increasing number of shoot and spikes. Yield is possibly greater, but grain in its mass may be small, whereas in malting practice more important is share of proper developed grain.

Table 2

Brewing barley grain of choice quality (well-filled fraction)

Barley cultivar	Nitrogen fertilization [kg N·ha^{-1}]	Choice quality [%]			
		2008	2009	2010	Mean
Interaction cultivar × fertilization					
Sebastian	0	98.0 a	93.2 a	93.9 abc	95.3 a
	20	97.6 a	92.8 a	93.8 abc	95.1 a
	40	97.7 a	94.4 a	93.3 bc	95.2 a
	60	97.3 a	94.4 a	93.3 bc	95.4 a
	60 (20+40I)	97.6 a	93.5 a	92.9 c	94.9 a
	60 (20+40II)	97.2 a	93.3 a	92.9 c	94.9 a
Mauritia	0	97.9 a	93.4 a	95.7 ab	95.7 a
	20	97.8 a	93.9 a	95.7 ab	96.0 a
	40	97.6 a	94.0 a	95.2 abc	95.8 a
	60	97.8 a	93.4 a	95.5 ab	95.6 a
	60 (20+40I)	97.7 a	94.0 a	95.9 a	96.0 a
	60 (20+40II)	97.6 a	93.2 a	95.0 abc	95.4 a
$LSD_{0.05}$		1.02	2.98	2.15	1.03
Means for variables					
Sebastian		97.5 a	93.6 a	93.4 b	95.1 b
Mauritia		97.7 a	93.7 a	95.5 a	95.7 a
$LSD_{0.05}$		0.42	1.22	0.88	0.42
0		97.9 a	93.3 a	94.8 a	95.5 a
20		97.7 a	93.3 a	94.7 a	95.5 a
40		97.7 a	94.2 a	94.2 a	95.5 a
60		97.6 a	93.9 a	94.4 a	95.5 a
60 (40+20I)		97.6 a	93.7 a	94.4 a	95.4 a
60 (40+20II)		97.4 a	93.3 a	93.9 a	95.1 a
$LSD_{0.05}$		0.72	2.11	1.52	0.73
Years		97.7 a	93.9 c	94.4 b	95.3
$LSD_{0.05}$		0.52			–

Yield of well-filled (>2.5 mm of thickness) grain is dependant from level of total yield as well as choice quality of specific cultivar. In technological estimation of barley grain usability it is important indicator defining amount of grain useful for malting.

Nitrogen fertilization effected in increase of choice quality yield of barley grain, independently from season (Tab. 3). Other authors [Koziara et al. 1998, Liszewski 2008] research also shows, that yield of barley grain is mostly affected from the number of spikes per unit of surface. Many authors [Fatyga et al. 1995, Liszewski 1998, Liszewski et al. 1995] confirm dependency between nitrogen fertilization and values of yield structure elements and yield of grain. Significant increase of well-filled grain yield in comparison to control object was observed alongside nitrogen fertilization with 20 or 40 kg·ha^{-1} dose (depending from cultivar and climatic conditions). Fertilization increase from 40 to 60 kg·ha^{-1} did not reflect in expected increase of usable yield.

Table 3

Yield of brewing barley grain of choice quality

Barley cultivar	Nitrogen fertilization [kg N·ha^{-1}]	Yield of brewing barley grain of thickness over 2.5 mm [t·ha^{-1}]			
		2008	2009	2010	Mean
Interaction cultivar × fertilization					
Sebastian	0	2.96 e	4.61 d	5.84 c	4.47 c
	20	3.55 cd	5.15 bcd	6.00 bc	4.90 b
	40	3.96 bc	5.62 abc	6.53 abc	5.37 a
	60	4.14 ab	6.07 a	6.76 abc	5.66 a
	60 (20+40I)	4.21 ab	5.98 ab	6.49 abc	5.56 a
	60 (20+40II)	4.16 ab	6.05 a	6.88 ab	5.69 a
Mauritia	0	3.42 d	4.65 d	5.90 c	4.66 bc
	20	3.99 b	4.85 cd	6.09 abc	4.98 b
	40	4.47 a	5.63 abc	6.67 abc	5.59 a
	60	4.56 a	5.70 abc	6.61 abc	5.63 a
	60 (20+40I)	4.29 ab	5.92 ab	6.62 abc	5.61 a
	60 (20+40II)	4.24 ab	5.98 ab	7.01 a	5.74 a
$LSD_{0.05}$		0.41	0.77	0.81	0.39
Means for variables					
Sebastian		3.83 b	5.58 a	6.42 a	5.27 a
Mauritia		4.16 a	5.45 a	6.49 a	5.37 a
$LSD_{0.05}$		0.17	0.31	0.33	0.16
0		3.19 c	4.63 b	5.87 c	4.56 c
20		3.77 b	5.00 b	6.05 bc	4.94 b
40		4.22 a	5.62 a	6.60 ab	5.48 a
60		4.35 a	5.89 a	6.69 a	5.64 a
60 (40+20I)		4.25 a	5.95 a	6.56 ab	5.58 a
60 (40+20II)		4.20 a	6.01 a	6.94 a	5.72 a
$LSD_{0.05}$		0.29	0.54	0.57	0.27
Years		4.00 c	5.52 b	6.45 a	5.32
$LSD_{0.05}$		0.19			–

LSD – last significant difference
a, b, c, d, e – homogeneous groups ($\alpha = 0.05$)

Vegetation season of 2010 favored to achieve highest yield of well-filled grain (on average 6.45 t·ha^{-1}), which were higher than 2008 and 2009 seasons, respectively by 44.5 and 38%.

Investigated cultivars yielded on similar level. Only 2008 season effected in higher yield choice quality grain for 'Mauritia' cultivar, on average by 8.7%. Those results confirm observations made by Pecio [2002], that new barley cultivars are characterized by high variation in yielding and plumpness of grain.

Mass of malt obtained from unit of surface contained within 1.63 to 5.37 t d.m.·ha^{-1} range and was mostly dependant from weather conditions and dose of nitrogen fertilization used (Tab. 4).

Table 4

Weight of barley malt

Barley cultivar	Nitrogen fertilization [kg N·ha^{-1}]	Weight of malt [t d.m.·ha^{-1}]			
		2008	2009	2010	Mean
Interaction cultivar × fertilization					
Sebastian	0	1.63 d	3.92 c	4.39 d	3.18 d
	20	1.96 bc	4.23 abc	4.74 bcd	3.56 bc
	40	2.11 abc	4.83 abc	4.81 abcd	3.82 ab
	60	2.18 abc	5.12 a	5.13 abc	4.08 a
	60 (20+40I)	2.29 a	5.07 a	4.80 abcd	3.98 a
	60 (20+40II)	2.28 a	5.11 a	5.11 abc	4.10 a
Mauritia	0	1.91 c	3.96 c	4.48 d	3.28 cd
	20	2.16 abc	4.02 bc	4.53 cd	3.47 cd
	40	2.38 a	4.64 abc	5.12 abc	3.94 a
	60	2.41 a	4.63 abc	5.10 abc	3.97 a
	60 (20+40I)	2.32 a	4.93 ab	5.37 a	4.12 a
	60 (20+40II)	2.21 ab	5.03 a	5.22 ab	4.09 a
$LSD_{0.05}$		0.26	0.84	0.54	0.27
Means for variables					
Sebastian		2.08 b	4.71 a	4.83 a	3.79 a
Mauritia		2.23 a	4.54 a	4.97 a	3.81 a
$LSD_{0.05}$		0.11	0.34	0.22	0.11
0		1.77 c	3.94 b	4.44 c	3.23 d
20		2.06 b	4.12 b	4.63 bc	3.51 c
40		2.25 ab	4.74 a	4.97 ab	3.88 b
60		2.30 a	4.87 a	5.12 a	4.02 ab
60 (40+20I)		2.31 a	5.00 a	5.09 a	4.05 ab
60 (40+20II)		2.25 ab	5.07 a	5.17 a	4.10 a
$LSD_{0.05}$		0.19	0.60	0.38	0.19
Years		2.17 c	4.60 b	4.90 a	3.89
$LSD_{0.05}$		0.14			–

LSD – last significant difference
a, b, c, d – homogeneous groups (α = 0.05)

Independently from applied factors, from grain of investigated cultivars, in the result of malting, similar amounts of malt were obtained. Only in 2008 season, 'Mauritia' cultivar were producing malts of average mass higher by 7.5%.

2010 vegetation season also favored obtainment of highest malt mass from unit of surface (on average 4.90 t d.m.·ha^{-1}), they were significantly higher in comparison to 2008 and 2009, respectively by 133 and 2.58% ('Sebastian' cultivar) and by 123 and 9.6% ('Mauritia' cultivar).

With increase of nitrogen dose used simultaneously increase of obtained malt mass was observed. Increase of nitrogen dose from 40 to 60 kg·ha^{-1}, did not effect in expected increase of mass.

Efficiency of malting from unit of surface is mainly conditioned by weather and cultivar predispositions, that are linked with yield of well-filled grain and susceptibility of specific cultivar to mass loss, in the form of so called natural loss. Depending on used factors malting efficiency per surface area was from 58.7% to 92.9% d.m.·ha^{-1} (Tab. 5).

Table 5

Malting productivity of brewing barley grain

Barley cultivar	Nitrogen fertilization [kg N·ha^{-1}]	Malting productivity [% d.m.·ha^{-1}]			
		2008	2009	2010	Mean
Interaction cultivar × fertilization					
Sebastian	0	60.9 a	89.8 ab	86.0 ab	78.3 a
	20	60.4 abc	89.4 ab	86.4 ab	78.3 a
	40	60.3 abcd	92.9 ab	84.1 ab	78.2 a
	60	59.9 bcde	93.1 a	84.4 ab	78.5 a
	60 (20+40I)	60.9 a	90.7 ab	82.7 b	77.8 a
	60 (20+40II)	60.8 ab	92.6 ab	82.7 b	78.2 a
Mauritia	0	59.6 cdef	88.9 ab	86.9 ab	77.9 a
	20	59.5 def	89.9 ab	88.5 a	78.4 a
	40	59.1 ef	89.5 ab	85.9 ab	77.5 a
	60	58.8 f	88.8 ab	86.9 ab	77.2 a
	60 (20+40I)	58.7 f	87.0 b	88.9 a	77.8 a
	60 (20+40II)	59.1 ef	90.6 ab	83.8 ab	77.5 a
$LSD_{0.05}$		0.84	5.18	4.42	1.87
Means for variables					
Sebastian		60.5 a	91.4 a	84.4 b	78.2 a
Mauritia		59.1 b	89.1 b	86.8 a	77.7 a
$LSD_{0.05}$		0.34	2.11	1.81	0.76
0		60.2 a	89.3 a	86.4 ab	78.1 a
20		59.9 ab	89.6 a	87.4 a	78.3 a
40		59.7 ab	91.2 a	85.0 ab	77.9 a
60		59.4 ab	91.0 a	85.7 ab	77.9 a
60 (40+20I)		59.8 ab	88.9 a	85.8 ab	77.8 a
60 (40+20II)		59.9 ab	91.6 a	83.3 b	77.9 a
$LSD_{0.05}$		0.59	3.66 a	3.13	1.32
Years		59.9 c	90.4 a	85.6 b	78.6
$LSD_{0.05}$		0.94			–

LSD – last significant difference
a, b, c, d, e, f – homogeneous groups ($\alpha = 0.05$)

Independently from weather conditions in researched years, increase of fertilization level did not effect in substantial decrease nor increase of malting efficiency for tested spring brewing barley grain cultivars.

Barley cultivars in diversified way responded on various vegetation conditions. In 2008 and 2009 seasons superior malting efficiency (average by 2.5 pp) was presented by 'Sebas-

tian' cultivar, while in 2010 season by 'Mauritia' cultivar. Highest efficiency per surface area were attained in 2009 season (average 90.4% d.m.·ha^{-1}), abundant in rainfalls during vegetation season, and lower in 2008 (by about 30 pp) and 2010 season (by about 5 pp).

From malster's point of view only valid parameters are yield of choice quality grain for specific cultivar and its quality. Divergence of goals set by cultivators (maximization of total yield) and malsters is main reason of Polish shortages of good quality brewing barley grain.

Results of conducted research however show good substantiation on introduction of sprinkling to technology of brewing barley grain cultivation, which may solute many raw material problems in vegetation seasons with agricultural drought periods, that substantially worsen malting traits of brewing barley grain. Most of research of other authors, irrigation contributed to raise of malting parameters of grain, including lower overall protein content in grain, increase it mass and choice quality and improved germinative energy [Błażewicz et al. 2011, Koszański et al. 1995, Nowak et al. 2005, Wojtasik 2004, Żarski et al. 2011].

Conclusions

It was found that the tested cultivars of brewing barley were characterized by diversified resistance to the adverse impact of variable weather conditions and cultivation level.

Unfavourable weather conditions during barley plants growing season were the most important factors of grain economic usefulness aggravation, determined on the basis of well-filled grain yield, malts weight and malting productivity.

Optimal level of nitrogen fertilization for tested cultivars of brewing barley, in respect of usable grain yield and weight of obtained from them malts is 40 kg·ha^{-1}. Increasing the nitrogen dose to 60 kg·ha^{-1}, did not result in significant increases in weight of choice quality grains and malts.

Acknowledgements

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2

DEGRADATION OF RAPESEED BIOACTIVE COMPONENTS DURING RAPESEED STORAGE UNDER ADVERSE CONDITIONS

Introduction

Poland's accession to the European Union has contributed to an increased profitability of rapeseed production. This has resulted in a systematic expansion of rape cultivation area and growing yields of this crop. In 2007 rapeseed yields were exceptionally good, amounting to 2 million tones [Rosiak 2008]. Rapeseed harvested in Poland is typically of 7–17% moisture content, while 80–90% seed need to be cleaned and force-dried to moisture content of approx. 7% [Rybacki et al. 2001], since such moisture content is a pre-condition of appropriate storage. A higher seed moisture content significantly reduces the time of safe storage [Pronyk et al. 2006]. This is connected with the fact that an elevated water content in seeds intensifies seed respiration processes, enhances activity of the enzymes they contain and promotes the development of microflora, which under specific conditions results in seed self-heating. A consequence of increased temperature is an acceleration of adverse chemical and biochemical changes occurring in seeds [Niewiadomski 1993]. Moreover, an increase in storage temperature to 30°C contributes to an increase in contents of lipid hydrolysis and oxidation products in rapeseed and it affects the fatty acid profile [Krasucki et al. 2002]. An increase in rapeseed contamination with mould fungi also causes several adverse changes in the technological quality of this raw material and obtained products; moreover, it possess a risk of rapeseed contamination with secondary metabolites of these microorganisms, i.e. mycotoxins, which exhibit toxicity in relation to humans and animals [Hussein and Brasel 2001].

Rapeseed oil is considered to be one of the most valuable plant fats. It is a rich source of mono- and polyenoic acids [Sanders 2002] and natural oxidation inhibitors – tocopherols, phenolic compounds and sterols [Piironen et al. 2002, Khalique et al. 2005]. Tocochromanols and plastochromanol-8 (PC-8) determine lipid stability in stored seeds and account for an adequate nutritive value of produced oils [Hofius and Sonnewald 2003]. An important group of native antioxidants found in rapeseed comprises tocopherols (-T). Among tocopherols found in rapeseed four homologues of tocopherols are mentioned, i.e. α-, β-, γ- and δ-, with α-T and γ-T contents amounting to 800 mg kg^{-1} oil, and the other two being found in trace amounts [Ratnayake and Daun 2004]. Antioxidant activity of homologous tocopherols *in vivo* is as follows α-T > β-T > γ-T > δ-T, while their activity *in vitro* is found to be in the opposite order α-T < β-T ≈ γ-T < δ-T [Eitenmiller and Lee 2004, Yanishlieva and Marinova 2001]. Antioxidant activity of tocochromanols depends on their concentration, type of substrate, other chemical compounds exhibiting pro-oxidative and synergistic action, solvent, light and temperature, at which they act [Kamal-Eldin and Appelqvist 1996]. This is confirmed by numerous studies conducted with the application of different fat substrates, under different conditions (temperature, light) [Lampi et al. 1999]. Tocopherol amounts in plants are probably related to the content

of unsaturated fatty acids. Canola oil contains relatively high levels of tocopherols [Przybylski and Mag 2002]. Lipid peroxy radicals react with tocopherols several orders of magnitude faster than with other lipids. A single molecule of tocopherol can protect about 103 to 106 molecules of polyunsaturated fatty acids (PUFA) in the living cell. This explains why the ratio of tocopherols to PUFA in the cells is usually 1:500 and still sufficient protection is provided [Patterson 1981]. Plastochromanol-8 (PC-8) is a derivative of γ-tocotrienol, which has a longer side chain. This compound was detected in canola and linseed oils and its antioxidant activity was established to be similar to that of α-tocopherol [Przybylski and Mag 2002, Zambiazi 1997]. Inadequate rapeseed preservation may contribute to a decrease in contents of these compounds and thus to a deterioration of nutritive value of produced oil. For this reason the aim of this study was to investigate the affect of adverse rapeseed storage conditions on the rate of degradation of contained native tocopherols.

Analysis of the content of tocopherols and plastochromanol-8 in rapeseed.

Materials and Methods

The material used for tests was canola cv. *Californium* obtained directly after harvest from the Zlotniki Experimental Station owned by the Poznań University of Life Sciences, Poland. Prior to the onset of the experiment rapeseed was processed to obtain the assumed storage conditions (three moisture contents of approximately 10.2, 12.4 and 15.4% wet mass basis (w.b.) and two temperatures of 25±1 and 30±1°C) by spraying seeds in a batch of 4 kg with a specific amount of distilled water. The amount of water required to obtain seeds with the assumed moisture content was determined using mass balance. The assumed seed moisture content (10.2, 12.4 and 15.4%), which was determined based on Halsey's equation, corresponded to water activity of 81, 85 and 91%. Seeds after being moistened in order to equalize moisture content throughout seed bulk were packaged in polyethylene bags and conditioned for 24 h at 5°C. Seeds after being moistened were stored in a thermostatic chamber equipped with three hygrostatic apparatuses, used to maintain constant relative humidity (φ). Assumed relative humidity of ambient air surrounding seeds placed in containers was maintained constant with the use of saturated solutions of salts, i.e. NaCl, KCl and $BaCl_2$, placed in cells of hydrostatic apparatuses. Relative humidity in containers with seeds was monitored using probes of relative humidity with capacity sensors. Temperature in seed bulk was monitored using Cu-Konstantan thermocouples (type EE21-FT6B53/T24). Relative humidity in interested spaces and temperature were measured online using the I-7018 data acquisition system by ICP-CON and ICP computer software for the recording, visualization and storage of data.

During seed storage samples for analyses were collected every 6 days. Germinative energy and contents of tocopherols and plastochromanol-8 were determined in samples. Each of the experiments was run until the moment when the seed germinative energy dropped below 75%.

Determination of germinative energy. Germination was determined by placing 50 seeds on filter paper (90mm diameter) placed in a Petri dish with distilled water. The plates were covered and incubated in a growth chamber at 25°C for 4 days, after which the plates were uncovered and incubated for another 3 days. At this time the number of seeds that had germinated were counted and recorded.

Determination of seed moisture content. The seed moisture content was determined with the use of an electronic moisture analyzer (based on a precision weighing balance by drying a 5 g sample at the temperature of 115°C to constant mass) and was treated as the reference. The measuring accuracy of the analyzer is 0.05% w.b. (wet basis). The moisture analyzer was verified using the oven method according Current Protocols in Food Analytical Chemistry [2003].

Determination of tocochromanols and plastochromanol-8. In order to determine contents of tocochromanols and PC-8 the collected rapeseed samples were comminuted in a laboratory mill. For further analyses, 2g of a sample and 0.5 g pyrogallol were weighed and placed in a round-bottomed flask, where saponification was performed by adding 20 ml anhydrous ethyl alcohol and 2 ml of 60% KOH. After 30 min heating at the solvent boiling point, 50 ml of 1% NaCl solution were added to the samples, which were then cooled. Subsequently 50 ml *n*-hexane with a 10% addition of ethyl acetate were added. Tightly sealed flasks were shaken (at 300 rpm) for 30 min. Next, approximately 2 ml saturated NaCl solution were added. After 15 min from the top layer (non-saponified substances) an amount adequate for HPLC injection was collected. Recovery of tocopherol standards, saponified using this method, was 99.9%. Tocopherols and PC-8 were identified qualitatively and quantitatively using an HPLC apparatus (Waters 600 Milford, MA) in a system comprising a Waters 600 pump, a LiChrosorb Si 60 column (200 x 4.6 mm, 5 µm, Merck, Darmstadt, Germany) and a fluorimetric detector. The whole amount was analyzed using the Millenium 32 program. The mobile phase comprised a mixture of *n*-hexane with *1,4*-dioxane (97:3, v/v). Flow rate was 1.5 ml/min. The fluorimetric detector (Waters 474 Milford, MA) ran at excitation l=290 nm and emission l=330 nm for tocopherols. Concentrations of individual tocopherol homologues were calculated from a previously prepared calibration curve [PN-EN-12822, 2002; PN-EN-ISO 9936, 2006, Ryynänen et al. 2004]. Exemplary chromatogram of tocopherols and plastohromanol-8 separation is shown at Figure 1.

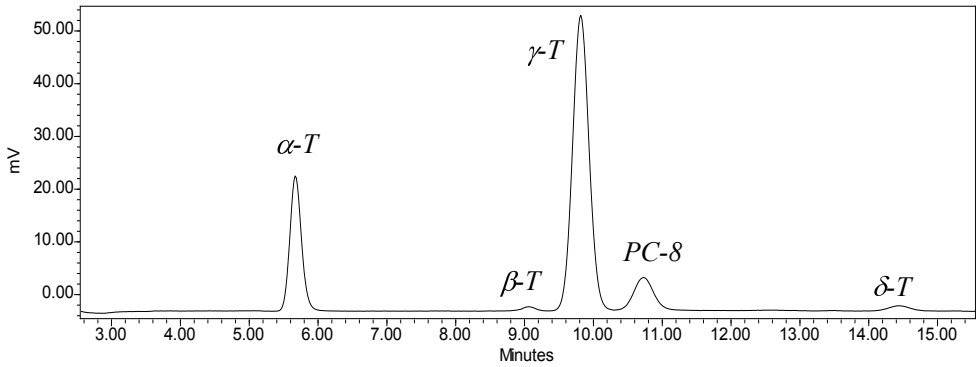

Fig. 1. Chromatographic separation of tocopherols: α-tocopherol (α-T); β-tocopherol (β-T); γ-tocopherol (γ-T); δ-tocopherol (δ-T) and plastochromanol-8 (PC-8)

Statistical Analysis. The results obtained were subjected to statistical analysis. Linear models were established, on the basis of which constant degradation rates of tocopherols (k_D) were estimated. Results are presented as means of three replications ± standard deviation. One-way analysis of variance and post-hoc Tukey's tests for the significance level $p < 0.05$ were carried out using a Statistica (version 7.1) program.

Results and Discussion

Degradation of tocopherols during rapeseed storage

Rapeseed harvested from the field had germinative energy of 96%. Changes in germinative energy during storage of individual rapeseed samples are presented in Figure 2. Rapeseed storage time in the conducted experiment, in which germinative energy of seeds dropped below 75%, and seeds showed signs of moulding, was the longer, the lower seed moisture content and the lower storage temperature were. Germinative energy of seeds with moisture content of 15.4% during storage at 25°C dropped below 75% after 18 days, while at a temperature of 30°C it was after 12 days. For rapeseed with moisture content of 12.4% storage time after which germinative energy of seeds dropped below 75% was 36 days, irrespective of the applied storage temperature, while for seeds with moisture content of 10.2% it was 78 days at 30°C and 108 days at 25°C, respectively.

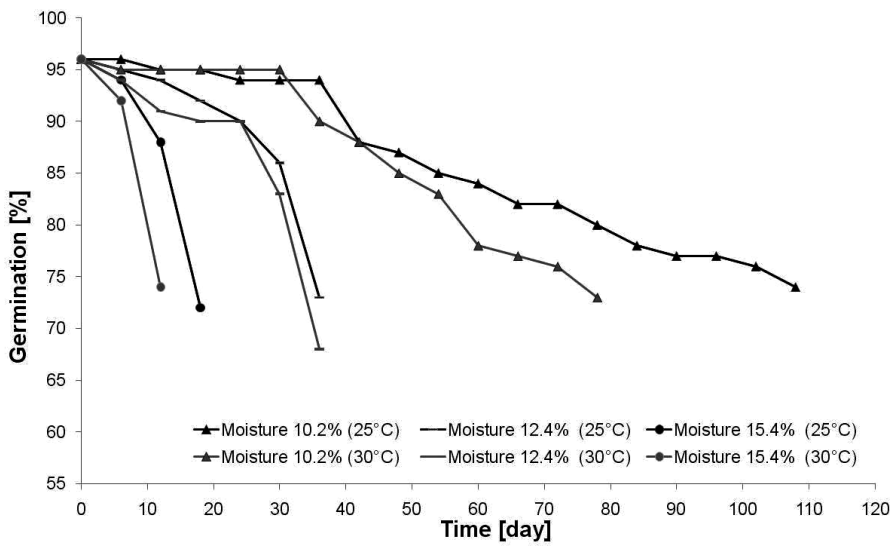

Fig. 2. Change of the germination in rapeseed stored at 25 and 30°C depending on the moisture content

Initial total tocopherol content in tested rapeseed was 548.8 mg/kg, while literature data show that it may range from 80 to 1000 mg kg^{-1} [Abidi et al. 1999, Dolde et al., 1999]. According to Dolde et al. [1999] total tocopherol content in seeds is dependent to a considerable degree on environmental and technological conditions. The percentage composition of tocopherol fractions contained in tested seeds was characteristics of rapeseed. The dominant tocopherol was homologue γ-T (291.1 mg/kg), which accounted for 53% total tocopherol content. The content of homologue α-T was 251.1 mg/kg (46%), while those of homologues β-T and δ-T in tested samples were 1.5 and 5.1 mg/kg, respectively. Similar proportions of individual tocopherol homologous in rapeseed were recorded by Marwede et al. [2004]. The same authors also analyzed the a-T to g-T ratio in rapeseed and obtained values range from 0.54 to 1.70. In seeds collected for storage the α-T/γ-T ratio was 0.86 (Tab. 1–2). Conducted

analyses showed that during rapeseed storage the total tocopherol content decreases (Fig. 2), with the rate of losses of these compounds being affected by the temperature of storage and seed moisture content. After the completion of the experiments the total content of tocopherols in stored seed samples, in which germination energy dropped below 75%, was reduced by 8–16%. The biggest losses (16%) were recorded for seeds with moisture content of 10.2% stored both at 25 and 30°C, for which storage time was longest. Content of tocopherols in these seeds after the completion of the experiment was 463 and 459.2 mg/kg, respectively. The smallest losses were recorded in seeds with moisture content of 15.4%, stored at 25°C (8%) and 30°C (11%), in which germination energy dropped below 75% already after 18 and 12 days (Fig. 2). Seeds with moisture content of 12.4% were characterized by a decrease in tocopherol content by 11–13%, while – similarly as in case of seeds with moisture content of 15.4% – bigger losses of these compounds were recorded for a temperature of 30°C. Homologue β-T was the compound most susceptible to degradation. Its losses amounted to 61% for seeds with moisture content of 15.4%, stored at 25°C. For seeds with moisture content of 10.2%, for which storage time at 25°C was much longer, i.e. 78 days, these losses amounted to 72%. The initial content of homologue α-T in tested seeds was 251.1 mg/kg. At the moment when seed germinative energy decreased below 75% losses of α-T in seeds with moisture content of 10.2% both at 25°C and 30°C were approx. 17%, while in seeds with moisture content of 15.4% stored at 25°C they amounted to 9%. The content of homologue γ-T at the onset of the experiment was 291.1 mg/kg. For seed storage times assumed on the basis of a decrease in germinative energy, similarly as in case of α-T, the biggest losses of γ-T were recorded in seeds with moisture content of 10.2% (14–15%), whereas they were smallest in seeds with moisture content of 15.4% stored at 25°C. It needs to be observed here that in each experiment the degradation of homologue α-T occurred faster than that of homologue γ-T. It resulted in a decrease of the α-T/γ-T ratio during storage from 0.86 to 0.81, depending on the type of the experiment (Tab. 1 and 2). The biggest change in the α-T to γ-T ratio was recorded in seeds with moisture content of 12.4%. After the completion of the experiment the α-T/γ-T ratio amounted to 0.81 for seeds stored at 30°C and 0.82 for seeds stored at 25°C. Investigations conducted by Gawrysiak-Witulska et al. [2009] showed that during the storage of seeds with moisture content of 7% at 10±2°C the value of coefficient α-T/γ-T increased, thus during the storage of seeds with an appropriate moisture content a faster degradation of homologue γ-T was found than it was for α-T. This indicates that adverse storage conditions (elevated moisture content and too high temperature) significantly affect the acceleration of degradation of homologue α-T, i.e. the homologue with the highest biological activity. Tocopherols belong to the most efficient inhibitors of the free radical chain reaction, protecting against lipid oxidation by quenching superoxide anion radicals [Schneider 2005]. Their presence in blood serum and tissues, where they are the main lipophilic antioxidants, makes it possible to eliminate peroxide radicals, thus preventing damage to membrane protein and unsaturated fatty acids [Wang and Quinn 1999, Quinn 2004]. Activity of tocopherols is first of all connected with supplying protons to free lipid radicals [Schneider 2005].

Analysis of the content of plastochromanol-8 (Tab. 1–2) showed the biggest decrease in the content of this compound in seeds with moisture content of 10.2% (23%), stored at 25°C, for which storage time was longest. For the same seeds stored at 30°C the decrease in PC-8 content was much smaller, amounting to 10%. In seeds with moisture content of 15.4% during storage the content of PC-8 deceased by 15%, irrespective of storage temperature. In seeds with moisture content of 12.4% during storage similar dependencies were observed as

for seeds with moisture content of 10.2%. Higher losses (14%) occurred during storage at 25°C than during storage at 30°C.

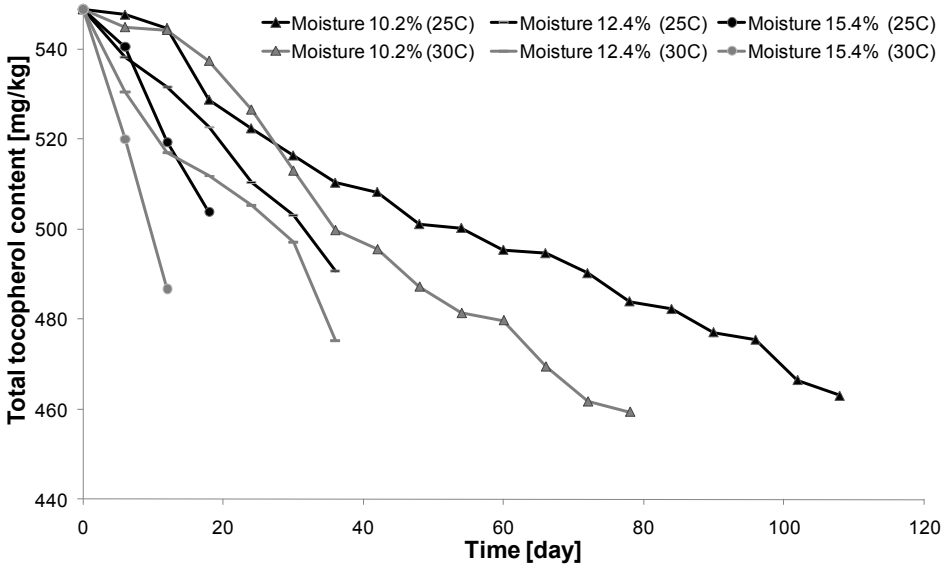

Figure 3. Change of total tocopherol content in rapeseed stored at 25 and 30°C depending on moisture content

A detailed analysis of recorded results showed that the degradation of tocopherols progressed fastest in seeds with moisture content of 15.4% stored at 30°C. In the first 6 days of storage a statistically significant decrease in the content of these compounds amounted to 5%, while in the next 6 days it was a further 6%. In the same seeds, this time stored at 25°C, degradation of tocopherols during individual 6-day storage periods progressed by 2, 4 and 3%, while these changes were also statistically significant. The trend was similar during storage of seeds with moisture content of 12.4%. At 30°C tocopherol degradation during individual 6 days of storage amounted to 1–5%, while at 25°C it was smaller, of 1–2%. During individual 6 days of storage in seeds with moisture content of 10.2% tocopherol degradation amounted to 0–2%.

Intensity of biological and chemical processes occurring in rapeseed is dependent on storage conditions. Optimal moisture content of seeds for long-term storage should be 7%. The European Union regulations accept (as a commercial standard) seed moisture content of 9%. Storage of seeds with an elevated moisture content leads to an increased activity of lipases, first native and then of microbiological origin, which in turn results in an increase in the amounts of free fatty acids. Oxidation and degradation of these compounds to a considerable degree contribute to reduced shelf life of produced oil [Jędrychowski et al. 1993, Tańska and Rotkiewicz 2003]. Temperature is a very important storage factor in case of rapeseed. Its elevation to 30°C causes an increase in contents of lipid hydrolysis and oxidation products in seeds. It also has a considerable effect on the fatty acid profile [Krasucki et al. 2002]. According to Pronyk et al., [2006] storage of seeds with moisture content of 12

and 14% at temperatures exceeding 25–35°C causes considerable development of micoflora and an increased production of CO_2. Apart from these changes, inappropriate seed storage may result in considerable losses of biologically active compounds, such as sterols, tocopherols and phenolic compounds [Rudzińska et al. 2006, Siger et al. 2006]. In a study by Siger et al. [2006] in the course of the first 12 months of storage in case of rapeseed dried at high and near-ambient temperature conditions the content of phenolic acids was observed to decrease by 22% in cv. Lisek and by 24% in cv. Kronos. Along with a decrease in the content of native antioxidants those authors showed a lower antioxidant activity in the $DPPH^{\bullet}$ test. Investigations conducted by Kalucka et al. [2006] showed that losses of tocopherols during 12 months of storage in case of seed with moisture content of 7% at 20°C amounted to 50%. According to Gawrysiak-Witulska et al. [2009] storage of seed with moisture content of 7% for 12 months at lower a temperature (10°C) resulted in losses of these compounds were smaller (23–30%).

Conclusions

Experiments conducted within this study concerning inappropriate storage conditions of rapeseed clearly indicate that a too high moisture content as well as storage temperature have an adverse effect on contents of phytochemicals, such as native tocochromanols and plastochromanol-8. In rapeseed oil produced from the seed, contents of native tocopherols have a significant effect both with respect to nutritive value (as a natural source of vitamin E) and from the technological point of view (as they inhibit autoxidation of fatty acids, thus extending shelf life of oil). By meeting respective parameters of postharvest procedures we may significantly affect the rate of degradation of tocopherols contained in rapeseed. Insight into the scale of changes in tocopherol contents in rapeseed for different storage conditions may be of significant importance for the optimization of seed preservation.

Table 1

Tocopherol and PC-8 content [mg kg^{-1}] in rapeseed stored at 25°C depending on moisture content

Time [days]	Tocopherol content				a-T/g-T	PC-8
	a-T	b-T	g-T	d-T		
Moisture 10.2%						
0	251.10 ± 0.19^{n}	1.53 ± 0.04^{h}	291.12 ± 0.06^{o}	5.07 ± 0.05^{j}	0.86	125.38 ± 0.27^{n}
6	250.60 ± 0.35^{n}	1.54 ± 0.01^{h}	290.89 ± 0.18^{o}	4.55 ± 0.03^{i}	0.86	123.55 ± 0.56^{m}
12	249.41 ± 0.45^{m}	1.13 ± 0.02^{g}	289.61 ± 0.32^{n}	4.49 ± 0.06^{i}	0.86	122.56 ± 0.40^{l}
18	243.16 ± 0.31^{l}	1.12 ± 0.01^{g}	280.19 ± 0.22^{m}	4.25 ± 0.03^{h}	0.87	120.73 ± 0.57^{k}
24	238.65 ± 0.40^{k}	0.90 ± 0.02^{f}	278.67 ± 0.28^{l}	4.13 ± 0.02^{g}	0.86	119.88 ± 0.72^{j}
30	234.75 ± 0.20^{j}	0.88 ± 0.02^{f}	276.71 ± 0.41^{k}	4.00 ± 0.02^{f}	0.85	119.26 ± 0.25^{j}
36	231.69 ± 0.08^{i}	0.77 ± 0.02^{e}	274.05 ± 0.18^{j}	3.78 ± 0.04^{e}	0.85	114.47 ± 0.80^{i}
42	228.45 ± 0.29^{h}	0.66 ± 0.02^{d}	275.40 ± 0.40^{i}	3.73 ± 0.09^{e}	0.83	111.64 ± 0.63^{h}
48	225.45 ± 0.44^{g}	0.65 ± 0.03^{d}	271.63 ± 0.35^{h}	3.36 ± 0.04^{d}	0.83	108.24 ± 0.10^{g}
54	224.99 ± 0.35^{f}	0.63 ± 0.03^{d}	271.32 ± 0.25^{g}	3.32 ± 0.08^{d}	0,83	107.65 ± 0.12^{f}
60	222.41 ± 0.23^{e}	0.60 ± 0.09^{c}	269.14 ± 0.32^{f}	$3.26 \pm 0.06^{c,d}$	0,83	104.99 ± 0.25^{e}
66	221.40 ± 0.34^{d}	0.57 ± 0.02^{c}	269.46 ± 0.43^{f}	3.22 ± 0.02^{c}	0.82	104.60 ± 0.21^{e}
72	220.69 ± 0.61^{d}	0.54 ± 0.04^{b}	265.78 ± 0.33^{e}	3.20 ± 0.07^{c}	0,83	102.56 ± 0.31^{d}
78	218.41 ± 0.33^{c}	0.51 ± 0.01^{b}	261.86 ± 0.24^{d}	$3.17 \pm 0.01^{b,c}$	0.83	100.73 ± 0.58^{c}
84	217.54 ± 0.34^{c}	0.50 ± 0.06^{b}	261.06 ± 0.12^{d}	$3.16 \pm 0.06^{b,c}$	0,83	100.10 ± 0.42^{c}
90	214.12 ± 0.12^{b}	$0.45 \pm 0.07^{a,b}$	259.41 ± 0.62^{c}	3.12 ± 0.05^{b}	0,82	98.45 ± 0.12^{b}
96	213.58 ± 0.40^{b}	0.44 ± 0.04^{a}	258.32 ± 0.25^{c}	3.11 ± 0.01^{b}	0.82	97.93 ± 0.66^{b}
102	209.41 ± 0.74^{a}	0.43 ± 0.06^{a}	253.65 ± 0.34^{b}	3.01 ± 0.02^{b}	0,82	96.40 ± 0.32^{a}
108	208.49 ± 0.43^{a}	0.43 ± 0.03^{a}	251.30 ± 0.26^{a}	2.78 ± 0.01^{a}	0.83	96.38 ± 0.13^{a}
Moisture 12.4%						
0	251.10 ± 0.19^{g}	1.53 ± 0.04^{f}	291.12 ± 0.06^{g}	5.07 ± 0.05^{f}	0.86	125.38 ± 0.27^{f}
6	246.88 ± 0.26^{f}	1.24 ± 0.03^{e}	285.57 ± 0.48^{f}	4.59 ± 0.04^{e}	0.86	118.38 ± 0.73^{e}
12	242.48 ± 0.43^{e}	1.05 ± 0.04^{d}	283.53 ± 0.46^{e}	4.40 ± 0.09^{d}	0.86	114.42 ± 0.70^{d}
18	238.39 ± 0.34^{d}	0.92 ± 0.01^{c}	279.28 ± 0.49^{d}	4.03 ± 0.05^{c}	0.85	$113.57 \pm 0.21^{c,d}$
24	230.48 ± 0.40^{c}	0.91 ± 0.01^{c}	275.28 ± 0.48^{c}	3.67 ± 0.02^{b}	0.84	112.62 ± 0.89^{c}
30	227.33 ± 0.47^{b}	0.79 ± 0.02^{b}	271.38 ± 0.46^{b}	$3.59 \pm 0.12^{a,b}$	0.84	110.64 ± 0.79^{b}
36	219.65 ± 0.50^{a}	0.60 ± 0.02^{a}	266.99 ± 0.20^{a}	3.42 ± 0.03^{a}	0.82	107.60 ± 0.44^{a}
Moisture 15.4%						
0	251.10 ± 0.19^{d}	1.53 ± 0.04^{d}	291.12 ± 0.06^{d}	5.07 ± 0.05^{d}	0.86	125.38 ± 0.27^{d}
6	246.46 ± 0.40^{c}	1.19 ± 0.01^{c}	288.43 ± 0.38^{c}	4.39 ± 0.02^{c}	0.85	120.19 ± 0.66^{c}
12	234.53 ± 0.58^{b}	0.93 ± 0.03^{b}	280.23 ± 0.67^{b}	3.63 ± 0.06^{b}	0.84	115.41 ± 0.34^{b}
18	227.59 ± 0.29^{a}	0.59 ± 0.01^{a}	273.33 ± 0.32^{a}	2.29 ± 0.13^{a}	0.83	109.62 ± 0.54^{a}

*values (means ± SD) with different index letters are statistically significantly different ($p<0.05$)

Table 2

Tocopherol and PC-8 content [mg kg^{-1}] in rapeseed stored at 30°C depending on moisture content

Time [days]	Tocopherol content				a-T/g-T	PC-8
	a-T	b-T	g-T	d-T		
Moisture 10.2%						
0	251.10 ± 0.19^{j}	1.53 ± 0.04^{g}	291.12 ± 0.06^{k}	5.07 ± 0.05^{j}	0.86	125.38 ± 0.27^{k}
6	248.76 ± 0.31^{i}	1.48 ± 0.03^{f}	289.29 ± 0.25^{j}	5.30 ± 0.08^{i}	0.87	125.63 ± 0.55^{j}
12	247.70 ± 0.27^{i}	$1.43 \pm 0.03^{e,f}$	288.51 ± 0.44^{j}	$5.23 \pm 0.20^{h,i}$	0.85	123.63 ± 0.54^{i}
18	244.51 ± 0.44^{h}	1.31 ± 0.04^{e}	286.51 ± 0.16^{i}	$5.03 \pm 0.03^{g,h}$	0.85	117.92 ± 0.38^{h}
24	239.39 ± 0.53^{g}	1.11 ± 0.01^{d}	280.94 ± 0.81^{h}	5.08 ± 0.06^{h}	0.85	116.32 ± 0.28^{g}
30	231.45 ± 0.47^{f}	1.08 ± 0.06^{d}	275.56 ± 0.48^{g}	$4.87 \pm 0.06^{f,g}$	0.84	113.95 ± 0.34^{f}
36	224.48 ± 0.42^{e}	1.09 ± 0.12^{d}	269.41 ± 0.91^{f}	$4.76 \pm 0.05^{e,f}$	0.83	112.61 ± 0.53^{e}
42	223.59 ± 0.72^{e}	$1.02 \pm 0.08^{c,d}$	266.24 ± 0.23^{e}	4.65 ± 0.04^{e}	0.84	111.02 ± 0.20^{d}
48	218.71 ± 0.58^{d}	$0.92 \pm 0.02^{b,c}$	263.15 ± 0.85^{d}	4.44 ± 0.03^{d}	0.83	110.15 ± 0.36^{d}
54	216.99 ± 0.34^{c}	0.88 ± 0.06^{b}	259.34 ± 0.51^{c}	4.12 ± 0.09^{c}	0.84	109.02 ± 0.51^{c}
60	216.54 ± 0.47^{c}	0.87 ± 0.02^{b}	258.22 ± 0.23^{c}	4.11 ± 0.01^{c}	0.84	108.53 ± 0.46^{c}
66	212.34 ± 0.54^{b}	0.79 ± 0.02^{b}	252.52 ± 0.38^{b}	3.81 ± 0.01^{b}	0.84	105.40 ± 0.35^{b}
72	209.21 ± 0.51^{a}	$0.51 \pm 0.09^{a,b}$	248.57 ± 0.65^{a}	3.41 ± 0.09^{a}	0.84	103.54 ± 0.38^{a}
78	208.12 ± 0.41^{a}	0.46 ± 0.03^{a}	247.52 ± 0.72^{a}	3.33 ± 0.02^{a}	0.84	103.00 ± 0.57^{a}
Moisture 12.4%						
0	251.10 ± 0.19^{f}	1.53 ± 0.04^{f}	291.12 ± 0.06^{g}	5.07 ± 0.05^{f}	0.86	125.38 ± 0.27^{f}
6	239.47 ± 0.41^{e}	1.60 ± 0.08^{e}	282.55 ± 0.48^{f}	4.89 ± 0.03^{e}	0.85	119.11 ± 0.99^{e}
12	231.49 ± 0.42^{d}	1.42 ± 0.07^{d}	277.53 ± 0.46^{e}	4.60 ± 0.06^{d}	0.84	115.67 ± 0.89^{d}
18	230.34 ± 0.54^{d}	1.16 ± 0.03^{c}	275.79 ± 0.18^{d}	4.50 ± 0.09^{d}	0.84	114.10 ± 0.84^{c}
24	226.55 ± 0.48^{c}	1.02 ± 0.02^{b}	273.50 ± 0.43^{c}	4.15 ± 0.13^{c}	0.83	113.54 ± 1.05^{c}
30	221.38 ± 0.33^{b}	0.55 ± 0.03^{a}	271.34 ± 0.57^{b}	3.82 ± 0.11^{b}	0.82	108.39 ± 0.86^{b}
36	210.48 ± 0.64^{a}	0.44 ± 0.04^{a}	261.00 ± 0.13^{a}	3.30 ± 0.01^{a}	0.81	102.61 ± 0.81^{a}
Moisture 15.4%						
0	251.10 ± 0.19^{c}	1.53 ± 0.04^{c}	291.12 ± 0.06^{c}	5.07 ± 0.05^{c}	0.86	125.38 ± 0.27^{c}
6	236.80 ± 0.34^{b}	1.20 ± 0.03^{b}	278.08 ± 0.50^{b}	3.91 ± 0.20^{b}	0.85	114.50 ± 0.48^{b}
12	220.23 ± 0.40^{a}	0.62 ± 0.05^{a}	262.37 ± 0.54^{a}	3.50 ± 0.08^{a}	0.84	109.48 ± 0.44^{a}

*values (means ± SD) with different index letters are statistically significantly different ($p<0.05$)

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3

INTERMEDIATE PRODUCTS OF PLUM PROCESSING AS RAW MATERIALS FOR FRUIT DISTILLATES PRODUCTION

Introduction

Brandies – natural fruit vodkas (okowita, eau de vie), are a particularly interesting group of alcoholic beverages, which – according to the Regulation (EC) No 110/2008 of the European Parliament and the Council of 15 January 2008 on the definition, description, presentation, labelling and protection of geographical indications of spirit drinks and repealing Council Regulation (EEC) No 1576/89 – are the spirit drinks with the minimum alcoholic strength by volume of 37.5% $v \cdot v^{-1}$, made exclusively by distillation process to the strength of alcohol at less than 86% $v \cdot v^{-1}$ of: fermented fruit pulp, fermented fruit mash, fruit macerate (also partly fermented) in ethyl alcohol, vodka or distillate. Moreover, the minimal usage of raw material should amount: in case of vodka obtained by fermentation and distillation – 5 kg of fruit per 20 dm^3 ethanol 100% vv^{-1}, while for vodka obtained by maceration and distillation – 100 kg per 20 dm^3 ethanol 100% $v \cdot v^{-1}$. There are no rules concerning addition of water and sugar to fruit mashes, however, usage of fruits in minimal amount (5 $kg \cdot 20\ dm^{-3}$ pure alcohol) can imply significant addition of those substances. Such beverages are defined as "vodka" and complemented by the name of fruit, for example: apple vodka, cherry vodka, pear vodka etc. In the case of spirit drink obtained from maceration and distillation, description of the product has to be elaborated by the information "produced on the way of maceration and distillation".

Almost all kind of fruits can be processed into spirits: seed fruits (apples, pears), stone fruits (cherries, plums, apricots, peaches), berry fruits (raspberries, currants, blackberries, strawberries, rowan berries) as well as wild fruits.

Very popular stone fruit brandies in Eastern and Central Europe are plum brandies (slivovitz) prepared from fresh Węgierka plums. In a submontane region of Poland with specific climatic conditions Śliwowica Łącka is produced of Węgierka Zwykła plums [Satora and Tuszyński 2008]. In the manufacturing process plums and a liberal proportion of the ground kernels are first crushed, then sugar may be added and then mixture is allowed to ferment. Distillation results in obtaining the raw product which needs aging in order to develop its finer qualities. Its flavor is due in part to plum kernels which contain considerable amount of amygdalin, the characteristic component of bitter almonds. Some producers have obtained a Hechsher for their slivovitz to certify that it is kosher.

This study aimed at evaluation of intermediate products of plum processing as potential raw materials for plum distillates production.

Material and Methods

Raw materials, microorganisms and supplements

The raw materials for production of fruit distillates were intermediate products of plums var. Węgierka processing, i.e. pulp, concentrate and syrup after candisation of fruit obtained from Polish fruit processing factories.

Fermentations were started by addition of raisins (150 g·100 dm^{-3}) as a source of microorganisms or by using dried wine yeast *Saccharomyces bayanus* (Fermentis, Division of S.I. Lesaffre, France) (40 g d.m.·100 dm^{-3}). Dried wine yeast was re-hydrated before being added to mash. Activit preparation (Institut Oenologique De Champagne, France) (20 g·100 dm^{-3}) and $(NH_4)_2HPO_4$ (40 g·100 dm^{-3}) were added as nutrient for yeast.

Preparing of fermentation wort

Fermentation wort were prepared through the dilution of semi-products of plum processing with water initially in terms of weight 1:1 and then to obtain the wort by the extract ca. 17–18ºBlg. Selected samples of wort were supplemented with sucrose from 6 to 12% w·w^{-1}.

Fermentation

Fermentation trials were carried out in stainless/acid-resistant steel containers with a volume of 70 dm^3, each containing approximately 50 dm^3 of plum wort. After inoculation with yeast, which was earlier re-hydrated, the containers were closed with cover equipped with fermentation tubes filled with glycerol and kept in a room at 17–18°C, with occasional stirring and measurement of apparent extract as an index of fermentation dynamics. The process was continued until the apparent extract measured at several hours' intervals was not subject to changes.

Distillation

When fermentation was complete the whole ethanol was distilled from wort using according to the law of parallel-current working apparatus. Raw spirits containing 20–23% v·v^{-1} ethanol were then moved into the apparatus equipped with a birectifier (dephlegmator according to Golodetz), to remove 3% of heads and concentration up to the strength of ca. 75% v·v^{-1} ethanol.

Analytical methods

The chemical composition of raw materials was analysed by methods recommended in the fruit-vegetable industry [PN-90/A-75101]. Wort before fermentation were analyzed for total extract with use of an aerometer with Balling degrees scale (ºBlg) (equivalent to Brix degrees). It refers to the concentration of dissolved solids, mostly sugars, expressed as the weight percentage of sucrose. Total sugars (after acid hydrolysis) were estimated by Schoorl and Regenbogen method [Schoorl and Regenbogen 1917] and expressed in g invert sugar·100 cm^{-3} of wort. Also pH of wort by pehameter was determined.

On completion of fermentations, wort were analyzed for the apparent extract (in the presence of ethanol) and real extract (after distillation of ethanol), both expressed in ºBlg. Ethanol concentration in distillates was measured by using an aerometer with scale in % v·v^{-1} of ethanol [Suchodolski 1955]. Concentration of residual sugars was assayed by the Schoorl and Regenbogen method in worts after distillation of ethanol.

Distillates were analyzed by using Agillent 6890N gas chromatograph (USA), equipped with a flame – ionization detector (FID), a split/splitless injector and a capillary column HP--Innowax (60 m x 32 mm x 0.5 μm). The temperature at the injector (split 1:45) and FID was kept at 250°C. The temperature program was as follows: 40°C (6 min), a rise to 83°C (2°C/min) and then to 190°C (5°C/min) (2 min). The flow rate of carrier gas (helium) through the column was 2 ml/min.

All assays were carried out in triplicate.

Results and Discussion

Chemical composition of intermediate products of plum processing used in this study is shown in Table 1. The high content of total sugars expressed as invert sugar (from 38.20 g·100 g^{-1} of pulp to 67.88 g·100 g^{-1} of syrup after candisation) is advantageous from the technological point of view because it could provide a high yield of ethanol produced from raw material. It makes them attractive raw material for alcoholic fermentation and brandies production.

Table 1

Chemical composition of semi-products of plum processing

	Pulp	Concentrate	Syrup after candisation
Total extract [°Blg]	58.40	67.20	75.86
Total sugars [g invert sugar·100 g^{-1}]	38.20	45.30	67.88
pH	3.60	3.28	3.46

Chemical characteristic of prepared plum wort is shown in Table 2. Initial extract content varied between 17.4 and 18.7°Blg. The lowest concentrations of fermentable sugars were found in wort prepared from intermediate products of plum processing without addition of sucrose and ranged between 11.4 g invert sugar·100 cm^{-3} of plum pulp-based wort and 15.7 g invert sugar·100 cm^{-3} of syrup after candisation-based wort. The addition from 6 to 12% w/w of sucrose to the mashes (keeping the total extract of wort on the level of ca. 17–18°Blg) resulted in an increase of fermentable sugar concentration. The content of invert sugar ranged from 14.4 g to 16.8 g·100 cm^{-3} of wort supplemented with 6% of sucrose and from 16.8 g to 17.9 g·100 cm^{-3} of wort in which sucrose constituted 12% of the total mass of wort, respectively. pH of prepared mashes ranged between 3.40 (plum concentrate-based wort) and 3.94 in syrup after candisation-based wort, supplemented with 12% of sucrose.

The composition of wort significantly affected the fermentation duration. The shortest time of process, lasting approximately 7–8 days was observed for wort prepared from plum pulp (without and with addition of sucrose) fermented with wine yeast *S. bayanus*. Fermentation of plum pulp-based wort without addition of wine yeast but with the contribution of native microflora of raisins resulted in lengthening of fermentations and in consequence the overall process time was 11 days. Obtained apparent extract was on the level of 0.8°Blg i.e. higher than determined in wort fermented with wine yeast *S. bayanus* (0–0.5°Blg) (Fig. 1).

The completion of a majority of fermentations of worts prepared from plum concentrate and syrup after candisation (with wine yeast *S. bayanus*) was observed after 10 days.

Table 2

Results of fermentation of worts prepared from intermediate products of plums processing

Sample	Wort before fermentation			Wort after fermentation					Intake of sugars [%]	Ethanol yield [% of theoretical]
	Extract [°Blg]	Total sugars [g invert sugar ·100 cm^{-3}]	pH	Apparent extract [°Blg]	Real extract [°Blg]	Total sugars [g invert sugar ·100 cm^{-3}]	pH	Ethanol content in wort [$v \cdot v^{-1}$]		
Plum pulp, without addition of sucrose, *S. bayanus*	17.4	11.4	3.68	0.5	1.8	0.95	3.77	6.2	91.7	84.1
Plum pulp, addition of 6% sucrose, *S. bayanus*	17.8	14.4	3.77	0.3	1.2	0.58	3.92	8.6	96.0	92.0
Plum pulp, addition of 12% sucrose, *S. bayanus*	17.5	16.8	3.85	0.0	0.7	0.32	3.99	10.3	98.1	94.5
Plum pulp, addition of 12% sucrose, without yeast	17.5	16.8	3.85	0.2	1.1	0.68	3.90	9.5	95.9	87.2
Plum concentrate, without addition of sucrose, *S. bayanus*	17.5	11.8	3.40	1.5	1.9	0.72	3.53	6.5	93.9	86.6
Plum concentrate, addition of 6% sucrose, *S. bayanus*	17.5	14.9	3.43	0.5	1.1	0.75	3.55	8.9	94.9	92.4
Plum concentrate, addition of 12% sucrose, *S. bayanus*	17.6	16.8	3.45	0.0	0.6	0.36	3.57	10.4	97.9	95.4
Syrup after candisation of plums, without addition of sucrose, *S. bayanus*	17.5	15.7	3.48	0.8	1.9	0.53	3.76	8.8	96.6	86.5
Syrup after candisation of plums, addition of 6% sucrose, *S. bayanus*	18.7	16.8	3.68	1.1	2.5	0.75	3.80	9.2	95.5	84.6
Syrup after candisation of plums, addition of 12% sucrose, *S. bayanus*	18.7	17.9	3.94	1.0	2.5	0.73	3.99	9.8	95.9	84.8
Syrup after candisation of plums, addition of 12% sucrose, without yeast	18.7	17.9	3.94	2.1	3.9	1.85	4.11	8.4	89.7	72.5

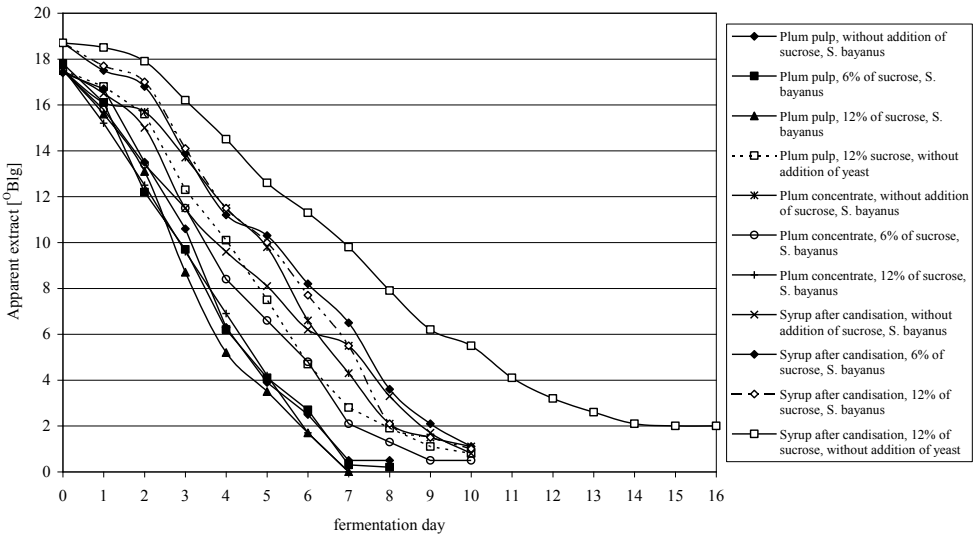


Fig. 1. Fermentation dynamics of intermediate products of plum processing-based worts

During spontaneous fermentation of wort prepared from syrup after candisation of plum a longer initial phase was observed and in a consequence fermentation of this wort stopped after 16 days. Also the apparent extract was relatively high (2.1°Blg) in comparison with observed in others made from syrup after candisation of fruit, as well as from plum pulp and concentrate (Fig. 1).

Table 2 also comprise the influence of initial extract of intermediate products of plum processing-based wort on ethanol contents, sugars intakes and ethanol yields. Depending of fermentation conditions, the degree of sugar assimilation varied between 89.7 and 98.1%.

Results obtained indicate that the addition of sucrose to the wort affected ethanol contents increase from 6.2–6.5% $v \cdot v^{-1}$ (reference wort prepared from plum pulp and from plum concentrate, fermented with wine yeast *S. bayanus*) to ca. 10.3% $v \cdot v^{-1}$ (wort supplemented with 12% of sucrose, also fermented with wine yeast *S. bayanus*). Consequently with the increasing fermentable sugars content, the gradual increase of ethanol yield was observed from 84.1–86.6% (reference wort without sucrose addition) to 94.5–95.4% of theoretical (wort supplemented with 12% of sucrose), except for fermentation trials prepared from syrup after candisation of plums. The lowest ethanol yields ranging between 72.5 and 87.2% of theoretical were achieved in wort fermented by addition of raisins as a source of microorganisms.

3.3. Analysis of chemical composition of distillates

The chemical composition of distillates obtained is shown in Table 3. All tested distillates were distinguished by a characteristic aroma and flavour but the best were obtained from plum pulp.

Basic volatile compounds contained in alcoholic beverages are aldehydes – intermediates of two-step processes of alpha-keto acids decarboxylation to alcohols [Kłosowski and Czupryński, 1993].

Acetaldehyde accounts for almost 90% of all carbonyl compounds in the distillates. Among other factors, the temperature, pH and nutrient content of the culturing medium as well as the activity of yeast and other microorganisms present in the fermenting solution influence its formation [Nielepkowicz-Charczuk and Kałuzka 1996, Nykanen and Suomalainene 1983]. The organoleptic properties of acetaldehyde vary, depending on its concentration, from the "classical" walnut aroma characteristic of sherry to an odour similar to that of overripe apples [Apostolopoulou et al. 2005].

Its concentrations in tested plum distillates varied greatly and ranged between 99.73 (plum pulp-based wort, 12% sucrose, *S. bayanus*) and 896.62 mg·dm^{-3} spirit 100% v·v^{-1} (plum pulp-based wort, 12% sucrose, *S. bayanus*). The plum brandies tested by Satora and Tuszyński [2008] contained relatively small amounts of acetaldehyde, with the lowest level being found in Slovakian Slivovica (74 mg·dm^{-3} spirit 100% vv-1) and the highest in commercial Śliwowica Paschalna (310 mg·dm^{-3} spirit 100% v·v^{-1}). Śliwowica Łącka samples were characterised by fairly uniform contents of acetaldehyde (92–175 mg·dm^{-3} spirit 100% vv-1) regardless of production year and origin, which may result from the use of uniform raw materials and similar conditions during fermentation (spontaneous process), distillation and maturation.

Esters have a significant effect on the organoleptic properties of alcoholic beverages and may contribute a pleasant, fruity fragrance to the general aroma [Satora et al. 2008, 2010, Satora and Tuszyński 2008]. Esters of acetic acid significantly affect wine, fruit distillate and vodka aromas, rendering them fruity and flowery. Ethyl acetate predominates, usually accounting for over 50% of all esters (over 90% in spirits and cognacs). At low concentrations, ethyl acetate contributes to the fruity aroma of wine, while higher concentrations (150–200 mg·dm^{-3} spirit 100% v·v^{-1}) may indicate excessively long storage of the product or infection by acetic acid bacteria [Apostolopoulou et al. 2005].

Ethyl acetate was the most abundant among esters that were quantified in plum distillates obtained, its concentrations varied between 81.13 mg (plum pulp-based wort, without addition of sucrose, *S. bayanus*) and 579.86 mg·dm^{-3} spirit 100% v·v^{-1} (plum concentrate-based wort, 12% sucrose, *S. bayanus*). Also low amounts of isoamyl acetate (2.75–74.99 mg·dm^{-3} spirit 100% v·v^{-1}) were found in tested spirits.

Higher alcohols dominate the group of volatile compounds in alcoholic beverages and have a significant effect on their sensory characteristics and quality [Satora et al. 2010]. Isobutanol (3-methyl-propanol) and amyl alcohols are synthesized mainly from corresponding amino acids (valine, leucine and isoleucine), other amino acids and sugars, while propanol is produced only from threonine and sugars [Vidrih and Hribar 1999].

All the plum distillates obtained were rich in higher alcohols irrespective of fermentation variant. Differences in concentrations of n-propanol were great and ranged between 89.08 (syrup after candisation-based wort, 12% sucrose, *S. bayanus*) and 442.97 mg·dm^{-3} spirit 100% v·v^{-1} (plum concentrate-based wort, without addition of sucrose, *S. bayanus*). The concentrations of 2-methyl-1-propanol ranged from 193.40 to 402.01 mg·dm^{-3} spirit 100% v·v^{-1}.

Contents of n-butanol in all the tested distillates were relatively small (1.98–26.69 mg·dm^{-3} spirit 100% v·v^{-1}). The most abundant of isoamyl alcohols detected in the distillates was 3-methyl-1-butanol (1744.31–3206.72 mg·dm^{-3} spirit 100% v·v^{-1}), while contents of 2-methyl-1-butanol ranged between 249.03 and 628.49 mg·dm^{-3} spirit 100% v·v^{-1} (Tab. 3).

Table 3

Concentration of volatile compounds in obtained plum distillates

Sample	Concentration [mg·dm^{-3} spirit 100% v·v^{-1}]								
	Acet-aldehyde	Ethyl acetate	n-propanol	2-methyl-1-propanol	Isoamyl-acetate	n-butanol	2-methyl-1-butanol	3-methyl-1-butanol	Sum of vola-tile compounds
Plum pulp, without addition of sucrose, *S. bayanus*	328.07	81.13	928.38	402.01	11.54	26.69	389.99	2 300.22	4 468.03
Plum pulp, addition of 6% sucrose, *S. bayanus*	896.62	124.93	455.58	304.02	15.34	11.28	444.64	2 601.58	4 853.99
Plum pulp, addition of 12% sucrose, *S. bayanus*	99.73	141.55	250.58	198.03	9.57	5.14	367.31	2 192.80	3 264.71
Plum pulp, addition of 12% sucrose, without yeast	510.10	227.56	242.62	383.85	9.77	7.37	628.49	3 206.72	5 216.48
Plum concentrate, without addition of sucrose, *S. bayanus*	253.90	190.81	442.97	267.08	43.21	10.49	260.19	2 005.68	3 474.33
Plum concentrate, addition of 6% su-crose, *S. bayanus*	317.30	514.17	329.35	266.54	74.99	11.98	265.86	2 174.75	3 954.94
Plum concentrate, addition of 12% sucrose, *S. bayanus*	115.56	579.86	377.51	193.40	63.22	6.25	249.03	1 842.60	3 427.43
Syrup after candisation of plums, with-out addition of sucrose, *S. bayanus*	222.44	192.83	259.38	238.99	30.45	3.08	339.72	2 034.18	3 321.07
Syrup after candisation of plums, addi-tion of 6% sucrose, *S. bayanus*	217.28	64.37	117.45	236.48	3.91	2.17	339.05	1 946.58	2 927.29
Syrup after candisation of plums, addi-tion of 12% sucrose, *S. bayanus*	556.91	111.92	89.08	299.80	2.75	1.98	396.43	2 090.72	3 549.59
Syrup after candisation of plums, addi-tion of 12% sucrose, without yeast	226.05	345.42	124.87	296.65	7.90	3.48	304.42	1 744.31	3 053.1

The highest concentration of volatile compounds, expressed as a sum of acetaldehyde, ethyl acetate, isoamyl acetate, n-propanol, 2-methyl-1-propanol, n-butanol, 2-methyl-1-butanol and 3-methyl-1-butanol (5214.48 mg·dm^{-3} spirit 100% v·v^{-1}) was determined in distillate obtained from plum pulp-based wort, fermented with participation of microorganisms present on the raisins surface. Concentrations of these compounds measured in the remaining distillates ranged between 2927.29 mg (syrup after candisation of plums, with addition of 6% sucrose, *S. bayanus*) and 4853.99 mg·dm^{-3} spirit 100% v·v^{-1} (plum pulp, with addition of 6% sucrose, *S. bayanus*).

Apart from numerous valued components, plum distillates also contains some undesirable ingredients, among which methanol has a special place. It is produced during hydrolysis of pectin substances under the influence of the specific pectolytic enzymes, pectin methylesterase in particular. A certain amount still has to be present in natural brandies in order to maintain the authentic fruit origin [Nikicevic and Tesevic 2005].

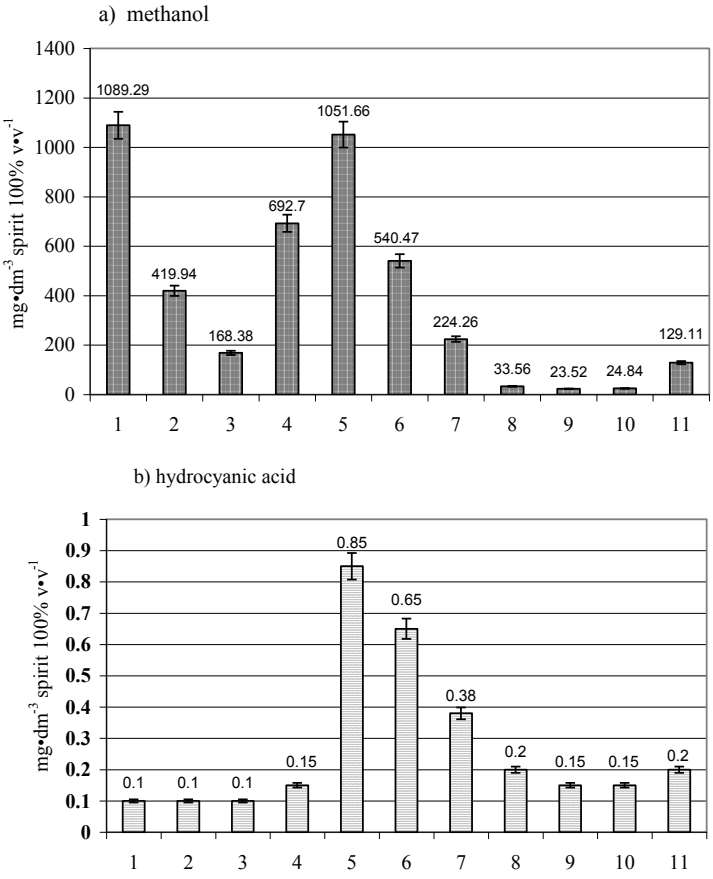
Methanol does not directly affect the product's aroma, because it has no specific odour. However, it is subjected to restrictive control owing to its high toxicity [Apostolopoulou et al. 2005, Tuszyński 1989]. According to EU directives for plum brandies, the concentration of methanol must not exceed 12 g·dm^{-3} spirit 100% v·v^{-1} [EC Regulation 110/2008].

Methanol contents in the tested samples of plum distillates differed significantly and varied over a wide range. The lowest amount was found in distillate obtained from syrup after candisation-based wort, supplemented with 6% of sucrose and fermented with wine yeast *S. bayanus* (23.52 mg·dm^{-3} spirit 100% v·v^{-1}), while the highest (1089.29 mg·dm^{-3} spirit 100% v·v^{-1}) in distillate originated from plum pulp-based wort, without addition of sucrose, fermented with wine yeast *S. bayanus* (Fig. 2). Satora and Tuszyński [2008] also found diversified concentrations of methanol in tested plum brandies, ranged from 346 mg·dm^{-3} spirit 100% v·v^{-1} in Slovakian Slivovica to 8741 mg·dm^{-3} spirit 100% v·v^{-1} in Śliwowica Łącka. The high methanol levels were probably associated with improper separation of heads, in which large amounts of the compound can be distilled. It is commonly known that methanol forms azeotropes and also transfers to the main fraction as well as to tails.

Products of cyanogenic glycoside decomposition are important components of the aroma of some alcoholic beverages. These constituents occur mainly in alcoholic beverages produced from stone fruits, lending them the characteristic aroma of bitter almonds. Fruits of *Prunus* genus plants (plums, cherries, sweet cherries, apricots, peaches, etc.) contain amygdaline in the stones and prunasine mainly in the vegetative parts of the plants.

Cyanogenic glycosides present in plants are relatively nontoxic until HCN is released. In an intact plant these compounds are accumulated in the cell vacuoles. Therefore, they are spatially separated from specific β-glucosidases which are located in the apoplast. Crushing the plant materials either by means of technical processes or chewing by animals results in cell disintegration and initiates the enzymatic hydrolysis of cyanogenic compounds by β-glucosidases (EC 3.2.1.21) resulting in the formation of sugars and cyanohydrin. Cyanohydrins (α-hydroxynitriles) can decompose spontaneously or in the process of enzymatic reaction catalyzed by hydroxynitrile lyase (EC 4.1.2.37) resulting in the formation of a ketone or an aldehyde and HCN [Brimer 2001].

Regulation (EC) No 110/2008 of both the European Parliament and the Council on the definition, description, presentation, labelling and the protection of geographical indications of spirit drinks, stipulates that the maximum hydrocyanic acid content in stone fruit spirits and stone fruit marc spirits shall amount to 7 grams per hectolitre of 100% v·v^{-1} alcohol (70 mg·dm^{-3}). The tested plum distillates contained very small amounts of HCN, ranging from ca. 0.1 to 0.85 mg·dm^{-3} spirit 100% v·v^{-1} (Fig. 2).



1. Plum pulp, without addition of sucrose, S. bayanus; 2. Plum pulp, 6% of sucrose, S. bayanus; 3. Plum pulp, 12% of sucrose, S. bayanus; 4. Plum pulp, 12% sucrose, without addition of yeast; 5. Plum concentrate, without addition of sucrose, S. bayanus; 6. Plum concentrate, 6% of sucrose, S. bayanus; 7. Plum concentrate, 12% of sucrose, S. bayanus; 8. Syrup after candisation, without addition of sucrose, S. bayanus; 9. Syrup after candisation, 6% of sucrose, S. bayanus; 10. Syrup after candisation, 12% of sucrose, S. bayanus; 11. Syrup after candisation, 12% of sucrose, without addition of yeast

Fig 2. Concentrations of undesirable compounds in obtained plum distillates

Conclusions

The results demonstrated that the concentrations of volatile compounds in the obtained plum distillates exceeding 200 grams per hectolitre of 100% $v \cdot v^{-1}$ (i. e. 2000 $mg \cdot dm^{-3}$) and low content of undesirable compounds (methanol and hydrocyanic acid) meet the requirements of the Council Regulation (EEC) No 110/2008 (repealing Regulation No 1576/89). Moreover their good aroma and flavour makes tested intermediate products of plum processing such as plum pulp, plum concentrate as well as syrup after candisation attractive raw materials for plum distillates production.

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4

PHENOLIC COMPOUNDS IN FERMENTED APPLE JUICE: EFFECT OF APPLE VARIETY AND APPLE RIPENING INDEX

Introduction

Apples are a highly favoured fruit to their unique flavour characteristics. Apple juice is the raw material of different fermented drinks, like apple wine and cider. Polyphenols is an important quality indicator in fermented drinks related to the color, bitterness and astringency, whose balance defines the overall mouthfeel of the beverage [Lea and Drilleau 2003, Alonso--Salces et al. 2004] and have been shown as the main contributors to the antioxidant activity of most beverages. The polyphenolic profile of apples and apple drinks is influenced by several factors: variety, climate, maturity, storage, processing [Van der Sluis 2001, Ruiz--Rodriguez 2008, Lata, 2007]. In apples, concentrations of polyphenols depend strongly on their cultivar, but in apple juices depends from processing technology [Eberhardt et al. 2000].

Polyphenols in apples can be classified into five major groups, i.e., hydroxycinnamic acids (5-caffeoylquinic acid and 4-p-coumaroylquinic acid) show the highest contents, flavan-3-ols which includes the monomeric (catechin) and polymeric forms (procyanidins), the latter constituted by (-)-epicatechin units; flavonols, dihydrochalcones and anthocyanins present in the apple peel [Tsao et al. 2005, Oszmianski and Wojdyło, 2008]. Also Wojdyło et al. [2008] conducting studies with new and old apple varieties and determined that flavanols (catechin and oligomeric procyanidins) are the major class of apple polyphenols, representing more than 80%, followed by hydroxycinnamic acids (1–31%), flavonols (2–10%), dihydrochalcones (0.5–5%), and in red apples, anthocyanins (1%). New varieties of apple had the same or higher value of bioactive compounds in comparison to the old varieties.

Biotransformations of cider apple fruits have to be carried out when they satisfy certain technological quality criteria, fruits have to be processed at their optimum maturity state, i.e., the time when they present an adequate chemical composition, which is responsible for their organoleptic and nutritional properties, as well as for the characteristics of their derived products [Rosa et al. 2004]. Apple producers follows the apple physical and chemical characteristics, because both of them are essential in the fermented beverage preparation − juice yield, flavour, sugar, acids phenolic etc. composition.

Not all ciders are made from ‘true’ cider apples. Many modern ciders have a high proportion of dessert and culinary apple varieties [Lea 1995]. In this work, special attention is drawn to the use of culinary apples for cider production. The apple variety ‘Lietuvas Pepins’ and ‘Auksis’ is popular commercially grown varieties in Latvia and are used as a culinary apples for juice production. The apple variety ‘Remo’ and ‘DI-93–4-14’ is new perspective apples varieties for Latvian growers decently for juice and wine production. While the scrab apple variety ‘Kerr’ is grown in small areas on the Latvian farms, it is characterized by a clear, fragrant juice with considerable tannins contents, suitabe for cider.

The aim of current research was to determine influence of apple variety and apple ripening index on the total phenolic compounds to fermented apple juice.

Materials and Methods

Raw materials

For experiments five variety apples ('Auksis', 'Lietuvas Pepins', 'DJ-41–7', 'Remo' and 'Kerr') grown in the Latvia State Institute of Fruit Growing and harvested in September and October 2010 were used.

Juice was obtained by mechanical press Voran Basket Press 60K (Voran Maschinen GmbH, Austria) after harvesting, and also after storage (relative humidity 90–95% and temperature +3±1ºC) of apples (two and four weeks) and yield was expressed as L kg $^{-1}$. Apple ripening index were determined before juice processing. For stabilization of juice 'Tannisol' (Enartis, Italy) was added (concentration 10 g 100 L^{-1}). Tannisol capsules consist of potassium metabisulphite (added amount to juice – 9.5 g 100 L^{-1}), ascorbic acid (0.3 g 100 L^{-1}) and tannin (0.2 g 100 L^{-1}). Fruit composition was assessed two and four week after the storage. Firmness was measured with a digital penetrometer TR 53 205 with 10 diameter probe (FT327), according to standard LVS NE 1131:2001 and expressed kg cm $^{-2}$. Soluble solids content (°Brix) was measured at 20°C with a digital refractometer ATAGO N20 according to ISO 2173:2003. The starch index was determined using a 0,1N iodine and potassium iodine solutation (scale 1–9). Streif index was calculated as follows:

$$Streif index = \frac{F}{SSC \times SI};$$

where
F – firmness, kg cm $^{-2}$,
SSC – soluble solids content, °Brix,
SI – starch index.
Physical and chemical parameters of apples are presented in Table 1.

Fermentation conditions

Fermentation was performed using commercial yeast *Saccharomyces bayanis* EC-1118 (Lalvin, Canada). The EC-1118 strain is recommended for all types of wines, including sparkling, and cider. Fermentation was carried out at 16±1°C for 28 days. The apple juice was fermented in a glass bottles (n=5). For analysis the average juice samples were combined from 5 bottles in equal proportions.

Determination of total polyphenolic contents by the Folin-Ciocalteu method

The total phenolic concentration was determined spectrophotometrically according to the Folin-Ciocalteu colometric method. Apple juice and fermented apple juice was diluted with ethanol/acetic acid solution (1:20 v/v). Ethanol/acetic acid solution was prepared using acetic acid water solution (2.5%) and ethanol (98% vol.) in ratio 10:90 (v/v). 0.5 ml of aliquet was mixed with 0.25 ml Folin-Ciocalteu reagents, after 3 minutes 1 ml 20% Na_2CO_3 and 3.25 ml distilled water were added. Samples were heated for 10 min. at 70°C and kept for 30 minutes at 18 ±2°C temperature. The absorbance was measured at 765 nm using a spectrophotometer

JENWAY 6300. Total phenols were expressed as gallic acid equivalents (mg L^{-1}). Each determination was performed in triplicate and results are expressed as mean ±SD.

Statistical analysis

Each determination was performed in triplicate and results are expressed as mean ±SD. Analysis of variance was perfoemed by ANOVA procedure and $p<0.05$ was considered statistically significant. A linear correlation analysis was performed with the software SPSS 14.00 for Windows.

Results and discussion

Characterization of apple juices

Apple composition is important for further apple processing. The juice yield, starch index, soluble solids, firmness and Streif index for the studied apple varieties are presented in Table 1. Steif index is an indirect measurement of the maturity, which is widely used by commercial growers in European countries, and is a combination of three fruit attributes: firmness, soluble solids content and starch index [Peirs et al. 2005]. In analysed apple varieties Streif index varied between 0.09 to 0.24 and it increased during storage. Juice yield varied from 51.9 to 72.5 L kg^{-1} of apples. Juice yield differed depending on Streif index, but there is not negative or positive correlation between these parameters. Higher juice yield for apples variety 'Kerr', 'Lietuvas Pepins' and 'DJ 93–4-14' were observed. Firmness and soluble solids content are two important internal quality attributes in determining fruit maturity and harvest time, and in assessing and grading post-harvest quality of apples [Peng and Lu 2008]. Contents of soluble solids ranged from 11.0 to 13.5 Brix%. 'DJ 93–4-14' was the variety with the highest content of soluble solids. Comparing soluble solids content depending on storage duration of apples, it is not possible to define one tendency, because for varieties 'Auksis', 'Lietuvas Pepins' soluble solids decreased during storage, whereas for 'Kerr' and 'Remo' apples it increased after two weeks of storage and then after four weeks decreased but for apple variety 'DJ 93–4-14' juice decreased after 2 weeks and increased after 4 weeks. The onset of ripening is associated with the conversion of starch into sugar during the pre- harvest and post harvest period of fruit [Hatfield and Knee 1998]. Firmness of apples decreased during storage, depending on the apple variety. The firmness of apples ranged between 12.0 to 16.9 kg cm^{-2} after harvesting, from 10.0 to 15.8 kg cm^{-2} after two weeks storage and from 8.8 to 13.3 kg cm^{-2} after four weeks storage. Fruit softening is induced by modifications of the cell wall pectic and hemicellulosic fractions, as the result of the combined action of several cell wall-modifying enzymes [Goulao et al. 2008].

Table 1

Physical and chemical parameters of apples

Parameters	Kerr			Auksis			Lietuvas Pepins			Remo			DJ 93–4-14		
	I	II	III	I	II	III	I	II	III	I	II	III	I	II	III
Juice yield, L kg $^{-1}$	69.1	72.5	69.3	73	60.3	51.9	63.3	65.2	68.9	65.4	61.9	66.4	65.6	70.4	63.2
Starch index [scale 1–9]	9	9	9	8.4	9	9	6.2	7.9	8.6	6.6	6.9	8.7	6.9	8.1	8.8
Soluble solids, Brix %	11.8	12.9	10.8	12	11.3	11.3	11.2	11	11	12	12.3	12	13.3	13	13.5
Firmnes kg cm $^{-2}$	16.2	15.8	12.9	15	13.4	8.8	16.9	12	9.7	12	10	9	16.5	15	13.3
Streif index	0.15	0.14	0.13	1.2	0.13	0.09	0.24	0.14	0.1	0.15	0.12	0.09	0.18	0.14	0.11

Total phenols of apple juices

The content of total phenols varied from 51.69 to 150.24 mg L^{-1} for the apple juices depending on variety and storage duration (Fig. 1). Total phenol content in analyzed samples was higher than reported Falguera et al. [2008], respectively, 2.55–10.57 mg L^{-1}.The highest content of total phenols in variety 'Kerr' juice was observed. 'Kerr' belongs to scrab apple varieties and are suitable for cider fermentation. These results are in accordance to Sanoner P. et al. [1999], study results show the polyphenol concentration is a higher in cider varieties than dessert apples. The total phenols content of apple varieties 'Kerr', 'Auksis' and 'Lietuvas Pepins' decreased by increasing apple storage time. While opposite tendencies for total phenol changes in variety 'Remo' and 'DJ 93–4-14' apple juices were observed. The lowest content of total phenols in apple variety 'Remo' juice obtained after two weeks storage of apples before juice pressing was determined. Whereas in apple variety 'DJ 93–4-14' juice after two weeks storage of apples before juice pressing the highest content of total phenols was determined. It is in accordance with results reported by Oliveira et al. [2011] who observed that both unripe and ripe fruits displayed similar content of total phenols, but the intermediate stage of ripening presented significant higher amounts of total phenols, almost doubling the former compounds in their composition.

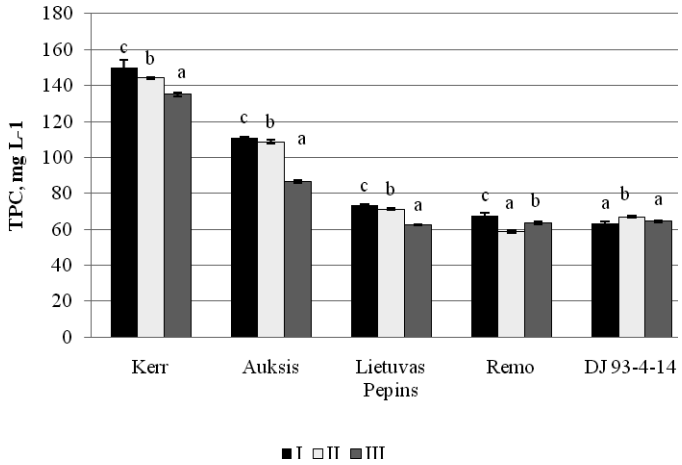

Fig. 1. Content of total phenolic compound in apple juices

Total phenols of fermented apple juices

The content of total phenols in fermented apple juices varied between 27.78 mg L^{-1} to 92.32 mg L^{-1} (Fig. 2) and it was 1.1 to 2.3 times lower than in corresponding apple juice. These results are in accordance to Berrueta et al. [2006], who reported polyphenol concentrations ranged from 0.28 to 217 mg L^{-1} in the ciders from French apples, and from 21 to 512 mg L^{-1} in ciders from Galician apples. But in other study Alonso-Salces et al. [2005] observed total polyphenol content of Basque ciders 200–1200 mg EC L^{-1} and it is approximately, one-half of the content in French ciders (300–3800 mg EC L^{-1}). The highest content of total phenols was in apple variety 'Kerr' juice and ranged between 69.90 mg L^{-1} to 92.32 mg L^{-1} depending on apple storage duration before pressing. Higher total polyphenol content was in all samples, which were fermented using juice pressed immediately after harvesting and after two weeks of storage.

Conclusions

This study revealed the great influence that apples varieties and harvest ripening have on the content of total phenolic compounds in fermented apple juice. In this particular case five apples variety juice were fermented using the three different processing times, depending on the apples storage period. In this sense, several aspects, such as apple varieties, apples maturity basic contents and total phenolic contents in apples juices and fermented apples juices, were analyzed. The phenol content of the fermented juice is higher in samples with higher phenolic content in raw materials. The polyphenol content is a higher in cider apples varieties than dessert apples varieties. Apples ripening seem to have impact on phenolic compound in juice.

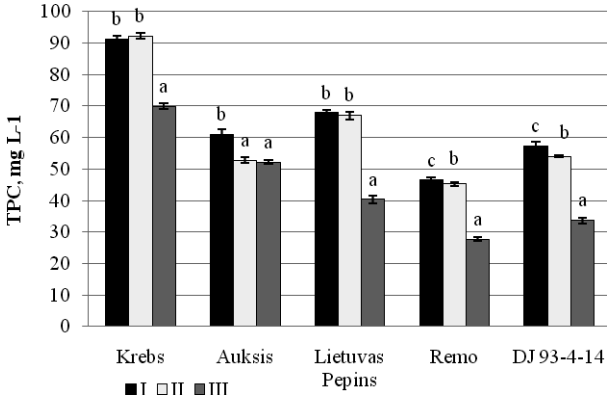

Fig. 2. Content of total phenolic compound in fermented apple juices after 28 fermentation days

These results are necessary for apple growers and processors, to enable optimum fruit harvesting and processing chain, which is, both the apple physical characteristics (firmness, juice yield) and chemical (soluble solids, phenolic compounds).

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5

INFLUENCE OF BIOTIN DEFICIENCY ON BIOMASS GROWTH, RISING POWER AND CHEMICAL COMPOSITION OF SELECTED STRAINS OF BAKER'S YEAST

Introduction

Biotin (vitamin H) is essential for all living organisms. This compound is a cofactor for a few essential enzymes of the carboxylase family (EC 6.4.1.-). Biotin is required for the biosynthesis of fatty acids and the metabolism of amino acids and carbohydrates. Some organisms, including higher plants, most fungi and bacteria, are prototrophic for biotin. Others, including most vertebrates and most of industrially employed *Saccharomyces cerevisiae* are biotin auxotrophs. The main substrate used in production of baker's yeast – beet molasses is low in biotin and it's analogues like desthiobiotin and aminopelargonic acids. Lack of this compounds may result in a more than 15 percent of decrease in the biomass yield of the commercial stage propagation, decrease of the rising power of baker's yeast were also observed [Lipińska 2001, Moszczyński and Pyć 1998, Oura 1974, Oura and Suomalainen 1978, Patelski and Szopa 2004, Szopa 1976]

One of more desirable features of baker's yeast, besides basic technological features, is low requirement for growth factors, including B-group vitamins. Industrial *Saccharomyces cerevisiae* strains are predominantly auxotrophic for biotin, and a lot of researches on vitamin biosynthesis and technological features of *S. cerevisiae* strains improvement were made [Patelski 2004, Rose and Harrison 1970, Stecka and Piasecka-Jóźwiak 1997, Suomalainen et al. 1965, Suomalainen and Keranen 1963, Szopa ans Ołtuszak 1987].

Aim of this work was to evaluate the influence of biotin deficiency on growth, chemical composition and rising power of selected strains of baker's yeast.

Methods and Materials

5 baker's strains of *Saccharomyces cerevisiae* designed as: R, D, W, B, P were used in trials. Saccharomyces cerevisiae "Y" – biotin-independent strain of *S. cerevisiae v. sake* and *S. cerevisiae "225"* – auxotrophic for biotin, used for its microbiological determination were used as reference. All strains were obtained from Pure Cultures Collection of the Institute of Fermentation Technology and Microbiology (Technical University of Lodz).

Propagation of strains in biotin-free medium (1) [Oura 1974] was evaluated. As a reference, cultivations in molasses medium (2) with/without biotin addition were also made.

Cultivations were carried out in 1L flasks containing 150 mL medium with use of reciprocal shaker for 48h at 30±1°C. Biomass concentration was determined gravimetrically.

Rising power, total protein, ash and phosphorus content were tested according to methods described in Polish Standards [PN-A-79005:1997].

All assays were carried out at least in triplicate. Statistical analysis (analysis of variance, determination of SD, Student's t test at the significance level a = 0.05) was carried out by using Origin 7.5 computer program.

Table 1

Media com

Biotin free medium (1) [Oura 1974]:	
Solution I:	
$(NH_4)_2Fe(SO_4)_2*6H_2O$	70 mg
$(NH_4)_2SO_4$	23.8 g
KH_2PO_4	6.45 g
salt A*	10 mL
salt B*	1 mL
distilled water	to 1 600 mL
Solution II:	
glucose	100 g
distilled water	to 400 mL
Ca- pantothenate	12.5 mg
mesoinositol	250 mg
tiamine	10 mg
pirydoxine	12.5 mg
nicotinic acid	10 mg
***) Salt A:**	
KCl	47.7 g
NaCl	25.5 g
$CaCl_2*2aq$	36.7 g
$MgCl_2*6aq$	20.9 g
distilled water	2 000 mL
Salt B:	
$MnSO_4*H_2O$	21.65 g
$CuSO_4*5H_2O$	2 g
$ZnSO_4*7H_2O$	9.85 g
$Na_2MoO_4*2H_2O$	13 mg
H_3BO_3	29 mg
KJ	7
$NiSO_3*7H_2O$	11.05 mg
distilled water	2 000 mL
Both solutions were mixed after preparation 20.4 potassium ftalate ($C_8H_5KO_4$) were added. pH of the medium was adjusted to 5.2±1 and sterilized by filtration through 0.22µm Millipore filter.	
Molasses medium (2):	
beet molasses solution 7.5°Blg	1 000 mL
$(NH_4)_2S0_4$	4 g
$(NH_4)_2HPO_4$	3 g
biotin (as an option)	20 µg
pH=5.5	
Sterilisation: thermal 121°C/20 min.	

Results and Discussion

Biomass yield of yeast strains after 48 h cultivation in biotin free-medium was shown at the Figure 1. Biomass yield after 48 h of cultivation in biotin-free medium ranged from 0.49 g d.m./L (strain W) to 0.76 g d.m./L (strain R), while for reference strain "225" biomass yield was 0.52 g d.m./L. Second reference – Strain Y was distinguished by sevenfold higher biomass gain (3.6 g d.m./L) as observed for industrial strains after cultivations in biotin-free medium. It can be clearly seen from Figure 1 that *S.cerevisiae* "Y" posses low requirement for biotin and may be called "biotin independent". Much lower yields observed for the rest of the strains suggest that, as predicted, none of tested industrial baker's strains had ability to biotin synthesis. Similar values of biomass yield in biotin free medium may be observed in researches described by Szopa and Ołtuszak [1987] and Szopa [1976] and also in results obtained by Patelski [2004] and Patelski and Szopa [2004].

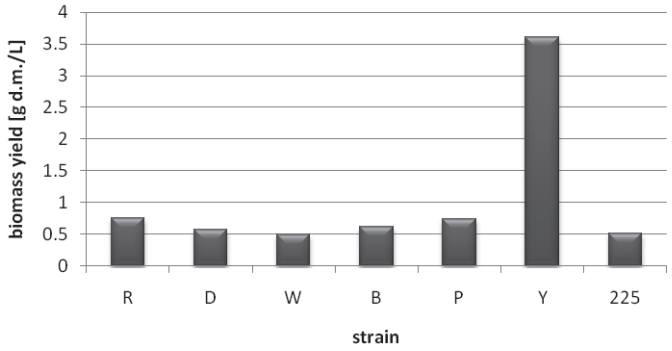

Fig. 1. Biomass yield of yeast strains after 48h cultivation in biotin free-medium

More important from the technological point of view is the biomass yield in molasses medium. Yield of the 48h shaken cultures of tested strains cultivated in molasses medium are shown in the Figure 2. Cultures were grown with (20 µg/L) and without biotin addition to molasses medium.

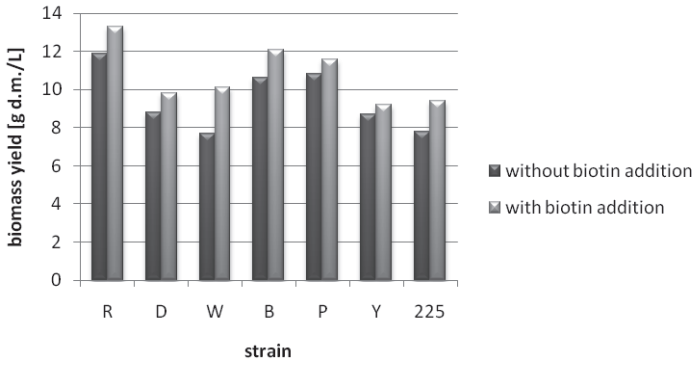

Fig. 2. Biomass yield of the 48 h shaken cultures of tested strains cultivated in molasses medium with and without biotin addition

After cultivation in molasses medium biomass yields for tested strains were less differentiated and varied from 7.67 g d.m./L to 13.3 g d.m./L. According to Figure 2 it can be seen that biotin addition increased the biomass yield. This phenomenon was observed for all strains and is only a confirmation of the data known from the literature [Patelski 2004, Patelski and Szopa 2004, Rose and Harrison 1970, Suomalainen et al. 1965, Suomalainen and Keranen 1963, Szopa and Ołtuszak 1987, Szopa 1976]. The strongest impact of the biotin on the growth was observed for W strain. The biomass yield after biotin addition increased from 7.67 g d.m./L to 10.1 g d.m./L (31% of biomass gain). Lowest biomass gain in a response to biotin addition was observed for biotin-independent "Y" strain – 3.4%. Results of the biomass yield are in range closely correlated with those obtained by Szopa [1976], Szopa and Ołtuszak [1987] and Patelski [2004] and Patelski and Szopa [2004].

One of the most important factors describing suitability of *S.cerevisiae* strains in bakery is rising power. This parameter describes ability of the yeast to act as a dough-leavening agent. From the scientific point of view this parameter may be expressed as sum of the three times needed to rise the dough for the known height when incubating the dough in tin (the baking tin dimensions and the dough recipe are standarised) or may be determined with use of fermentograph – measuring CO_2 emerging during dough fermentation [PN-A-79005:1997]. Total rising time of the dough prepared with yeast obtained with or without biotin addition is shown in the Figure 3.

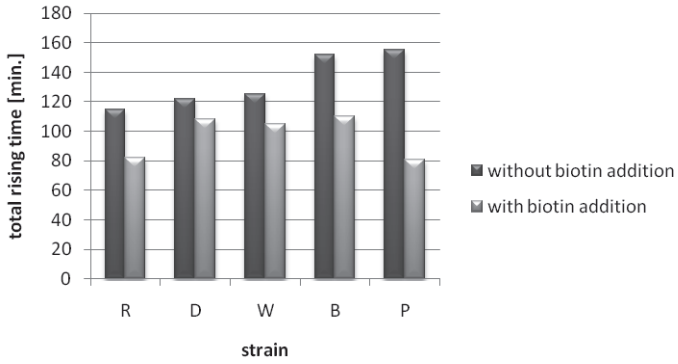

Fig. 3. Total rising time of the dough prepared with yeast obtained with or without biotin addition

It can be seen from the fig. 3 that biotin had a big impact on the rising time of the dough prepared with use of all tested strains.

Rising time values varied from 81 to 108 min. for biomass obtained with biotin addition (20 µg/l) and from 115 to 155 min. for unsuplemmented cultivations. The biggest difference in rising power was observed for strain P where biotin deficiency resulted in elongation of the rising time from 81 to 155 min. Similar results were obtained by Szopa and Ołtuszak [1987] and also find confirmation in our earlier investigations [Patelski 2004, Patelski and Szopa 2004].

Due to impact of protein content on the biological persistence ("shelf life") of the compressed baker's yeast the total protein content (by Kjeldahl's method) was determined and the results are shown in the Figure 4.

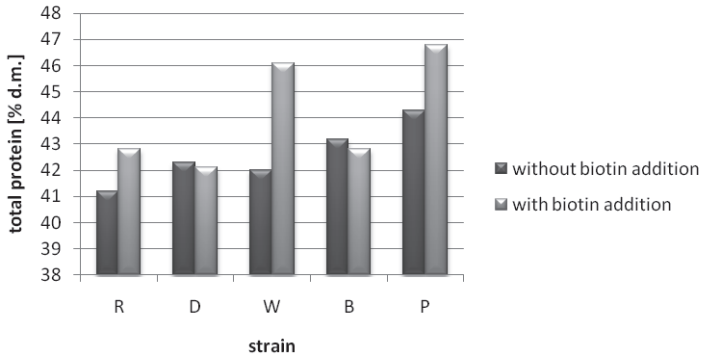

Fig. 4. Total protein content in the yeast biomass obtained with and without biotin supplementation

Total protein content varied between 41.2 and 44.3% d.m. for biomass cultivated without biotin addition and was in the range 42.1 to 46.8% d.m. for biomass obtained with biotin supplementation. It may be observed that there is slight tendency of potentiation of the protein synthese in the biotin abundance conditions.

To evaluate more detailed influence of the biotin deficiency on the yeast biomass cultivation and it's chemical composition, total minerals (ash) and phosphorus (as P_2O_5) were also tested. Results are shown in the Table 2.

Table 2

Total minerals and phosphorus content in the yeast biomass obtained with or without biotin addition

strain	Ash [% d.m.]		Phosphorus (P_2O_5) [% d.m.]	
	without biotin	with biotin	without biotin	with biotin
R	5.8	5.5	2.8	3.3
D	5.2	6.4	3.4	3.2
W	5.7	5.2	3.3	3.6
B	6.1	5.8	3.8	3.2
P	5.3	5.9	3.2	2.9

Minerals content varied between 5.2 and 6.1% d.m. for biomass cultivated without biotin addition and was in the range 5.2 to 6.4% d.m. for biomass obtained with biotin supplementation. Phosphorus content expressed as phosphorus pentoxide varied between 2.8 and 3.8% d.m. for biomass obtained without biotin and ranged from 2.9 to 3.6% d.m. for biomass obtained with biotin. However there are slight differences between strains in accumulation of phosphorus and minerals in total but close correlation between those values and biotin supplementation was not observed. Results of phosphorus, protein and total minerals content obtained in this work are typical to baker's yeast biomass obtained during laboratory cultivations, and were presented in many works [Lipińska 2001, Patelski 2004, Rose and Harrison 1970, Szopa and Ołtuszak 1987].

Conclusions

Biotin and it's some analogues seem essential for growth of the baker's yeast and it was proved in the technology of baker's yeast. Biotin participates in yeast metabolism in many reactions: carboxylation of pyruvate, in the synthesis of nucleotides, in nucleic acid synhesis, in protein and polysaccharides synthesis and in the synthesis of fatty acids.

Results of biotin defficiency like yield drop, protein content and rising power decrease observed for biomass obtained without biotin supplementation may be clearly elucidated by the involvment of the biotin-dependent enzymes in the crucial metabolic pathways.

Results obtained during investigations described above find confirmation in data presented in the literature [Lipińska 2001, Patelski 2004, Rose and Harrison 1970, Stecka and Piasecka-Jóźwiak 1997, Szopa and Ołtuszak 1987, Szopa 1976]. From our point of view those results are very important because helped us to select yeast strains distinguished by the properties we expect from the baker's strains of *Saccharomyces cerevisiae*. Selected strains may be used in the future for obtaining new yeasts with use of somatic hybridization with other strains possessing features complementary to yeasts described in this work.

Acknowledgements

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6

OBTAINING OF SACCHAROMYCES CEREVISIAE HYBRIDS FOR FERMENTATION OF SUGAR BEET THICK JUICE WORTS

Introduction

Ethanol is expected to be one of the dominating renewable biofuels within the coming 20–30 years. It can be easily obtained from sugar beet juices. Due to high sugar concentration, reduced storage volume and microbial stability it is more suitable to ferment worts prepared from concentrated sugar beet juice. Production of ethanol from sugar beet is not only easy way to obtain the sustainable transport fuel but also enables to activate local sugar beet growers and gives work in sugar factories. Industrial ethanol production is strongly dependent on yeast activity. *Saccharomyces cerevisiae* is one of the first domesticated organisms, and many centuries of use resulted in selection of strains optimized for specific applications, although technologist still are looking forward for new strains with improved technological properties. For over 50 years of breeding with use of mass mating of haploid cells with subsequent zygote selection, hybridization is often used as a tool to obtain a new yeast's strains. This method is quite labour – intensive but still in use due to the lack of disadvantages of the modern methods of yeast improving resulting in creating strains with new DNA not verified in nature [Akada 2002, Bertolini et al. 1991, Miklos and Spiczki 1991, Naumov et al. 2006, Randez-Gill et al. 1999, Sato et al. 2002, Stecka and Piasecka-Jóźwiak 1997, Szopa 1980, Szopa and Ołtuszak 1987, Szopa et al. 1990, 1993, Takano et al. 1994].

Aim of this work was to obtain and select *Saccharomyces cerevisiae* hybrids intended for fermentation of sugar beet thick juice-based worts.

Materials and Methods

65 haploid clones of distillery yeasts from Pure Cultures Collection of the Institute of Fermentation Technology and Microbiology (Lodz Technical University) were used as a biological material. Haploids were obtained according to the procedure of Johnston and Mortimer by using incubation in sporulation media (5) and digesting asci with enzymatic extract of Helix pomatia [Johnston and Mortimer, 1959]. Spores were isolated from tetrads with micromanipulator by method described by Fowell [1969]. Agar film with isolated spores were transferred on the surface of solidified YPG (2) medium and incubated for 48 h at 30°C. Mating type of isolated yeast clones were determined on solidified YPG medium after mixing with marker strains possessing known mating type.

First step of the hybrids obtaining was to select parental haploid strains possessing desired features of distillery yeast. Criteria for selection of haploid clones were: morphological features, ability to fermentation and assimilation of selected sugars (glucose, galactose, maltose,

sucrose, lactose, melibiose), ability to assimilate glycerol. The most important feature expected from haploid strains was the ability to conduct vigorous fermentation of high density worts with high ethanol yields and it was also checked during fermentation of the wort (1) in test tubes equipped with Durham tubes (Table 1).

Fermentation of the wort

Initial selection of haploids suitability for fermentation of thick juice worts was carried out in the test tubes filled with wort (1) and equipped with Durham tubes. After 20 hours from inoculation, level of the gas in the Durham tubes was evaluated and strains possessing strongest fermentative ability were selected for further examinations.

Alcoholic fermentations of wort with use of selected haploids were carried out in 1 l flat-bottomed flasks, containing 0.5 L of thick juice-based medium (1). Worts were inoculated with 2 g d.m./L of yeast cream. The flasks were plugged with stoppers, equipped with fermentation pipes to avoid aeration. Fermentations were conducted for 5 days at 30°C±2. Fermentation progress was controlled gravimetrically based on the fact that decrease in the mass of the fermenting sample was related to emission of carbon dioxide.

Ethanol concentration

Separation of the ethanol from worts after fermentation was carried out by using the distillation unit. Ethanol concentration was assayed in distillates by means of hydrometer scaled in ethanol volumetric %.

Hybridisation and hybrids separation

Hybrids were obtained by using "mass matting" method based on the yeast life-cycle and natural affinity of haploid *Saccharomyces cerevisiae* cells possessing opposite matting types to matting and zygotes formation. Zygotes were isolated on agar films with use of micromanipulator equipped with glass needle – Figure 1.

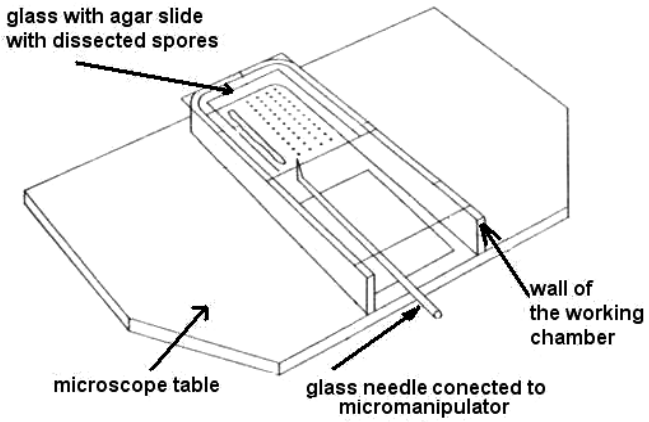

Fig. 1. The main part of the micromanipulation set used to tetrad's dissection and zygotes separation

Films with separated zygotes were immediately transferred on the solidified YPG medium and were incubated in 30°C to form diploid colonies. Hybrids were transferred on agar slants and selected in further steps of this work.

Criteria in selecting hybrid strains were morphological features, sporulation, ability to ferment and assimilate selected sugars (glucose, galactose, maltose, sucrose, lactose, melibiose), ability to assimilate glycerol. Dynamics of fermentation of 25°Blg worts (1) prepared from sugar beet juice were also tested.

Table 1

Media composition

Fermentation medium (1)	
thick juice obtained during sugar beet processing tap water $(NH_4)_2HPO_4$ $MgSO_4*7H_2O$ $ZnSO_4*7H_2O$ pH 4,8 sterilization – 121°C/20 min.	to obtain 25°Blg wort to obtain 25°Blg wort 0,2 g/L 0,01 g/L 0,005 g/L
YPG medium (2)	
yeast extract bacto-peptone glucose distilled water agar (for solidified forms) pH 5,2 sterilization – 121°C/20 min.	10 g/L 10 g/L 20 g/L to 1 L 30 g/L
Assimilation ability medium (3)	
base mineral medium prepared according to Lodder and Kreger van Rij [1971]. selected sugar or glycerol: sterilization: filtration (Sartorius 0,22 μm)	 10 g/L
Fermentation ability medium (4)	
yeast extract selected sugar distilled water pH=5.5 sterilization: 121°C/21 min.	10 g/L 20 g/L 1 L

Table 1 cont.

Media composition

Sporulation media (5)	
Presporulation:	
glucose	20 g/L
yeast extract	5 g/L
$(NH_4)_2SO_4$	3 g/L
KH_2PO_4	3 g/L
distilled water	1 L
pH = 5.5	
sterilization: 121°C/21 min.	
Sporulation:	
potassium acetate	10 g/L
yeast extract	2,5 g/L
glucose	0,2 g/L
agar	25 g/L
distilled water	1 L
pH = 6	
sterilization: 121°C/21 min.	

Statistical analysis (analysis of variance, determination of SD, Student's t test at the significance level a = 0.05) was carried out by using computer program Origin 7.5. All assays were carried out in triplicate.

Results and discussion

Haploids selection

65 haploid clones of *Saccharomyces cerevisiae* with known mating type were tested for: morphological features, ability to fermentation and assimilation of selected sugars (glucose, galactose, maltose, sucrose, lactose, melibiose), ability to assimilate glycerol. All tested strains were able to assimilate glucose and sucrose. 11 haploids were not able to assimilate galactose. 12 clones were not able to assimilate maltose. None strain was able to assimilate lactose. Only 6 monospore clones were able to assimilate melibiose. Glycerol was not assimilated by 10strains. Fermentation patterns were similar. For further tests 34 strains were selected – all able to assimilate and ferment: glucose, galactose, maltose and sucrose.

Fermentation and assimilation properties are typical for *Saccharomyces cerevisiae* distillery strains and find confirmation in the literature [Pielech-Przybylska and Balcerek 2008, Szopa et al. 1990]

The most important feature expected from haploid strains was the ability to initiate and conduct vigorous fermentation of high density worts – test tubes filled with wort (1) and equipped with Durham tubes were used to evaluate this feature and the results are shown in Figure 2.

Method with use of Durham tubes cannot provide a detailed information about fermentation properties of the strain but seems fair good when selecting large amount of yeast strains. 8 strains distinguished among haploids by vigorous fermentation (filling of Durham tubes and strong fizzing) were selected for further evaluation during fermentation of thick juice wort in 1L-flask fermentation scale.

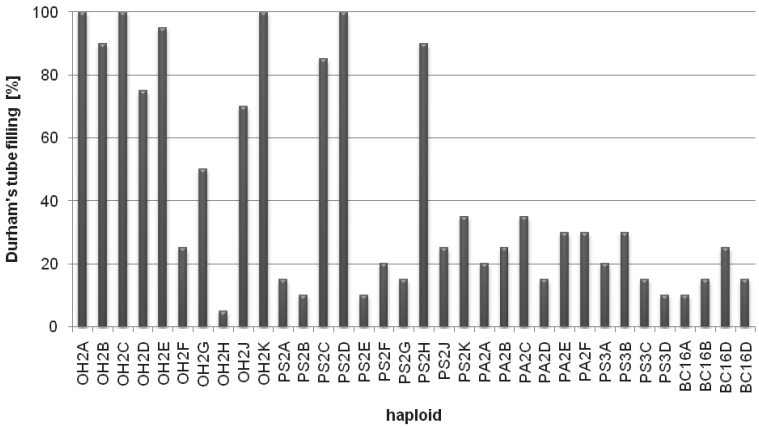

Fig. 2. Durham tube filling after 20h fermentation of thick juice wort with use of selected haploids

Results of fermentation yield after 5 days of fermentation are shown in the Figure 3. Dynamics of fermentation was controlled gravimetrically based on the fact that decrease in the mass of the fermenting sample is related to carbon dioxide formed from sugars during fermentation.

Fermentations of thick juice diluted to 25°Blg enriched with mineral salts and nitrogen (1) were carried out for 5 days at 30°C. Initial phase of fermentations lasted for approximately 48–72 hours and ethanol yield approached 55÷86% of the theoretical. Those results are close to those obtained by Szopa et al. [1990] 4 best fermenting haploids – PS2D, OH2J, OH2C and OH2K possessing desired features were selected to obtain fusants.

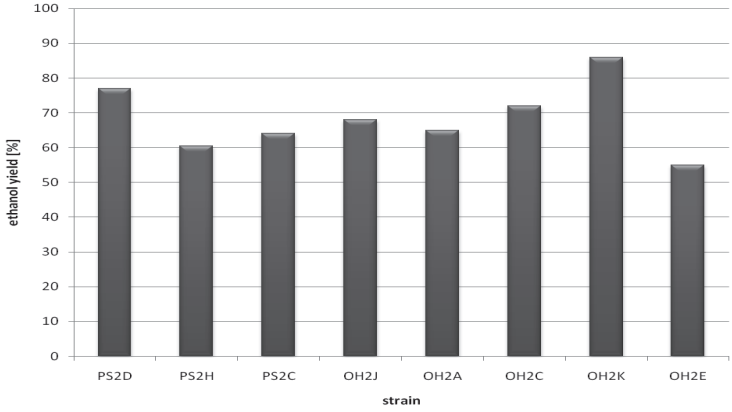

Fig. 3. Ethanol yield after 5 day fermentation of thick juice wort with use of selected haploids

Hybridisation and initial fusants selection

Hybrid strains were obtained with use of "mass mating" method [Fowell 1969, Szopa et al. 1990, Szopa 1980]. According to mating types – strain PS2D ("alpha") was mixed with OH2J("a") or with OH2K("a") or with OH2C("a" mating type). Parent strains, mating scheme and names of hybrids are given below in Table 2.

Table 2

Mating scheme of parent strains and hybrids designations

parent 1, type "alpha"	parent 2, type "a"	hybrid designation	isolated strains
PS2D	OH2j	1P2J…	26
PS2D	OH2K	1P2K…	14
PS2D	OH2C	1P2C…	18

Zygotes were isolated with use of micromanipulator equipped with glass needle. After incubation (30°C/48h) hybrids were transferred to agar slants. 58 hybrids were isolated for evaluation.

Criteria in selecting hybrid strains were: morphological features, ability to ferment and assimilate selected sugars (glucose, galactose, maltose, sucrose, lactose, melibiose), ability to assimilate glycerol. Dynamic of fermentation of 25°Blg wort (1) prepared from sugar beet juice and sporulation ability in appropriate medium (4) were also tested. All strains were able to assimilate glucose and sucrose. 5 hybrids were not able to assimilate galactose. 10 were not able to assimilate maltose. None strain was able to assimilate lactose and melibiose. Glycerol was not assimilated by 14 strains. Fermentation patterns were similar. 7 strains unable to sporulate were removed from selection process. For further tests 22 hybrids able to assimilate and ferment: glucose, galactose, maltose and sucrose were selected.

Assimilation and fermentation ability may be subject of changes even as a reason of long-term storage of the strain so it's hard to compare the results with obtained by others but it may be stated that the results obtained in this work are closely correlated to obtained by Szopa et al. [1990] and are typical for *Saccharomyces cerevisiae* strains according to Lodder and Kreger van Rij [1971].

The main aim of whole work was to obtain new strains with ability to conduct vigorous fermentation of high density wort. To evaluate this feature test tubes filled with wort (1) and equipped with Durham tubes were used and the results are shown in Figure 4.

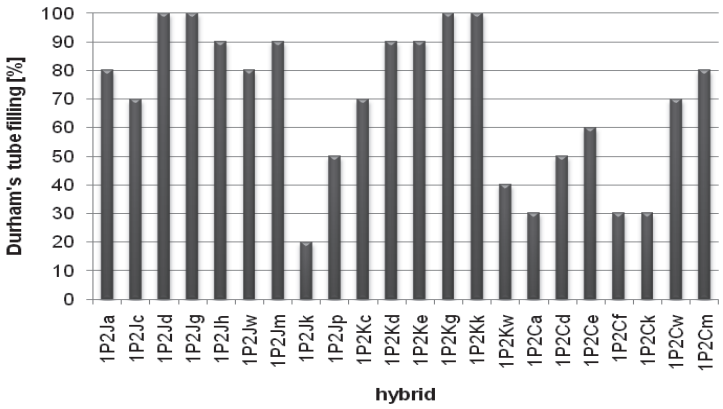

Fig. 4. Durham tube filling after 20h fermentation of thick juice wort with use of selected hybrids

11 hybrids were distinguished by vigorous fermentation in test tubes and were selected for tests in larger-scale fermentations of thick juice 25°Blg wort (1) in 1L flasks. Ethanol yield after 5 days of fermentation of sugar beet juice medium (1) in 30°C is shown in Figure 5.

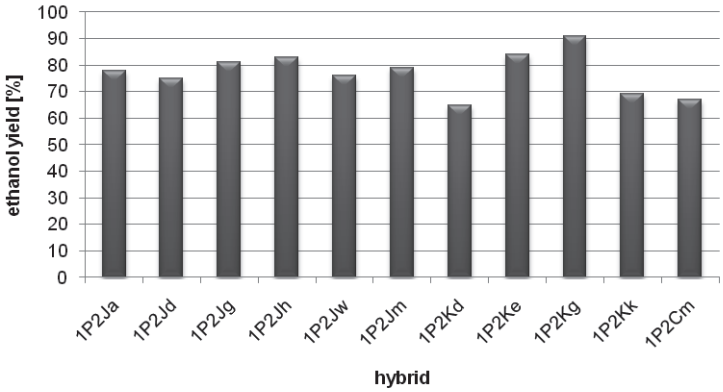

Fig. 5. Ethanol yield after 5 day fermentation of thick juice wort with use of selected haploids

It can be seen from the Figure 5 that fermentation yield for 8 strains exceeded 75%. The lowest value – 65% was observed for hybrid 1P2Kd; highest yield, equal to 91%, was obtained after fermentation with use of 1P2Kg hybrid. It may be seen in the literature [Pielech-Przybylska and Balcerek 2008, Szopa et al. 1993] that fermentation yield in the range 80–90% is fair good in laboratory-scale fermentations Fermentation course is as much important as its total yield therefore the weight-loss (relative to CO_2 liberation) was also tested. Initial phase of fermentation observed for 6 strains was shorter than 45 hours. Duration of initial fermentation phase corresponds with results obtained by Pielech-Przybylska and Balcerek [2008] – when using D2 distillery yeast during 25°Blg fermentations of sugar beet thick juice-based worts.

Conclusions

Hybridization is very time-consuming and labour-intensive multi-step process of strains improving however, if carefully done, leads usually to awaited goals. During this work a few new strains with ability to carry vigorous fermentation of thick juice-based worts were obtained. Those results need to be proved in next steps of studies, including quarter- or half-technological scale at the distillery. Sometimes only scale-enlargement induces such amounts of unexpected problems that such verification is a necessary step during obtaining new strains useful in the real industry.

The most perspective strains were then selected for detailed investigations during larger-scale fermentations in our quarter industrial scale distillery.

Acknowledgements

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7

THE EFFECT OF SUPPORTIVE ENZYMES ON HIGHER ALCOHOLS SYNTHESIS DURING RYE MASHES FERMENTATION

Introduction

The activities of α-amylase and glucoamylase are absolutely essential for the starch distilling industry. Apart from starch the cereals contain many compounds (proteins, non-starch polysaccharydes, phytates), which can be used by yeasts in the fermentation process after previous enzymatic hydrolysis. It seems legitimate to use the enzymes, which could support the degradation of the compositions above mentioned, for example protease and phytase.

The ability of degradation of the proteins into amino acids is a common feature of protease, which are carriers of nitrogen necessary for the proper action of the yeast. Distillery yeasts are not proteolytic and hence cannot break down proteins [Russel 2003].

The protein content in cereal grains is about 10% of its dry weight. Therefore the release of nitrogen from natural resources can improve the yeast nutrition; it accelerates their multiplication and improves the dynamics of fermentation, thereby reducing the risk of bacterial infections. The increase of ethanol efficiency is the measurable effect. The enzyme manufacturers have introduced the proteolytic enzymes both as the separate preparations and as a component in the saccharification preparations.

The phytic acid and phytates are the major storage forms of phosphorus in plant seeds [Ciereszko et al. 2009], for example cereals (rye, triticale, wheat, corn) used in distilling industry (accounting for 18–88% of the total phosphorus present in seeds) [Chichester 1982, Kumek 2008, Wikiera 2004]. The quantity of phytate phosphorus will depend on the conditions of vegetation, the method of fertilization, harvest date, etc. [Lee et al. 2000, Łambucki 2008].

The phytic acid has a large chelating potential [Duliński and Żyła 2009, Russel 2003, Żebrowska and Ciereszko 2009]. It can form a wide variety of insoluble salts (phytates) with divalent and trivalent metal cations (Ca, Mg, Fe, Zn, Cu), both within a single phosphorus group and between the two phosphorus groups (the same or different molecules). The phytic acid has also the ability of bonding amino acids, proteins and starch [Kumek 2008].

The feed manufacturers and their customers, namely livestock farmers, have expressed their interest in the phytates for many years. Phosphorus in fact, is essential for the proper functioning of living organisms. It is a component of many organic compounds: phospholipids, nucleic acids, ATP [Kumek 2008]. The phytate phosphorus is also very important for the environment since it aims to minimize the adverse feed ingredients excreted in the faeces to the environment, especially of the phosphorus compounds [Kapela 2007].

Phytates are also attractive for the distilling industry. Phosphorus is an essential element for the yeast, being present in the DNA, RNA, phospholipids, and phosphorylated compounds (ATP, glucose-6-phosphate) [Russel 2003].

Magnesium is the most significant intracellular divalent cation in yeast. The multidirectional biological activity of magnesium is associated with the activation of numerous enzymes involved in the synthesis of proteins, nucleic acids, the lipids metabolism and thermoregulation [Błażejak et al. 2002].

The presence of magnesium ions in the base (for example distilling mash) stimulates and prolongs the life of yeast by stabilizing the plasma membrane, mitochondrial membranes, ribosomes and remaining the proper chromosome structure [Walker 1994, Walker and Birch 1996]. It exerts a protective effect on the yeast cultures subjected to the stress conditions such as the temperature and osmotic pressure, as well as playing a role in alcohol tolerance.

Calcium stimulates the growth of yeasts but the most important is its participation in the proper activation of the liquefaction enzyme – thermostable bacterial α-amylase which forms characteristic enzyme complexes with calcium ions, ensuring a high enzyme activity and stability of the structure [Radzanowski 2003, Kapela 2007]. Zinc is the element required for proper growth and it acts as the cofactor of many enzymes of reactions taking place inside the cell of yeast. One of the most important enzymes involved in the etanol fermentation – the alcohol dehydrogenase is the metalloenzyme containing just zinc [Jelski et al. 2006]. Its deficiency may therefore lead to low biomass growth and slowing of ethanol fermentation [Russel 2003, Russel 1997].

Copper and iron ions are also the essential nutrients for yeast, which act as cofactors in enzymes including the redox pigments of the respiratory chain [Russel 2003].

The enzymatic hydrolysis of phytic acid and phytate by phytase is the solution of the above mentioned. It catalyzes the removal of all six phosphate groups, with releasing orthophosphate groups to the fermented medium and the associated metal ions, amino acids and starch. The activity of this enzyme (of microbial origin) is widely used in the feed industry (used commercially in swine and polutry feed to reduce the need for inorganic phosphorous and to improve the digestion of proteins and minerals) [Konieczny-Janda et al. 2001]. There are three types of phytase: an endogenous-enteric, feed native and exogenous-microbial. The plant phytase is present almost in all grains. However, there are significant differences in its activity. The highest phytase activity characterizes cereals: rye, triticale and wheat (over 1 200 $U \cdot kg^{-1}$) [Fretzdorff and Weipert 1986, Lee et at. 2000, Żebrowska and Ciereszko 2009], the lowest – grain corns in turn (below 0.2 $U \cdot kg^{-1}$) [Lee et al. 2000]. However, the microbial phytases attract the greatest attention, due to the high catalytic activity, and in case of bacterial enzymes – to the high thermal stability and resistance to proteolysis [Kapela 2007]. The use of phytase seems to be justified in distilling industry. In addition, the hydrolysis of phytate can improve the quality of spent-wash significantly, increasing its nutritional value.

An objective of the presented study was the determination of the effect of supportive enzymes: protease and phytase on dynamic fermentation of rye mashes and synthesis of higher alcohols. The scope of this study embraced:

– physicochemical analysis of raw materials (rye variety Dańkowskie Diament),
– preparing of sweet mashes by pressureless starch liberation (PLS) method,
– controlled fermentations of mashes,
– gas chromatography analysis of mashes before, during and after fermentation.

Materials and Methods

The following materials were used throughout the study:

1. Grain of winter rye variety Dańkowskie Diament, purchased from "Danko" Plant Breeding Ltd.,
2. Distillery yeast *Saccharomyces cerevisiae* – strain I-7–43, selected in the Institute of Biotechnology of Agriculture and Food Processing in Warsaw, purchased from the Yeast Factory in Maszewo Lęborskie (Poland).
3. Commercial enzyme preparations: Termamyl S.C. (α-amylase, EC 3.2.1.1), SAN Extra L (glucan 1,4-α-glucosidase, EC 3.2.1.3), Neutrase 0.5L (*Bacillus* neutral protease) and Shearzyme 500L (endo-1,4-β-xylanase) were purchased from Novozymes (Denmark). Ronozyme P-(L) preparation (phytase) was purchased from DSM Nutritional Products (USA).

Preparing of sweet mashes

Sweet mashes were prepared by using a cylindrical vessel, which was placed in a water bath and equipped with a double blade impeller and a thermometer.

Prior to mashing, rye grains (concentration of starch was 66.6%) were ground in a disk mill to particles with dimensions below 1.5 mm. Then 3.5 L of water was added per each 1 kg of ground rye grains and the temperature of this suspension was gradually increased to 90°C with continuous agitation. Simultaneously, a starch-liquefying enzymatic preparation (Termamyl S.C.) was added (according to manufacturer's recommendation: 0.13 $mL \cdot kg^{-1}$ starch) and the first portion of xylanase (Shearzyme 500 L – 0.1 $mL \cdot kg^{-1}$ starch). Starch liquefaction was carried out for 90 minutes. Then the temperature of the hydrolysate was decreased to 66 ÷ 67°C and it was supplemented with a saccharifying enzyme preparation (SAN Extra L) in a dose recommended by its manufacturer (0.6 $mL \cdot kg^{-1}$ starch) and the second portion of xylanase (0.1 $mL \cdot kg^{-1}$ starch).

Neutrase 0.5L (0.05 $mL \cdot kg^{-1}$ starch) and Ronozyme P (0.09 $mL \cdot kg^{-1}$ starch) preparations were added to rye mashes on completion of starch liquefaction and after addition of saccharifying enzyme, and adjustment of temperature of mash to 50°C. Then the mash was cooled to the temperature at which fermentation was started (30°C).

Yeast cream preparation

Fermentation processes were conducted by using dried distillery yeast (strain I-7–43) in a dose of 0.3 g d.m.$\cdot L^{-1}$ of sweet mash. To eliminate microbial contaminations, portions of dried yeasts suspended in small water aliquots were acidified with approximately 30% sulfuric acid to pH ≈ 2.5 and kept for 15 ÷ 20 minutes.

Fermentation

Alcoholic fermentation was carried out in 8 L laboratory fermenter containing 5.5 L of mash. pH was adjusted to 4.8 and then the mash was supplemented with diammonium phosphate (0.2 $g \cdot L^{-1}$) and inoculated with portions of yeast cream in a dose of 0.3 g d.m.$\cdot L^{-1}$ of mash. Fermentation was carried out at 28–30°C for 72 h.

Gas chromatography

During fermentations mashes were quantitatively analyzed for higher alcohols content such as: n-propanol, 2-methyl-1-propanol (isobutanol), 2-methyl-1-butanol (amyl alcohol), 3-methyl-

-1-butanol (isoamyl alcohol) and n-butanol using headspace gas chromatography (Agilent 6890N, USA) equipped with flame-ionisation detector (FID) and Split/Splitless injector.

During fermentations 10 mL of mash sample was take and transferred into a headspace vial and closed tight. Vials were termostated with 50°C for 30 min.

Headspace analyzer conditions were as follows:

– pressurization time 0.2 min.
– loop fill time 0.2 min.
– loop equilibration time 0.05 min.
– injection time 0.7 min.
– loop temperature 65°C
– transfer line temperature70°C.

GC conditions were as follows:

– injector: splitless (200°C),
– detector: FID (250°C),
– oven: initial temperature program was 40°C. This temperature was held for 6 min and then ramped up 2°C·min^{-1} to 90°C, then increased 10°C·min^{-1} to 200°C and held for 2 min at this temperature,
– flow rate of carrier gas (helium) through the column: 2 mL·min^{-1}.

Separation was performed on capillary column HP-Innowax (Agilent) with polyethylene glycol phase (60 m x 0.32 mm I.D. x 0.5 μm film thickness).

Results of this analysis are shown in Table 1 presenting concentrations of higher alcohols reported as mg per L of mash.

Determination of ethanol and extract concentration

During fermentations, the mashes samples (after filtration) were analyzed for ethanol concentration by using an aerometer with scale in % v·v^{-1} of ethanol. Ethanol was collected by distillation of 100 mL of mash in a laboratory system consisting of 500 mL distillation flask, Liebig cooler, 100 mL volumetric flask (used to collect ethanol) and a thermometer.

The sweet and after fermentation (after distillation of ethanol) mash extracts were measured by using an aerometer with scale in Balling degrees (°Blg; it refers to the concentration of dissolved solids, mostly sugar, as the weight percentage of sucrose or maltose).

Results of this analysis are shown in Table 1.

Evaluation of fermentation

The efficiency of fermentation EF (the percentage yield of sugar consumption during fermentation) was calculated as a ratio of sugars used during fermentation to their content in the mash prior to this process and expressed in %. The yield of ethanol synthesis was calculated in relation to total sugars presented in sweet mash and expressed as the percentage (%) of theoretical yield.

Table 1

Contents of ethanol and higher alcohols in fermenting rye mashes with application of different supportive enzymes

VARIANT	COMPOUNDS	CONCENTRATION								
		Time of fermentation [h]								
		0	**17**	**21**	**25**	**41**	**45**	**49**	**65**	**72**
WITHOUT PROTEASE AND PHYTASE	Ethanol [% v·v^{-1}]	0	1.80	2.92	3.11	6.05	6.33	6.41	8.74	8.76
	n-propanol [mg·L^{-1}]	nd	6.09	5.99	6.75	8.37	8.87	9.16	9.87	9.95
	Isobutanol [mg·L^{-1}]	nd	35.69	45.50	57.57	87.64	89.14	96.59	109.17	111.56
	n-butanol [mg·L^{-1}]	nd	nd	nd	nd	0.04	0.15	0.06	0.09	0.11
	Amyl alcohols[1] [mg·L^{-1}]	nd	58.52	77.38	97.89	159.49	168.15	181.31	203.45	205.86
Extract of sweet mash – 17.2°Blg; Real extract of mash after fermentation – 4.6°Blg; EF – 85.9%; Ethanol yield (% theoretical) – 95.9%										
PROTEASE	Ethanol [% v·v^{-1}]	0	2.50	3.27	4.33	6.64	6.82	7.28	7.53	7.97
	n-propanol [mg·L^{-1}]	nd	8.66	11.32	12.52	19.49	22.58	23.21	27.47	28.05
	Isobutanol [mg·L^{-1}]	nd	43.51	79.88	104.30	162.47	178.50	182.05	217.68	206.13
	n-butanol [mg·L^{-1}]	nd	nd	nd	nd	0.03	0.06	0.06	0.12	0.12
	Amyl alcohols[1] [mg·L^{-1}]	nd	54.30	87.34	107.70	175.89	196.00	200.98	226.56	227.58
Extract of sweet mash – 17.0°Blg; Real extract of mash after fermentation – 5.8°Blg; EF – 86.0%; Ethanol yield (% theoretical) – 96.8%										
PHYTASE	Ethanol [% v·v^{-1}]	0	1.73	2.13	2.74	4.66	4.86	5.54	6.73	7.44
	n-propanol [mg·L^{-1}]	nd	6.89	7.11	7.41	7.66	7.78	7.85	8.74	10.41
	Isobutanol [mg·L^{-1}]	nd	23.12	31.23	35.18	61.49	63.76	65.90	80.09	95.32
	n-butanol [mg·L^{-1}]	nd	nd	nd	nd	nd	0.01	0.02	0.10	0.17
	Amyl alcohols[1] [mg·L^{-1}]	nd	38.01	54.51	61.11	126.49	138.09	139.22	174.54	208.97
Extract of sweet mash – 17.3°Blg; Real extract of mash after fermentation – 6.3°Blg; EF – 79.9%; Ethanol yield (% theoretical) – 79.4%										
PROTEASE AND PHYTASE	Ethanol [% v·v^{-1}]	0	2.73	3.64	4.26	6.34	6.76	7.24	7.58	7.77
	n-propanol [mg·L^{-1}]	nd	7.24	6.95	8.51	13.38	14.79	15.51	22.34	22.70
	Isobutanol [mg·L^{-1}]	nd	49.54	60.22	75.98	128.71	136.51	173.11	176.47	177.08
	n-butanol [mg·L^{-1}]	nd	nd	nd	0.02	0.09	0.11	0.14	0.28	0.29
	Amyl alcohols[1] [mg·L^{-1}]	nd	67.92	81.76	104.54	183.54	196.65	203.64	247.93	248.54
Extract of sweet mash – 17.1°Blg; Real extract of mash after fermentation – 4.3°Blg; EF – 87.9%; Ethanol yield (% theoretical) – 81.8%										

[1] sum of 2-methyl-1-butanol and 3-methyl-1-butanol; nd – not detected; EF – efficiency of fermentation

Results and Discussion

The research shows that the use of protease and phytase enzymes affects significantly the dynamics and efficiency of fermentation and higher alcohols content. Using of protease affects the liberation of amino acids to the mash – the yeast-assimilable nitrogen. In both variants, which the protease was used in, the fermentation efficiency exceeded 96% (protease) and 81% (protease with phytase) of theoretical yield and in the first case only it was comparable to the yield of the fermentation mash without of the supportive enzymes: protease and phytase – 95.9% of theoretical yield. The protease favourably affects on the dynamics of fermentation compared with fermentation mash without tested enzymes: in both samples with a protease, concentration of ethanol after 24 h of fermentation was about 4.3% $v \cdot v^{-1}$ – ie about 38% more in relation to the control sample. The combined effect of phytase and protease had a positive impact on the dynamics of fermentation, both in relation to the sample with phytase and without tested enzymes (control sample).

The results of GC analysis of mashes during fermentation showed significant differences in the concentrations of higher alcohols. Their content in mashes after 72 hours of fermentation was the highest in the samples with protease: 461.9 $mg \cdot L^{-1}$ mash (protease) and 425.9 $mg \cdot L^{-1}$ mash (protease with phytase). In comparison with the control fermentation, the increase of concentration in the mash with protease, concerned n-propanol, isobutanol and amyl alcohols, by 182, 85 and 11% respectively. The increase of all determined higher alcohols, in turn, was observed in the sample where the protease activity combined with phytase had been applied.

The proper yeast nutrition, the acceleration of their multiplication, and consequently alcoholic fermentation carried out dynamically represents the measurable effect of a protease activity as a supportive enzyme. However, the presence of higher concentrations of fusel oils, in relation to the control sample without protease, is the adverse phenomenon. According to the theory of Ehrlich yeasts released ammonia from molecules of amino acids and incorporated them into yeast proteins while the higher alcohols resulting from this metabolic process.

The solution might be found in the reduction or elimination of mashes supplementation with the inorganic nitrogen source. However, the tests of mash fermentations with protease without the mineral nitrogen addition carried out so far (unpublished data) have not succeeded to reduce the content of higher alcohols, in relation to the sample with its participation.

Considering the effect of phytase on yeasts growth and fermentation efficiency it has been noted that the lowest, in relation to other variants, ethanol yield (79.4% of theoretical) was in case of mash fermentation with the presence of phytase and it approached to the efficiency of mash fermentation with both protease and phytase. The results also showed a slight decrease of the higher alcohols concentration, in relation to the control sample. A similar relationship has been observed while comparing the two trials, with using: protease and protease with phytase. The release of calcium ions into the mash, as one of the possible effects of enzymatic degradation of phytate, could inhibit the uptake of amino acids and reduce the efficiency of ethanol fermentation in case of excessive concentrations (above 1 mM and 25 mM respectively) [Russel 2003].

Conclusions

Taking into account the theory of Ehrlich, a certain pattern can be observed in case of the higher alcohols synthesis during the fermentation of mashes, with the application of the protease as a supportive enzyme. The presence of high concentrations of amino acid nitrogen, released by the action of proteases, increases the fusel oil content in fermented mashes and consequently also in the spirits. The use of exogenous phytase (of microbiological origin) seems to be an attractive proposal for the distilling industry, especially in pressureless liberation of starch technology with simultaneous saccharification and ethanol fermentation of native starch and in pressure cooking method. Optimum temperature of cereal phytases ranged from between 45–55°C [Chichester 1982]. Some strains of yeasts have also an ability of phytate degradation, due to the presence of intercellular and extracellular phytase activity. The first positive results for the presence of phytase in yeast brewing were received by Nuobariene et al. [2011].

Yeast requires a number of inorganic ions for optimum growth and fermentation. Important cations are zinc, manganese, magnesium, calcium, copper, potassium and iron. The role played by many of these ionic species is both enzymatic and structural. For example, a proper ratio between calcium and magnesium positively influences fermentation rates. Greater participation of Ca ions in these proportions is detrimental to yeast growth and ethanol productivity through Ca antagonism of essential biochemical functions of Mg [Russel 2003].

Supportive enzymes can greatly increase spirit production. However, their application should be conducted with great care and knowledge of their mechanism of action and possible adverse events which may happen, resulting even from the wrong proportions of metal ions released into the medium during the degradation of phytate.

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8

COW MILK CASEIN AS A SOURCE OF SELECTED BIOLOGICALLY ACTIVE PEPTIDES

Introduction

Many dietary proteins may be a source of biologically active peptides. These peptides, inactive within the sequence of the native protein, may be released both during digestion in the gastrointestinal tract and during food processing [Korhonen et al. 1998, Korhonen and Pihlanto-Leppälä 2001].

Molecules of bioactive peptides usually contain from 3 to 20 amino acid residues. Their effect on the body most frequently relates to the nervous, immune, cardiovascular and digestive systems [Haque and Chand 2008].

Food protein hydrolysates are commonly used as food supplements for persons requiring specific protein diet They are a valuable source of peptides with diverse biological activities. The type of activity manifested after hydrolysis is determined not only by precursor proteins and their primary structure, but also by the nature of the enzyme used in the hydrolysis. Many commercial food products containing bioactive peptides, available on the market in many countries, are often obtained from different milk proteins – casein and whey proteins. Their hydrolysis is usually carried out using commercially available proteolytic enzymes of animal (digestive enzymes: pepsin, trypsin and chymotrypsin), plant (papain, ficin, bromelain) or microbial origin. The technologies based on the use of these enzymes can have a significant commercial application associated with their low production costs.

Antimicrobial peptides

One of the most important, though rarely detected activity identified during the analysis of biologically active peptides, seems to be their antimicrobial activity. Peptides with antibacterial properties are considered to be an important component of the innate immune response, acting as the "first line of defense", especially in tissues such as lung mucosa or small intestine which often are exposed to a contact with pathogens. In recent years, particular attention has been paid to the antibacterial peptides derived from natural sources.

Until now, more than 500 different peptides with antibacterial activities have been identified and characterized after isolation from protein substrates of bacterial, mammal, or plant origin [Haque and Chand 2008]. These are mainly linear peptides, containing the residues of proline, arginine, and sometimes tryptophan residues, as well as disulfide bonds [Iwaniak and Minkiewicz 2007]. Lipophilic and hydrophilic properties, manifested both by molecules (amphiphilicity) and their positive charge are considered to be the primary factor in the interaction of peptides with biological membranes of bacteria. As has been shown, some milk-derived antimicrobial peptides have the ability to penetrate into the internal cellular structures [Haque and Chand 2008].

The antimicrobial activity of milk is associated mainly with the content of immunoglobulins, lactoferrin, lysozyme and lactoperoxidase and peptides produced as a result of their hydrolysis. As shown by Haque and Chand [2008], also casein may be an important source of peptides with antimicrobial properties. These peptides, derived from α_{s1}-casein, called caseicidins, were first identified in 1971 [Lahov et al. 1971]. They were obtained during casein hydrolysis by chymosin at neutral pH.

Isolated caseicidins showed activity against *Staphylococcus spp*., *Sarcina spp*., *Bacillus subtilis*, *Lactobacillus spp.*, *Diplococcus pneumoniae* and *Streptococcus pyogenes*. Another fragment of alpha$_{s1}$-casein (f1–23), known as isracidin demonstrated in vivo an antibiotic-like activity against: *S. aureus*, *Candida albicans*, *Streptococcus pyogenes* and *Listeria monocytogenes,* while *in vitro* it inhibited the growth of bacteria from the Lactobacilli and other Gram-positive bacteria. Isracidin given to dairy cattle with diagnosed mastitis exhibited antibiotic-like effects [Haque and Chand 2008].

Also α_{s2}-casein may be a source of peptides with antimicrobial properties. The first described peptide was caseicidin-I, positively charged fragment of f150–188 α_{s2}-casein, isolated from fermented milk. This peptide showed inhibitory effect on the growth of Gram negative bacteria, e.g. *E.coli*, and Gram positive bacteria, e.g. *Staphylococcus carnosus* [Pihlanto and Korhonen 2003, Haque and Chand 2008].

Antimicrobial properties are also observed for peptide isolated from α_{s2}-casein hydrolysate, i.e. fragment f 183–207 and f164–179, albeit the latter has a higher activity. This type of peptides was also identified in a rennin hydrolysate of the sodium caseinate. These are fragments from the C-terminal α_{s2}-casein: f181–207, f175–207 and f164–207. These peptides are active against many species of Gram (+) and Gram (-) bacteria. The pepsin hydrolysate was shown to have four other antimicrobial peptides: fragments f165–170, f165–181, f184–208 and f203–208 of α_{s2}-casein [Haque and Chand 2008].

An example of antimicrobial peptide derived from κ-casein is capacin. It is a carbohydrate-free phosphorylated form of caseinomacropeptide (CMP), which inhibits the growth of Gram (+) bacteria, e.g. *Streptococcus mutans* and Gram (-) bacteria, e.g. *Porphyromonas gingivals*. Another peptide with antimicrobial activity is a pentapeptide isolated from the trypsin hydrolyzate of κ-casein, known as κ-cathelicidin, a fragment f17–21 of the protein. This peptide inhibits the growth of pathogens such as *S. aureus*, *E. coli* and *S. typhimurium*. There were also reports of its cytotoxic activity against certain cancer cells, such as human leukemic cell line. Six other antimicrobial peptides are produced during the digestion of κ-casein, and the most active fragments are: f18–24, f139–146, f30–32, showing activity against *Listeria innocua*, *Staphylococcus carnosus* and *E. coli* [Haque and Chand 2008].

Despite the increasing number of studies on the antimicrobial activity of peptides derived from milk proteins, there are still few in vivo experiments using animal models or clinical tests on humans [Haque and Chand 2008].

Antioxidative peptides

The importance of oxidative processes in the human body and in food products is the subject of intensive research. Oxidation reactions in living organisms play an important role in old age diseases such as Alzheimer's disease, rheumatoid arthritis and atherosclerosis [Pihlanto 2006]. In food products, oxidative processes associated with oxidation of lipids cause deterioration of quality – rancid smell, unacceptable taste and shorter shelf-life.

Many peptides and protein hydrolysates can reduce the rate of lipid auto-oxidation. They also act as heavy metals acceptors and free radical scavengers. The antioxidative activity

of peptides released from different proteins is caused by the presence in their sequence the amino acid residues, such as histidine or tyrosine, known for their antioxidant properties. Similar properties are also shown by the residues of methionine, lysine and tryptophan. The amino acid composition, and the sequence and configuration of peptides, also have an impact on the antioxidant properties of peptides. The presence of proline residues increases the activity of the peptides and hydrophobic groups contribute to their interaction with the linolenic acid [Iwaniak and Minkiewicz 2007].

Recent studies have shown that hydrolysis of casein using digestive enzymes or by the action of proteolytic enzymes of lactic acid bacteria can produce antioxidative peptides [Korhonen and Pihlanto 2006]. A high ability to inhibit the oxidation of linolenic acid was observed for a fraction containing fragments: f169–176 and f33–48 of β-casein [Pihlanto 2006]. It is widely accepted that in the future antioxidant peptides may be used as additives to prevent oxidation of many food products, cosmetics and drugs. However, it needs to be found if peptides produced during fermentation or hydrolysis may prevent damage induced by oxidation in systems in vivo [Korhonen and Pihlanto 2006].

Anti-hypertensive peptides

One of the main risk factors of cardiovascular diseases is hypertension (e.g. in myocardial infarction, heart failure, etc.). Arterial blood pressure is partly regulated by the renin-angiotensin system [FitzGerald et al. 2004], which consists of angiotensin converting enzyme (ACE) and renin [Haque and Chand 2008]. The inhibition of ACE (dipeptidyl carboxypeptidase-I, EC 3.4.15.1) usually lowers blood pressure, but can also affect other regulatory systems, related to the immune system and nervous system [Haque and Chand 2008].

ACE is a multifunctional enzyme that occurs in various tissues and plays an important role in regulating blood pressure [Haque and Chand 2008]. ACE is an exopeptidase that removes the dipeptide from the C-terminus of various peptide substrates. It is a zinc metallopeptidase with broad substrate specificity *in vitro* [Ondetti and Cushman 1984]. Renin (angiotensinase, EC 3.4.23.15) affects angiotensinogen (plasma protein), an inert precursor, thereby releasing a decapeptide – angiotensin I. Angiotensin I is a substrate for ACE, which produces angiotensin II by removing the C – terminal dipeptide (HL) from angiotensin I. Then angiotensin II, by causing severe muscle contraction in small blood vessels, significantly raises blood pressure and increases heart rate. ACE also removes the C-terminal dipeptide of bradykinin (a vasodilator), resulting in the creation of an inactive peptide fragment.

The first reports of exogenous inhibitors of ACE concerned systems in vivo with observed pressure-lowering effect of snake bites [Ferreira et al. 1970]. ACE-inhibiting peptides in the venom, however, had limited application due to the lack of pharmacological activity when administered orally [Haque and Chand 2008]. There are many drugs in the market that are based on ACE inhibitors, e.g. captopril that regulates hypertension. However, synthetic drugs can cause some side effects, e.g. hypotension, increased potassium levels, reduced renal function, cough, rash, deformation of the fetus, and therefore bioactive peptides generated from casein and whey proteins can be an attractive alternative [Haque and Chand 2008].

ACE inhibitors derived from milk proteins include different fragments of casein and are called casokinins [Pihlanto and Korhonen 2003]. Casokinin sequences were found in all fractions of casein, particularly in the α_{s1}- and β-casein. ACE inhibitors were obtained during pepsin, trypsin or chymotrypsin hydrolysis of casein [Maruyama and Suzuki 1982, Maruyama et al. 1985, Maruyama et al. 1987]. These include fragments: f23–34, f23–27 and f194–199 of α_{s1}-casein and fragment f177–183 β-casein. Casokinins may also be produced during lactic fermentation.

Peptides released by the protease activity of lactobacilii are ACE inhibitors. In addition, two tripeptides with the characteristics of ACE inhibitors, Val-Pro-Pro and Ile-Pro-Pro, were obtained from the fermentation of milk using *Lactobacillus helveticus* and *Saccharomyces cerevisiae* [Pihlanto and Korhonen 2003]. Several peptides with the properties of ACE inhibitors have also been found in cheeses (parmesan, gouda, manchego, italico, gorgonzola) [Silva and Malcata 2005].

Anti-hypertensive properties of peptides derived from milk proteins have been subject only to a small number of clinical tests on humans. The ability of lowering blood pressure by milk fermented by *L. helveticus* and *S. cerevisiae* and containing two tripeptides VPP and IPP, was confirmed in patients with hypertension (the study conducted in Japan) [Hata et al. 1996]. This product is available under the trade name Calpis® (Calpis Co. Ltd, Japan). There have also been some clinical tests on some other drugs, with observed expected effect of lower blood pressure [Haque and Chand 2008].

Peptides as metal carriers

Some peptides act as biocarriers due to their ability to bind calcium or other minerals, thereby enhancing their bioavailability. Among them are phosphopeptides, with a strongly anionic character which makes them resistant to further proteolysis. Their negatively charged side chains, e.g. glutamic acid and aspartic acid, and in particular phosphate groups, are the binding sites for minerals. Phosphoserine residues are attributed the ability to chelate calcium by fragments of caseinophosphopeptides (CPPs) in systems in vitro [Pihlanto and Korhonen 2003]; dephosphorylated peptides do not have the ability to bind minerals [Sato et al. 1986]. The role of phosphoserine residues in binding minerals has been confirmed by studies which showed that the chemical phosphorylation of α_{s1}- and β-casein increases the binding capacity of Ca^{2+} [Yoshikawa et al. 1981]. Regardless of the presence of vitamin D, casein-derived phosphorylated peptides increase the calcification of bones in children and infants with rickets-related changes [Silva and Malcata 2005]. They may be very important in the prevention osteoporosis in adults.

Many CPPs have been identified in vitro in casein hydrolysates produced with the use of enzymes such as trypsin or alkalase, for example fragments: f43–58, f59–79 and f43–79 of α_{s1}-casein, fragments: f1–24 and f46–70 of α_{s2}-casein, and fragments: f1–28, f2–28, f1–25 and f33–48 of β-casein. Some CPPs were discovered in the fluid from the intestine fistula, which confirms their ability to last in the gastrointestinal tract. Caseinophosphopeptides may be also released by proteolytic enzymes of lactic acid bacteria during ripening of cheeses (Grana Padano, Comté) [Silva and Malcata 2005].

CPPs are capable of binding macronutrients such as Ca, Mg and Fe, as well as micronutrients – Zn, Ba, Cr, Ni, Co and Se. However, the calcium-binding capacity of casein phosphopeptide fractions may be different – the differences can be attributed to the activity of additional amino acids surrounding the phosphorylated binding sites [Silva and Malcata 2005].

Introduction of phosphopeptides as dietary supplements can also help reduce one of the significant nutritional problems – iron deficiency. Binding iron by phosphopeptides prevents the production of poorly digestible iron hydroxides with a high molecular weight. In vitro studies on rats have shown that binding iron by the phosphoserine residues of CPP of low molecular weight (fragment f1–25 of β-casein), compared with iron-binding capacity of casein alone or inorganic salts, improves the ability to overcome anemia and restores the ability to store iron in tissues [Ait-Oukhatar 1999]. In further studies on an in vivo model using radioactive iron, researchers determined the absorbed dose of this mineral by the tissue. It was shown that the iron was utilized for metabolic purposes [Ait-Oukhatar 2002].

It has been shown that binding to caseinophosphopetides (f1–25 fragment of β-casein) may also improve the absorption of zinc.

Although CPPs are used mainly due to their mineral-binding abilities, they are also attributed anticaries properties related to their ability to locate high concentrations of amorphous calcium phosphate on the tooth surface and consequently promote recalcification of enamel [FitzGerald 1998].

Caseinophosphopeptides do not have negative sensory characteristics and can therefore be used as anticaries additives [Shah 2000]. CPPs may also be used as buffering agents against the acidic environment of dental plaque. Thanks to these properties CPPs have become the ingredients of products for oral hygiene, especially toothpaste [FitzGerald 1998]. Currently, there are available other commercial products containing caseinophosphopetides, such as beverages, dietary supplements for children and sweets [Severin and Wenshui 2005]. The anti-caries effect may also be induced by the consumption of cheese, as there seems to be a significant correlation between high levels of casein in cheese and the protective effect against dental caries [Pause and Lembke 1993].

Some peptides derived from milk proteins may reveal more than one function. For example, peptides generated from the fragment f60–70 of β-casein are immunostymulators, opioids, and ACE inhibitors. Due to the high hydrophobicity and the presence of proline residues, the fragment is resistant to proteolysis. ACE-inhibiting peptides can also stimulate the immune system. Various reports shows that fragment f194–199 of α_{s1}-casein has an immunomodulatory activity and inhibitory activity against ACE. Also opioid peptides exhibit an ACE-inhibiting activity. An opioid antagonist peptide derived from kappa-casein, casoxin C, shows an activity against C3a receptors and phagocytosis-stimulating properties [Pihlanto and Korhonen 2003].

Conclusions

The development of many branches of the food industry significantly depends on the changing tastes and preferences of consumers, the introduction of modern technologies and also the search of attractive functional additives. Current trend among consumers is to buy healthy food and to reduce consumption of products containing significant quantities of synthetic additives such as preservatives or antioxidants. The search for the natural alternatives of such substances, those that could also enhance the value of food, focuses on the use of various biotechnological methods – one of them is enzymatic hydrolysis.

An analysis of production costs of many food products shows a great relative cost of functional additives. Taking into account the dominant position of foreign companies on the Polish market and high prices they charge for functional additives, it seems reasonable to seek easier and cheaper ways of obtaining them. Offering new preparations that could substitute commonly used synthetic preservatives would help promote domestic producers of the new generation of products.

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9

ENZYMATIC PROTEIN HYDROLYSATES AND THEIR USE IN FOOD AND NON-FOOD ITEMS

Introduction

In food processing, proteins are often subject to various modifications. The most practical and important in terms of quality of food products are enzymatic reactions, thanks to their mild conditions and easy control. Enzymatic reactions affect the conformation of molecules and the biological activity. Selective hydrolysis leads to improved functional properties of proteins, improving their organoleptic quality and nutritional value. Under the influence of proteolytic enzymes, proteins also release peptides that exhibit specific biological activity [Chrzanowska 1998, Zambrowicz and Trziszka 2010, Darewicz et al. 2000].

Dietary protein hydrolysates, mixtures of peptides with varying molecular weight and free amino acids are more easily absorbed by the human body than native proteins. They are widely used in the production of dietary supplements and foods for particular nutritional uses [Clemente 2000]. Hydrolysates with improved functional properties may be used as food additives, which may allow the preparation of new attractive products, e.g. gel foods, while others may be used as pharmaceuticals and functional food ingredients [Chrzanowska 1998].

Enzymatic hydrolysis of proteins

The hydrolysis of proteins is the cleavage of peptide bonds in chains of proteins, polypeptides or oligopeptides, into peptides and free amino acids using proteolytic enzymes. The rate of hydrolysis depends on many factors such as specificity, enzyme and substrate concentration, the rate of mass transfer in the E/S system, the degree of denaturation of proteins in the substrate, pH, temperature, reaction medium ionic strength, the presence of activators and inhibitors [Kołakowski et al. 2005]. The process of hydrolysis can take place via either of two mechanisms: "one-by-one" and "zipper". In the initial stage of the "one-by-one" reaction (i.e. a stage that determines the overall degree of hydrolysis), it is necessary to partially unfold native protein molecules allowing the enzyme access to the hydrolyzed peptide bond. As a result, the protein loses its stability and an increasing number of peptide bonds are exposed on the outside of the molecule (intermediates). In the next stage, these products are rapidly degraded to small peptides. As long as the reaction equilibrium is on the "native side," protein molecules are degraded one after the other, resulting in no significant quantities of intermediate products; there are only native proteins and final products. In contrast, in the "zipper" type reaction, native protein is rapidly converted into intermediate products. The limiting stage of the reaction is the slow hydrolysis of intermediate forms of protein into final products [Kunst 2003].

Proteolysis may be conducted in one- or two-step systems. One-step hydrolysis proceeds in a continuous manner, and the pH of the mixture changes spontaneously as a result of the

reaction, or is kept constant by the addition of acids or bases. During two-step hydrolysis, various enzymes are used in subsequent stages of proteolysis, most frequently exopeptidase first and endopeptidase later [Kolakowski et al. 2005].

Functional hydrolysates of food proteins

In food technology, one very important role of proteins is their participation in shaping the quality of food products [Korhonen et al. 1998]. Thanks to their properties such as solubility, emulsifying capacity, water and fat binding, formation of foams and gels, and participation in shaping the flavour of food, they improve the technological process and have a decisive impact on the organoleptic characteristics of the final product [Korhonen et al. 1998, Léonil et al. 2000]. Due to changes in the tertiary structure of proteins and molecular weight reduction [Vinnars and Wilmore 2003]. Most enzymatic modifications of proteins are carried out with the participation of enzymes such as pepsin, bromelain, trypsin, chymotrypsin, papain or ficain [Chrzanowska 1998]. The degree of hydrolysis has a decisive influence on organoleptic characteristics, and depending on the direction of use of the it may be beneficial to obtain a smaller or greater degree of degradation [Chrzanowska 1998]. Non-specific, high-degree hydrolysis may lead to the loss of functional properties and the appearance of bitter peptides [Chrzanowska 1998]. Limited hydrolysis may lead to radical changes in the physicochemical characteristics of the protein.

The coagulation of casein using chymosin consists of the cleavage of one peptide bond [Fen-105-Met-106] in the λ-casein, which leads to the precipitation of paracasein [Chrzanowska 1998].

Selective enzymatic hydrolysis is used, inter alia, to achieve the improved solubility, emulsifying and foaming properties of wheat gluten [Agyare et al. 2008] and fish protein [Shahidi et al. 1995]. Through hydrolysis reactions, some waste products obtained in industrial processes become a source of complete protein and can be used as ingredients in food products, such as byproducts of marine animals [Schwenke 1994, Vioque et al. 1999]. For example, hydrolysis using alkalase of proteins remaining after the production of rapeseed oil, leads to an improvement in emulsifying properties and water binding capacity. The subsequent application of endopeptidase facilitates hydrolysates with very high solubility [Schwenke 1994, Vioque et al. 1999].

A negative aspect of the enzymatic hydrolysis of proteins can be, for example, increased bitterness of foodstuffs. The bitter taste can be reduced to an acceptable level by means of ultrafiltration, activated carbon processing and plastein reaction [FitzGerald and O'Cuinn 2006, Gawędzki 2003].

Protein hydrolysates are currently sourced from a variety of products such as milk, eggs, fish, rice, legumes, meat, potatoes [Schaafsma 2009]. They have found application, among others, as ingredients increasing the share of nitrogen in specialty drinks, foods for pregnant women, nutritional and bactericidal ingredients in cosmetics, or dietary supplements. They can also be found in foods for people with an impaired ability to digest, foods for athletes, and in hypoallergenic products [Hernell and Lonnerdal 2003, Neklyudov et al. 2000, Schaafsma 2009]. In addition, hydrolysates are also useful as a nutrient medium in order to improve the growth of microorganisms or the production of recombinant proteins [Duarte de Holanda and Netto 2006, Quitain et al. 2001].

Bioactive peptides and their application

Diet has a vital role in shaping human health. Bioactive peptides, released from food proteins by the action of proteolytic enzymes, have varying health effects [Hartman and Miesel 2007,

Zambrowicz and Trziszka 2010]. They interact with the respective receptors of the body, thus regulating the functions of the circulatory, digestive, nervous and immune systems [Hartman and Miesel 2007, Kołakowski et al. 2005]. Peptide characteristics include opioid, immunostimulating, lowering blood pressure, inhibiting platelet aggregation, antioxidant activity, antimicrobial and carriers of metal ions [Hartman and Miesel 2007, Mine and Kovacs-Nolan 2006, Meisel 2004, Meisel and FitzGerald 2003].

The specific activity of peptides is determined by the type and location of amino acid residues contained in their primary structure [Park et al. 2001]. Bioactive peptides may be distinguished into simple and complex dipeptides, linear or cyclic metal oligopeptides and polypeptides [Hartman and Miesel 2007, Mine and Kovacs- Nolan 2006]. The precursors of biopeptides may be nutritional proteins of animal origin such as milk proteins (casein, whey protein) or muscle proteins (myosin, collagen), or of vegetable origin such as soy protein (glycinin, β-conglycinin) and wheat protein [Gibbs et al. 2004, Ibrahim et al. 1998, Kim 2007, Kong et al. 2007, Li et al. 2007, Saiga et al. 2003]. Egg proteins are also a rich source of bioactive peptides [Pellegrini et al. 2000, Tsuge 1991, Zambrowicz and Trziszka 2010] (Tab. 1). Because of their positive affect on human health, as well as the high safety profile, they can be used as components of functional foods and nutraceuticals**.**

Hydrolysates that are derived from wheat protein [Liyana-Pathiranaa and Shahidi 2007], maize protein [Kong and Xiong 2006] or blood porcine hemoglobin [Chang et al. 2007] have anti-oxidant properties. This activity depends largely on the type of proteases used for enzymatic degradation and also on the degree of hydrolysis [Kong and Xiong 2006]. The main components of the antioxidative peptides are amino acids such as tryptophan, tyrosine, cysteine, methionine, lysine, histidine and arginine, which determine their properties [Park et al. 2001, Marcuse 1962]. The presence of tyrosine in the structure of peptides is especially important for the ability to scavenge free radicals. Like other phenolic compounds, this amino acid has a hydroxyl bond in its aromatic ring, an electron donor to free radicals [Park et al. 2001, Davalos et al. 2004]. Kawashima et al. [1979] showed that some di-and tri-peptides containing aromatic amino acids also have a particularly strong antioxidant activity. Antioxidants prevent adverse changes resulting from the action of free radicals. These changes underlie the majority of pathological conditions [cardiovascular diseases, degenerative changes].

Protein hydrolysates with antioxidant activity are considered to be factors preventing diseases caused by oxidative stress, such as colon cancer, Alzheimer's disease and Parkinson's disease [Park et al. 2001].

Free radicals lead to adverse changes in food [rancidity, flavour and colour change] and hence antioxidant peptides may also be used as natural food preservatives [Saiga et al. 2003].

Biopeptides are also effectively used in the treatment of obesity, cardiovascular disease, lipid disorders, and neoplastic changes [Erdmann et al. 2008]. Cancer related changes in the human digestive tract are strongly determined by diet [Trock et al. 1990, Giovannucci and Violetta 2004, Kim and Milner 2007, Rose et al. 2007]. Egg yolk proteins and their hydrolysates exhibit antitumor activity by inhibiting tumor cell proliferation in the colon [Ishikawa et al.]. Antitumor activity is also exhibited by fish protein hydrolysates. Hydrolysis of cod and salmon muscle leads to the release of peptides that inhibit in vitro growth of cancer cells in two breast cancer cell lines [Picot et al. 2006].

Table 1

Examples of bioactive peptides derived from food

Effect	Origin	Encrypting protein(s)	Name/remarks/sequence (in single-letter code)
ACE inhibitory/	Soy	Soy protein	NWGPLV
Hypotensive	Fish	Fish muscle protein	LKP, IKP, LRP (sardine, bonito, tuna)
	Meat	Meat muscle protein	IKW, LKP
	Milk	α-LA, β-LG,	Lactokinins (e.g. WLAHK, LRP, LKP)
		α-, β-, κ-CN	Casokinins (e.g. FFVAP, FALPQY, VPP)
	Egg	Ovotransferin	KVREGTTY
		Ovoalbumin	Ovokinin (FRADHPPL)
			Ovokinin (2–7) (KVREGTTY)
Immunomodulatory	Wheat	Wheat gliadin	IAP
	Broccoli	Plant protein	YPK
	Rice	Rice albumin	Oryzatensin (GYPMYPLR)
	Egg	Ovalbumin	Peptides not specified
	Milk	a-, b-, k-CN, a-LA	Immunopeptides (e.g. aS1-immunocasokinin) (TTMPLW)
	Wheat	Wheat gluten	Immunopeptides
Cytomodulatory	Milk	a-, b-CN	a-Casomorphin (HIQKED(V)),
		b-Casomorphin-7	(YPFPGPI)
Opioid agonist	Wheat	Wheat gluten	Gluten-exorphins A4, A5 (GYYPT), B4 B5, and C (YPISL),
	Milk	a -LA, b-LG	a-Lactorphins, b-lactorphins
		a-, b-CN	Casomorphins
Opioid antagonist	Milk	Lactoferrin	Lactoferroxins
		k-CN	Casoxins
Antimicrobial	Egg	Ovotransferrin	OTAP-92 (f109–200)*
	Lyso-zyme	Peptides not specified	
	Milk	Lactoferrin	Lactoferricin
		a-, b-, k-CN	Casecidins, isracidin, kappacin
Antithrombotic	Milk	k-CN (glycomacropeptide)	k-CN (f106–116)a, casoplatelin
Mineral binding,	Milk	a-, b-CN	Caseinophosphopeptides
Hypocholesterolemic	Soy	Glycinin	LPYPR
	Milk	b-LG	IIAEK
Antioxidant	Fish	Sardine muscle	MY
	Wheat	Wheat germ protein	Peptides not specified
	Milk	a-LA, b-LG	MHIRL, YVEEL, WYSLAMAASDI

CN, casein; LA, lactalbumin; LG, lactoglobulin.
* f, fragment.

The source: Hartman R. and Miesel H., 2007. Food-derived peptides with biological activity: from research to food applications. *Current Opinion in Biotechnology* 18, 163–169.

Peptides derived from food proteins also exhibit antimicrobial activity. There are several mechanisms of peptide influence on a bacterial cell. One mechanism indicates that the activity of peptides, particularly in relation to gram-negative bacteria, is due to their ability to bind to the cell wall lipopolysaccharides. As a result of aggregation of peptides in the cytoplasmic membrane, channels are formed which destabilize the membrane and thus lead to cell death. This effect is shown for example by Lactoferricin B, comprising residues from 17 to 41 in the sequence of milk lactoferrin [Chrzanowska 1998]. Another mechanism of antimicrobial action of some peptides involves their passage through the membrane into the cytoplasm and binding to the DNA [Chrzanowska 1998, Powers et al. 2003, Chan et al. 2006]. The antimicrobial activity of peptides is enhanced by the presence of amino acids such as tryptophan and arginine [Chrzanowska 1998, Powers et al. 2003, Chan et al. 2006]. Tryptophan present in a peptide has an affinity to the cell membrane, promotes the folding of peptides in aqueous solutions, allowing them to adopt amphiphilic structures, necessary for transport through the cytoplasmic membrane. Arginine gives peptides a positive charge and forms hydrogen bonds with anionic components of the cell membrane, allowing interaction between the peptide and cytoplasmic membrane. [Powers et al. 2003, Chan et al. 2006]. A good example of an antimicrobial peptide is cysteine – rich peptide which inhibits the growth of both (G+) and (G-) bacteria. This peptide was obtained by hydrolysis of oyster alcalase and bromelain [Liu et al. 2008].

A rich source of opioid peptides are milk proteins. Opioid peptides are released from casein and whey protein. The largest group are β-casomorphins released from β-casein. They show the same amino terminal sequence: Tyr-Pro-Pro-Fen; their bioactivity is determined by the presence of tyrosine residues in the N-terminal position. Opioid peptides act on the central nervous system, affect pain perception and behaviour. These peptides also interact locally on the gastrointestinal system, e.g. extend the transit time of ingesta through the digestive tract [Chrzanowska 1998].

Metal ion binding ability of some peptides is also important from a nutritional standpoint. Peptides bound to micronutrients may serve as carriers, facilitating their absorption. Phosphopeptides released from the caseins of cow's milk allow the binding of divalent metal ions. As such, they are absorbed best by the body [Choi et al. 2005, Jiang and Mine 2000, Chrzanowska 1998].

The richest precursors of antihypertensive peptides are cow's milk casein, rice prolamins and ovoalbumin [Chrzanowska 1998, Miguel et al. 2006]. The action mechanism of most antyhipertensive peptides consists in inhibiting the activity of the angiotensin converting enzyme (ACE), a peptidyl-dipeptydase that cleaves C-terminal dipeptide from angiotensin I (decapeptide: DRVYIHPFHL) thereby causing its conversion to angiotensin II, a blood pressure booster. At the same time, inactivation of this enzyme by inhibitors is accompanied by the stimulation of bradykinin which lowers blood pressure [Miguel et al. 2006]. Antyhipertensive peptides may be used as natural pharmacological compounds. The currently available formulations are for example Vasotensin 120T ™, PeptACE ™ and Peptides 90. The active substance is oligopeptide LKPN "Katshuobushi Oligopeptide", released under the influence of tremolizin from bonito fish muscle protein, which after oral application, under the action of digestive enzymes, is converted to an active form of LKP [Hartman and Miesel 2007].

From food proteins may also release peptides that inhibit platelet aggregation, for example, peptides released under the influence of pepsin from sheep and human milk. Peptides derived from the N-terminus of the polypeptide chain of cow milk κ-casein are also recommended as ingredients in food products for people with cardiovascular diseases [Iwaniak and Minkiwicz 2007].

Immunostimulatory peptides: soymetide-4 and soymetide-13 were isolated, among other things, from trypsin hydrolysates of β-conglycinin. Soymetide-4 protected neonatal rats from etoposide-induced alopecia (hair loss). Alopecia induced by anticancer agents is a troublesome side effect. Soymetide-13 (Met-Ile-Thr-Leu-Ala-Ile-Pro-Val-Asn-Lys-Pro-Gly-Arg) stimulates phagocytosis in vitro by human polymorphonuclear leukocytes [Takahiro et al. 2004].

Egg proteins as precursors of bioactive peptides

In recent years, there has been increasing interest in using the contents of eggs to produce biopeptides via enzymatic hydrolysis with the possibility of subsequent use as nutraceutical or biomedical preparations. Researchers are looking for unconventional high-performance enzymes, and developing hydrolysis methods and process technologies for both the native proteins of egg yolk and white and the post-extraction proteins. Notable results in this regard have been achieved within the OVOCURA Project by the consortium of Wrocław University of Enviromental and Life Sciences and the Wrocław Medical University.

In one example, partial hydrolysis of native lysozyme with pepsin lead to a mixture of peptides exhibiting activity against *Bacillus* bacteria [Abdou et al. 2007]. These peptides retained their activity when heated for 30 min at 95°C in a pH range from 4.5 to 7, and thanks to these properties can be used as a natural preservative in food. Another precursor of antimicrobial peptides is ovalbumin, the main egg protein. Peptides released from this protein under the influence of trypsin and chymotrypsin exhibit bactericidal activity against (G+) bacteria such as *Bacillus subtilis, Micrococcus luteus, Staphylococcus aureus, Streptococcus epidermidis* and *Streptococcus zooepidermicus*, and (G-) bacteria *Bordetella bronchiseptica, Escherichia coli, Klebsiella pneumoniale, Pseudomonas aeruginosa*, and *Serratia marcesens*, and fungi such as *Candida albicans* [Pellegrini et al. 2004]. The bactericidal effect is also attributed to the 9.9 kDa fragment (f 109–200) of conalbumin (OTAP 92), active against (G+) and (G-) bacteria. The OTAP-92 peptide is derived from the N-terminal fragment of the polypeptide chain and has a cationic character [Gołąb and Warwas 2005, Ibrahim et al. 2000]. OTAP-92 peptide has three disulfide bonds, two of which are involved in stabilizing the structure. OTAP-92 peptide is also an anti-virulence factor, effective in the inactivation of the Marek's disease virus in chicken.

Many peptides derived from egg protein hydrolysates have antioxidant activity. For example, ovalbumin was used to obtain three antioxidant peptides containing histidine residues in the second position of their sequence [Tsuge et al. 1991]. Also two peptides with aromatic residues obtained under the influence of proteolytic Alcalase preparation from egg yolk proteins have the potential to inhibit the oxidation of linoleic acid [Park et al. 2001]. A characteristic feature of these peptides, and decisive for their bioactivity, is the presence of leucine residues at the N-terminal position [Park et al. 2001]. The same antioxidant effect is exerted by a formulation consisting of peptide fractions with molecular weights not exceeding 1 kDa, obtained by proteolytic action of bacterial enzymes (*Bacllus sp*) on yolk protein.

Antioxidant activity is also demonstrated by peptides released from ovalbumin under the influence of pepsin, which have the ability to scavenge AAPH free radicals [Davalos et al. 2004]. A similar antioxidant activity against 2,2-diphenyl-1-picrylhydrazyl (DPPH) radicals was observed for peptides released from protein by-products obtained in the isolation of lysozyme and cystatin from egg white [Graszkiewicz et al. 2007]. Hydrolysis of phosvitin with bovine trypsin results in the fraction of the peptide with the ability to inhibit oxidation of linoleic acid, scavenge DPPH free radicals and chelate iron ions (II). These peptides are characterized by a high content of phosphorus and amino acids such as histidine, methionine and tyrosine [Xu et al. 2007]. It has been shown that hydrolysis products obtained from

phosvitin, by inhibiting the iron-dependent reaction in the production of most reactive hydroxyl radicals, have protective properties towards DNA. Thus this protein and its peptides are considered as factors preventing the occurrence of oxidative stress-induced diseases such as colon cancer, Alzheimer's or Parkinson's disease [Matoba et al. 1999].

The property of binding metal ions can also be observed in phosphopeptides released from phosvitin under the influence of trypsin [Choi et al. 2005, Jiang and Mine 2000]. Similar to phosphopeptides released from the caseins of cow's milk, phosphopeptides derived from phosvitin are characterized by a high content of phosphoserine, which allows the binding of divalent ions of calcium [Choi et al. 2005, Chrzanowska 1998, Jiang and Mine 2004]. These peptides are most likely to find use in food technology as dietary and nutritional supplements, especially for people suffering from osteoporosis.

The best-studied antyhipertensive peptide derived from egg proteins is ovokinin, a fragment [f 358–365] of ovalbumin, released under the influence of pepsin [Fujita et al. 1995], as well as ovokinin (2–7), released from the precursor protein under the influence of chymotrypsin, differing from the previous ovokinin by the lack of individual residues in the N-and C-terminus [Matoba 1999]. Both peptides have the ability to lower blood pressure in hypertensive rats, and their effectiveness depends on the size of the dose [Fujita et al. 1995].

Oligopeptides, released from phosvitin, thanks to their ability to regulate the levels of antioxidant enzymes such as catalase and glutathione reductase, are considered to be antitumor factors in colon cancer [Ishikawa et al. 2009]. Another precursor of anticancer peptides is ovomucin; under the action of pronase, its fractions α and β release highly glycosylated oligopeptides, with weights: 220 (f β), 120 (f β) and 70 (f α) kDa [Hiidenhovi 2007, Watanabe et al. 1998]. Direct administration of these peptides into tumors in mice effectively inhibited growths [Watanabe et al. 1998].

Conclusions

Bioactive peptides are continuously produced in the body and play regulatory functions in all of the systems. Peptides with desired activities were also released in vitro in the processes of enzymatic hydrolysis, and their precursors can be for example food proteins. Egg proteins are a rich source of peptides with antimicrobial, antioxidant, immuno-stimulating and antihypertensive activity. These peptides may be applied in the food industry as natural substances preventing unwanted microbial and chemical transformations of food ingredients, and as nutraceuticals.

The dynamic development of chemical and biological methods to determine biological activity has helped discover many new peptides that are active *in vitro*. Unfortunately, one of the problems concerning the effectiveness of biopeptides is oral administration. In order to get into the bloodstream, they must pass through the body's natural barriers such as: highly acidic environment, pepsin, pancreatic enzymes and intestinal absorption [Gołąb and Warwas 2005].

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10

THE EVALUATION OF THE BIOLOGICAL ACTIVITY OF DENATURED EGG WHITE PROTEIN DEGRADED WITH NEUTRASE

Introduction

Egg white is known to have a high nutritional value and good functional properties. Many of its proteins exhibit antimicrobial and antitumoral activities, most notably lysozyme and cystatin. Ethanol extraction of these proteins [Sokołowska et al. 2007] results in large amounts of useful by-products – denatured proteins including ovalbumin, ovomucoid and conalbumin, which can be further processed using enzymatic hydrolysis.

Protein hydrolysates of animal, plant and microbial origin, obtained with the use of proteolytic enzymes, have a certain degree of hydrolysis and high nutritional value. They are a mixture of easily digestible peptides and free amino acids, making them extremely valuable products in supporting the treatment of many diseases, especially those of the digestive system [Clemente 2000, Gastaldi et al. 2003, Tunçtürk 2006, Yujie et al. 2006]. In the production of food, hydrolysates are also used as functional additives that improve the parameters of final products [Banach et al. 2010]. These properties are often accompanied by high biological activity associated with the release of biopeptides during the enzymatic degradation of proteins [Miguel et al. 2006].

Biologically active peptides are the fragments of proteins that influence life and health functions of the body through interaction with the circulatory, nervous, digestive and immune systems. They are categorized as biopeptides with the following activities: antihypertensive, immunomodulatory, antioxidant, opiate, bacteriocidal, cytomodulating, and peptides that bind and transport minerals [Iwaniak and Minkiewicz 2007, Fitzgerald and Murray 2006, Korhonen et al. 2006]. The attractive properties of biologically active peptides result in high interest from both the food and pharmaceutical industries. Biopeptides can be used as nutraceuticals in the production of functional foods and as therapeutic agents with defined pharmacological activity. Functional foods that contain biopeptides may constitute a new strategy in the prevention of many lifestyle diseases such as hypertension and cancer [Miguel and Aleixandre 2006].

The aim of this study was the assessment of potential biological activity of hydrolysates obtained from the degradation of ovalbumin preparation with a commercial proteolytic enzyme – Neutrase from *Bacillus amyloliqefaciens* (Sigma).

Material and Methods

Enzyme. In the study a commercial preparation of proteolytic enzymes – Neutrase (Sigma) was used.

Substrate. For hydrolysis ovalbumin preparation, a by-product in the isolation of lysozyme and cystatin from egg white using ethanol [Sokołowska et al. 2007] was used. Casein (BDH, Ltd. England) was used as the standard substrate.

Determination of protein content. Protein content was determined by Lowry et al., method [1951].

Determination of enzyme activity. Proteolytic activity was determined with the use 1% solutions of casein and ovalbumin at pH 7.5. The substrate with the enzyme was incubated for 10 minutes at 37°C. After this time the reaction was stopped by the addition of 5% trichloroacetic acid (TCA). The sample was then centrifuged and absorbance measured at λ = 280nm. One unit of enzyme activity was defined as the amount of enzyme giving an increase in absorbance of 0.1 under specified conditions.

Hydrolysis of the ovalbumin preparation. Enzymatic hydrolysis assays of 1% protein solution were carried out at 37°C for 5 hours using Neutrase at doses of 50 (W1), 100 (W2), 200 (W3), 400 (W4) $U*mg^{-1}$ in 0.1 M tris – HCl buffer at pH 8.0. The course of the hydrolysis was monitored by determination of the degree of hydrolysis and determination of free amino acid groups increase.

The degree of hydrolysis (DH) of the protein substrate was based on the determination of soluble peptide concentration in 5% TCA [Spellman 2003].

The content of free amino acid groups was determined using trinitrobenzene sulfonic acid (TNBS, Sigma) according to modified Kuchroo and Ramilly [1983] method.

Reversed – phase high performance liquid chromatography (RP-HPLC). Peptide profiles hydrolysates of the ovalbumin preparation were determined by RP-HPLC. Separation was performed using a Zorbax XDB-C_{18} Agilent column (250 x 4.5 mm) equilibrated with 0.1% TFA (trifluoroacetic acid) (phase A). The hydrolysates samples were suspended in phase A and than loaded on the column. Separation was performed at a flow rate of 1 $ml*min^{-1}$ at 30°C. The absorbed peptides were eluted with a gradient (0–100%) of phase B (0.1% TFA in acetonitrile). Absorbance measurement was made at λ = 230nm (Ardo and Polychroniadou 1999).

Determination of antioxidant activity as the ability to scavenge DPPH free radicals. Antioxidant activity was determined by a modified method of Yen and Chen [1995] as the ability to bind DPPH (2,2-di(4-*tert*-octylphenyl)-1-picrylhydrazyl) free radicals in an aqueous solution of peptides during 30 minute incubation. After this time, absorbance measurements were made at the λ=517 nm. The antioxidant activity of the 1 $mg*ml^{-1}$ protein solution was determined on the basis of the standard curve prepared for trolox.

Determination of antioxidant activity using the FRAP method [Benzie and Strain 1996]. Antioxidant activity was determined as the ability of the hydrolysate to reduce the oxidation of iron Fe (III) to Fe (II) ions in reaction with TPTZ (2,3,5-triphenyltetrazolium chloride). Absorbance measurement was made at λ=593 nm. The concentration of Fe^{2+} ions in 1 $mg*ml^{-1}$ the protein solution was determined on the basis of the standard curve for an $FeSO_4$ solution.

Determination of iron Fe (II) ion chelation [Xu et al. 2007]. Chelation of iron ions was determined by colometric measurement of the quantity of Fe (II) not bound with the hydrolysate in the reaction mixture with the participation of ferrozine (3-(2-pyridyl)-5,6-diphenyl-1,2,4-triazine-p,p′-disulfonic acid monosodium salt hydrate). Absorbance measurement was made at λ=562 nm. The ability to chelate iron ions was determined on the basis of the standard curve for a $FeCl_2$ solution.

Results and Discussion

This study was an attempt to optimize the enzymatic degradation of an ovalbumin preparation to obtain hydrolysates with the highest possible degree of hydrolysis and biological activity. The ovalbumin preparation, a by-product of lysozyme and cystatin extraction from egg white with ethanol, is characterized by the high degree of denaturation and hence does not have technologically attractive functional properties [Graszkiewicz et al. 2010]. They can be enhanced, however, by enzymatic hydrolysis. In this study, the degradation of the ovalbumin preparation was carried out using a commercial preparation of proteolytic enzyme (Neutrase). The enzyme preparation was introduced at four different doses: W1, W2, W3, W4.

The course of protein degradation was monitored quantitatively by determining the degree of hydrolysis (DH) and the increment of free amino groups; the results are shown in Figures 1 and 2 .

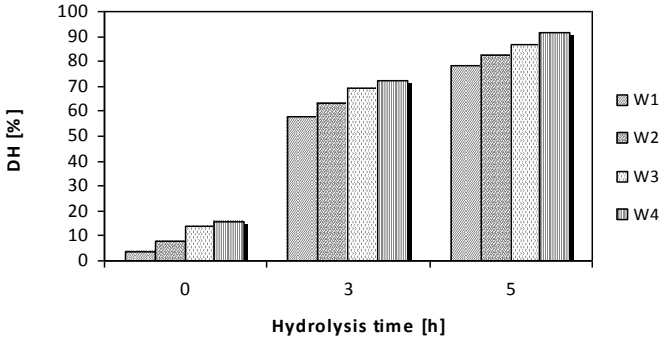

Fig. 1. Degree of hydrolysis (DH) of ovoalbumin preparation hydrolysed by proteolytic enzyme – Neutrase; W1–50 U*mg^{-1}, W2–100 U*mg^{-1}, W3–200 U*mg^{-1}, W4–400 U*mg^{-1}

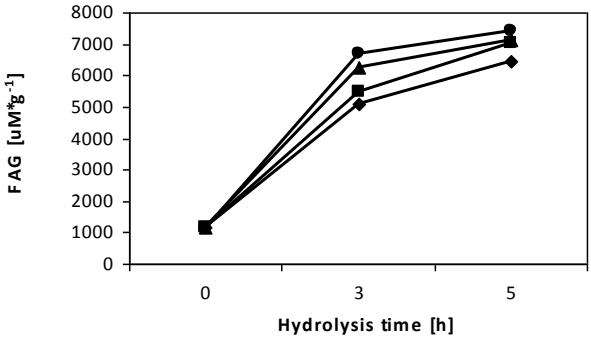

Fig. 2. Changes in free amino groups contents (FAG) during hydrolysis of ovoalbumin preparation by Neutrase*;* W1–50 U*mg^{-1}, W2–100 U*mg^{-1}, W3–200 U*mg^{-1}, W4–400 U*mg^{-1}

The greatest impact on the level of protein hydrolysis, expressed through the degree of hydrolysis (DH), was observed for each applied dose of the enzyme and the reaction time.

After 3 h of reaction in all the variants more than 50% degradation of the substrate was observed. Almost complete degradation, at 92%, was observed after 5hrs of incubation in the samples with the highest dose of enzyme preparation (400 U*mg^{-1}). The use of half lower dose, however, resulted in an almost equally high degree of hydrolysis (87%). Degradation of ovalbumin using Neutrase at a dose of 50 U*mg^{-1}protein resulted in about 20% lower DH. With regards to the dynamics of the degradation of the ovalbumin preparation systematic increase in the degree of protein degradation, irrespective of the dose of the enzyme was found. High activity of the tested enzyme towards the protein substrates was also shown by Je et al. [2007] who used the process of hydrolysis as a method of disposing of waste proteins in the fishing industry and received almost total degradation of the substrate. The obtained hydrolysates were observed to have antioxidant activity.

The highest level of free amino acid groups (7410 μM*g^{-1}) was determined in the hydrolysate obtained with the highest dose of Neutrase after 5 hrs of reaction. The use of lower concentrations of the enzyme resulted in slower degradation of the protein. The content of amino acid groups released after degradation of ovalbumin with protease from *Bacillus amyloliqefaciens* at 50 U*mg^{-1} was finally 6449 μM*g^{-1}.

Differences in the degradation of the ovalbumin preparation isolated from egg white with different doses of the enzyme were also determined on the basis of chromatographic separations (RP-HPLC). The obtained peptide profiles are shown in Figure 3.

During incubation of the enzyme preparation with the substrate, in all analyzed samples the presence of new, low molecular weight products of protein degradation, differing in hydrophobicity were observed which were not observed in the control. The highest number of obtained peptide fractions was observed after 5 hours of hydrolysis at enzyme doses of 200 U*mg^{-1} and 400 U*mg^{-1}. In these samples no peaks indicating the presence of substrate were found, however they were detected in hydrolysates W1 and W2. The use of higher doses of enzyme preparation also resulted in a greater share of peaks with short retention time (up to 10 minutes) that were not observed in the initial substrate.

In a further stage of the research, the hydrolysates were tested for their potential antioxidant activity. Antioxidants and free radicals are being widely discussed in many research fields. The formation of free radicals is an unavoidable consequence of oxidation processes – however, in many cases their effects are negative. They participate in lipid peroxidation, protein and DNA modifications, cause oxidative stress, and therefore may damage cells and tissues [Suetsuna et al. 2000]. It has been shown that free radicals are also involved in the occurrence of many chronic diseases such as cardiovascular disease, diabetes, neurological disorders and even Alzheimer's disease. In food, lipid peroxidation adversely affects the nutritional value of products. Hence the search for antioxidative substances of natural origin which could delay these processes [Je et al. 2007].

In this study the antioxidant activity of ovalbumin hydrolysates obtained using Neutrase by the determination of the ability to scavenge DPPH free radicals, reduce the oxidation of Fe (III) and to chelate Fe (II) was assessed. It was found that all the analyzed hydrolysates showed activity against DPPH radicals, although a much higher ability to scavenge free radicals was observed in samples after 5 hours of reaction (Fig. 4). Even the lowest dose of the enzyme, 50 U*mg^{-1}, generated the formation of protein degradation products that exhibited activity against DPPH radicals at a level of 0.3 uM trolox*mg^{-1}. Increasing the dose of Neutrase resulted in a nearly twofold increase in antioxidant activity.

In another test, an assessment of the reducing power of ovalbumin hydrolysates, expressed by their ability to decrease oxidation of Fe (III) to Fe (II) was also performed (Fig. 5).

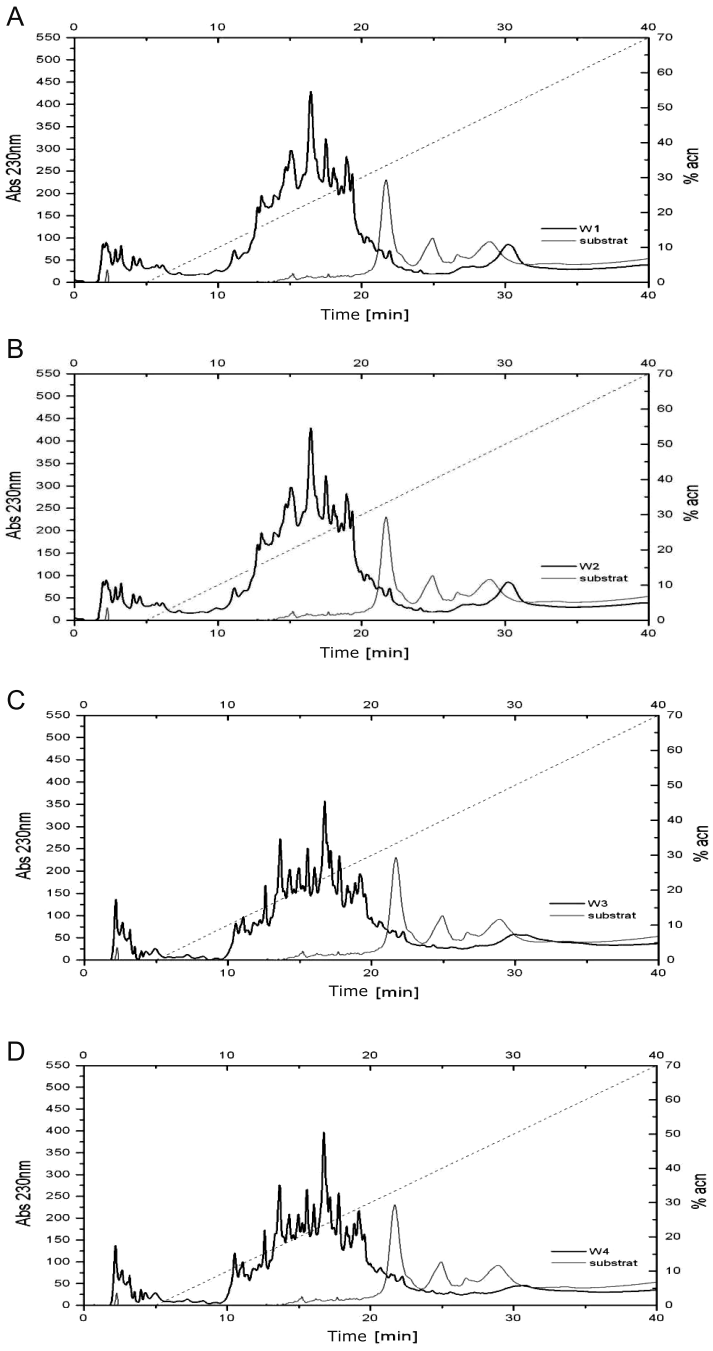

Fig. 3. Peptide profiles of ovoalbumin preparation hydrolysates (RP-HPLC). A) W1, B) W2, C) W3, D) W4

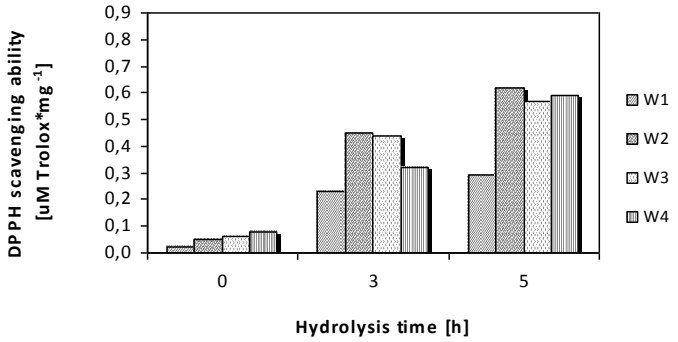

Fig. 4. DPPH scavenging activities of ovoalbumin preparation hydrolysates (W1–50 U*mg^{-1}, W2–100 U*mg^{-1}, W3–200 U*mg^{-1}, W4–400 U*mg^{-1})

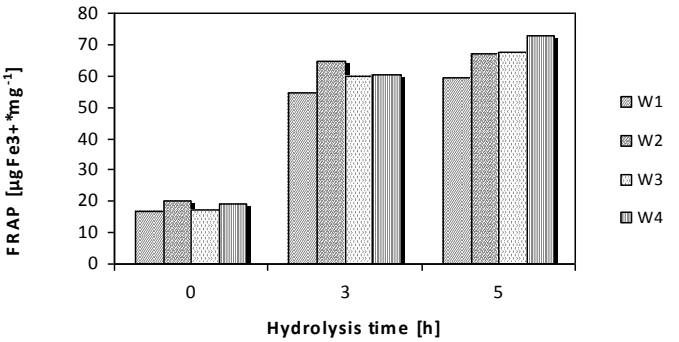

Fig. 5. The ferric reducing ability (FRAP) of ovoalbumin preparation hydrolysates (W1–50 U*mg^{-1}, W2–100 U*mg^{-1}, W3–200 U*mg^{-1}, W4–400 U*mg^{-1})

A significant increase in reducing power that accompanied the increasing degree of protein degradation was observed. In just over three hours of reaction, hydrolysates showed 3 times higher activity in comparison to initial activity. The highest values of these activity were observed for hydrolysates obtained after five hours of reaction. The use of increasing doses of the enzyme (100 U*mg^{-1}, 200 U*mg^{-1}, 400 U*mg^{-1}) resulted in a reduction of the oxidation of Fe (III) from 67 to 73 µg Fe^{3+}*mg^{-1}.

Enzymatic hydrolysates have also been studied in terms of their ability to chelate iron ions (II), catalysts for oxidation processes. These results are shown in Figure 6.

All hydrolysates showed chelating activity, increasing with time and the degree of protein degradation. A higher ability to bind Fe^{2+} ions was observed in protein preparations hydrolyzed with higher doses of Neutrase, yielding a maximum level of 240 µgFe^{2+}*mg^{-1}. In hydrolysates obtained from variant W1, the iron ion chelation value was lowest, amounting to maximum level of 104 µgFe^{2+}*mg^{-1}. Antioxidant activity of protein hydrolysates obtained from egg white using a variety of proteolytic enzymes has also been shown by Davalos et al. [2004] and Huang et al. [2010].

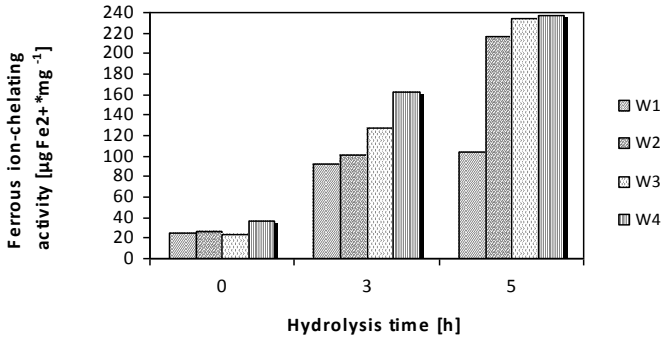

Fig. 6. Ferrous ion-chelating activities of ovoalbumin preparation hydrolysates(W1–50 U*mg^{-1}, W2–100 U*mg^{-1}, W3–200 U*mg^{-1}, W4–400 U*mg^{-1})

Conclusions

Hydrolysis of ovalbumin preparation using a commercial preparation of proteolytic enzyme (Neutrase from *Bacillus amyloliqefaciens*) resulted in an almost complete degradation of the substrate.

Peptides obtained as a result of hydrolysis showed biological activity.

The use of higher doses of enzyme preparation resulted in higher biological activity of hydrolysates.

The highest antioxidant activity, expressed as the ability to reduce the oxidation of Fe (III) (FRAP) and chelate Fe (II), was observed in hydrolysates obtained with enzyme doses of 200 U*mg^{-1} and 400 U*mg^{-1}.

Acknowledgements

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11

EGG-YOLK PROTEINS AS A SOURCE OF PEPTIDES WITH ANTIOXIDANT ACTIVITY

Introduction

Egg yolk is well documented as a versatile food ingredient that has functional and nutritional properties [Mine and Kovacs-Nolan 2004, Ahn and Ko 2006]. The main components of egg-yolk are phospholipids, triacylglycerols and proteins. Protein constitutes about 30% of the dried mass of egg yolk. The enzymatic hydrolysis of egg proteins yields physiologically important bioactive peptides that exhibit antimicrobial, immunomodulatory, antioxidant, mineral-binding, hypocholesterolemic, and antihypertensive [Mine and Kovacs-Nolan 2004, 2006, Sakanaka et al. 2004, 2006]. Many of the peptides are multifunctional and can exert more than one biological effect [Silva and Malcata 2005, Hartman and Miesel 2007].

Lipid oxidation is a significant problem to the food industry as it leads to the development of undesirable off-flavors, dark colors and potentially toxic reaction products [Graszkiewicz et al. 2007, Sakanaka et al. 2006]. Therefore the control of lipid oxidation in food products is desirable, and attempts to anticipate its occurrence via modeling or otherwise to ascertain the benefits of antioxidants in food storage is of great interest in research [Sakanaka et al. 2004, 2006, Rossini et al. 2009]. Special attention has been given to natural antioxidants [for example peptides] from a new trend to avoid the use of synthetic food additives [Sakanaka et al. 2004, 2006, Rossini et al. 2009].

Certain peptides and protein hydrolysates can reduce the auto-oxidation and the hydroperoxide content of fatty foods in rats. They act both as scavengers of heavy metals and promoters of the decomposition of hydroperoxides, which would otherwise serve as a source of free radicals [Gill et al. 1996].

An attractive source of antioxidant peptides are egg-yolk proteins [Sakanaka et al. 2004, 2006, Xu et al. 2007]. It has been reported that egg-yolk hydrolysates exhibit antioxidant capacities in a linoleic acid oxidation system [Sakanaka et al. 2004]. The hydroxyl radical and DPPH scavenging activity and suppression of discoloration of β-carotene have also been observed. In food modelling systems, peptides derived from egg-yolk hydrolysates effectively inhibited lipid oxidation processes in beef and tuna muscle homogenates [Graszkiewicz et al. 2007, Sakanaka et al. 2006]. Two peptides obtained from Lecitin-free egg yolk hydrolysate showed antioxidant activity in a linoleic acid model system. These peptides, composed of 10 and 15 amino acid residues, both contain a leucine residue at their N- thermal positions [Park et al. 2001]. Oligophosphopeptides derived from the partial tryptic hydrolysis of dephosphorylated phosvitin exhibited strong capability against the oxidation of linoleic acid, and also radical scavenging activity on DPPH free radicals [Xu et al. 2007]. Oligophosphopeptides also showed antioxidant activity against oxidative stress in human intestinal epithelial cells in an in vitro assay [Katayama et al. 2006, Xu et al. 2007].

Egg-yolk phosvitin, a highly phosphorylated protein [10% phosphorus], has a unique amino-acid composition [Xu et al. 2007, Kozłowski et al. 1988]. More than 55% of the amino-acids are serine residues and most of them are monoesterified with phosphporic acid. Only the C-terminal region, of approximately 15 amino acids, has hydrophobic characteristics [Castellani et al. 2003]. This primary structure make phosvitin one of the strongest metal-chelating agents – it binds metal ions: Fe [II], Fe[III],Co[II], Mn[II], Ca[II] and Mg[II]. Owing to this chelating property, phosvitin shows strong antioxidant activity [Kozłowski et al. 1988].

Egg-yolk also contains about 1% immunoglobulin Y – a polyclonal antibody [Tu et al. 2006, Lee et al. 2002]. The general structure of the Ig Y molecule is two light and two heavy chains. The MW of these protein was found by mass spectrometry to be 167.250 Da and pI of Ig Y is in the range of 5.7–7.6 [Schade and Chacana 2007]. Ig Y decreased the incidence of diarrhea caused by rotavirus or *E. coli*. IgY also conferred protection against dental caries formed by *Streptococcus mutans* [Lee et al. 2002].

The aim of our study was to determine the antioxidant activity of egg-yolk protein enzymatic hydrolysates.

Materials and Methods

Biological Material

The eggs were layed by Lohmann Brown Line hens. Phosvitin and immunoglobulin fractions were obtained after the lecithin isolation method described by Castellani et al. [2006].

Enzymatic activity

Pepsin (type A from pork stomach, Sigma) activity was determined in a reaction with 2% acid-denaturized hemoglobin as the substrate, according to Chrzanowska and Kołaczkowska [1998]. 650 µl of 0.2 M phosphate-citrate buffer [pH 3.0] was preincubated at 37°C for 5 min. Then 100 µl of enzyme solution (2–20 µg) was added. The reaction was started by adding 250 µl ml of hemoglobin. After 10 min the reaction was stopped by the addition of 1 500 µl of 10% trichloroacetic acid. The tubes were then centrifuged (5 500 rpm, 15 min, 20°C). The absorbance of the supernatants was measured at 280 nm.

Bovine trypsin (TPCK-treated, Sigma) activity was determined in a reaction with 1% casein as the substrate, according to Kakade et al. [1970]. The enzyme was dissolved in 20 mM HCl with 80 mM $CaCl_2$ to a final protein concentration of 4 to 20 µg · ml^{-1}. One ml of 1% casein [in 0.2 mM Tris-HCl buffer, pH 8.0] was preincubated at 37°C for 5 min and the reaction

The protein was started by adding 1 ml of enzyme solution. After 10 min the reaction was stopped by the addition of 3 ml of 5% trichloroacetic acid. The tubes were then centrifuged (4.500 x g, 15 min, 20°C). The absorbance of the supernatants was measured at 280 nm.

One unit of enzymatic activity of these enzymes (U) corresponded to the amount of enzyme capable of hydrolyzing 1% casein under reaction conditions and giving an increase in absorbance at 280 nm of 0.1.

Protein assay

Protein concentration was determined according to Lowry et al. [1951]. A standard curve was prepared for bovine serum albumin (BSA).

Enzymatic hydrolysis

Immunoglobulin and dephosphorylated phosvitin were dissolved in the reaction buffer (0.2 M phosphate-citriate, pH 3.5 and 0.2 M Tris-HCl contained 20 mM $CaCl_2$ pH 8.3 for pepsin and trypsin respectively) to a final concentration of 5.0 mg/ml. Hydrolysis was started by adding enzyme (20 U per 1 mg of hydrolyzed protein) and the reaction continued at 37°C for 3 h. It was stopped by heating at 100°C for 15 min. Then the hydrolysate was centrifuged and the supernatant was lyophilized.

Degree of hydrolysis [DH%]

DH was expressed as the percentage ratio of protein soluble in 5% trichloroacetic acid (TCA) to total protein [Silvestre 1997]. Five percent TCA was added to the hydrolysates (1:1). After 1 h of incubation at 4°C the samples were centrifuged (5500 rpm, 15 min, 20°C). The concentration of the trichloroacetic acid-soluble product in the supernatant was measured spectrophotometrically at 280 nm.

Determination of the free amino group content

The concentration of the free amino groups was determined according to Kuchroo et al. [1983]. 2 ml of the hydrolysate diluted with 0.1 M borate buffer was mixed with 50 µl of TNBS reagent (0.03 M). The samples were incubated in darkness for two hours at room temperature. The reaction was stopped by adding 2 ml of 0.1 M sodium phosphate containing 1.5 M sodium sulfate and the absorbance was measured at 420 nm. A blank was prepared with water instead of hydrolysate. The results were expressed as µM Gly · g^{-1} by reference to a standard curve prepared with defined concentrations of glycine.

Reverse-Phase High-Performance Liquid Chromatography (RP-HPLC)

The samples of hydrolysate and the peptide fractions were dissolved in the mobile phase A (1:1) and applied to a Zorbax XDB-C_{18} column (4.6 × 250 mm, Agilent). The operation conditions were – flow rate: 1 ml/min, gradient: 2% B/min, mobile phase A: 1 ml of trifluororacetic acid (TFA) per liter in bi-distilled water, phase B: 1 ml of trifluoroacetic acid (TFA) per liter in acetonitrile, T=30°C, and retention time: 5 min. The absorbed peptides were eluted by gradient phase B. The absorption of the eluents was monitored at 230 nm.

Antioxidant capacity as scavenging of the free radical 1,1diphenyl-2-picrylhydrazyl (DPPH)

The antioxidant capacity was measured according to the method described by Yen and Chen [1995] with slight modifications. The tested samples were dissolved in water to a final volume of 1 ml and mixed with 1 ml of ethanol (98%). The reaction was started by adding 0.5 ml of 0.3 µM DPPH in ethanol. The mixtures were left for 30 min at room temperature and the absorbance of the resulting solution was measured at 517 nm. Radical scavenging activity of the peptides was expressed as µM Trolox · mg^{-1} protein by reference to a standard curve.

Ferric reducing Activity (FRAP)

FRAP was measured according to the method described by Benzie and Strain [1996]. 3 ml of FRAP working solution (1A :1B: 10C, A – acetate buffer, 300 mM pH 3.6, B – 10 mM 2,4,6,tripyridyl-s-triazine (TPTZ) in 40 mM HCL, C – 20 m $FeCl_3$ x 6 H_2O in distilled water) was mixed with a 1 ml sample (water solution). After 10 mins of reaction, the absorbance was measured at 593 nm. The blank sample was FRAP reagent. Aqueous solution of known Fe (II) concentration was used for calibration (in a arrange of 100 1000 µM).

Chelating activity on iron (II)

Chelating activity was carried out by modification of the procedure of Xu et al. [2007]. A 250 µl sample was mixed with 11250 µl H_2O and 110 µl 1mM $FeCl_2$. After 1 min, 1 ml of 500 µM ferrozine aqueous solution was added and the mixture was allowed to react for 10 min. The absorbance of ferrous iron-ferrozine complex was measured spectrophotometrically at 562 nm. Sample blank was: 250 µl of sample, 2250 µl H_2O and 110 µl 1 mM $FeCl_2$. Control was all reagents without any sample. A known concentration of $FeCl_2$ (0–20 µg) was used as a standard curve. Chelating activity was expressed as the amount of iron linked with ferrozine complex.

Results and Disscusion

Hen egg is an excellent source of many bioactive substances which may find application in the medical and food industries [Trziszka et al. 2006]. In this study we investigated the possibility of applying enzyme hydrolysis to obtain phosvitin and immunoglobulin from hen egg yolk by generation of antioxidant peptides.

Before enzymatic hydrolysis, alkaline dephosphorylation of phosvitin was prepared. Jiang and Mine [2000] indicated that proteins containing phosphorylated amino acid residues are resistant to protease activity. It has been reported that tryptic digestion of phosvitin result in a large peptide fragment (Gln49-Arg 212) and a small one [Ala1-Arg 35] [Jiang and Mine 2000].

Phosvitin and Ig Y were hydrolyzed with porcine pepsin and bovine trypsin. The progress of hydrolysis was monitored by determining the degree of hydrolysis [DH, %] (Fig. 1) and the concentration of free amino-groups (Fig. 2). DH is an important parameter in the enzymatic modification of proteins and might be a factor controlling the composition and properties of the modified proteins [Ge and Zhang 1993].

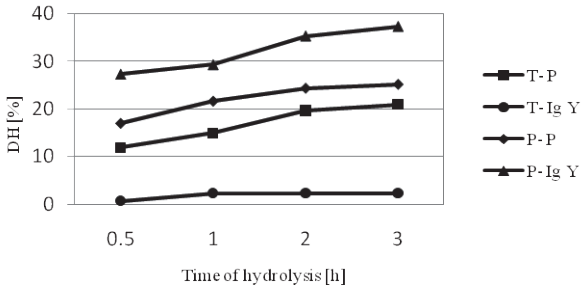

Fig. 1. Degree of hydrolysis of phosvitin and IgY with trypsin and pepsin after different times [T-P--tryptic hydrolysate of phosvitin, T-IgY- tryptic hydrolysate of immunoglobulin Y, P-P- peptic hydrolysate of phosvitin and P-IgY- peptic hydrolysate of immunoglobulin Y]

In our case, pepsin was very effective in the degradation of both proteins. DH increased to 37.3% and 25.1% during 3 h hydrolysis of Ig Y and phosvitin respectively. Ig Y was a very resistant protein for trypsin. It maintained a slow rate for four hours and the final DH reached: 2.3%, whereas hydrolysis of phosvitin lead to 20.9% DH.

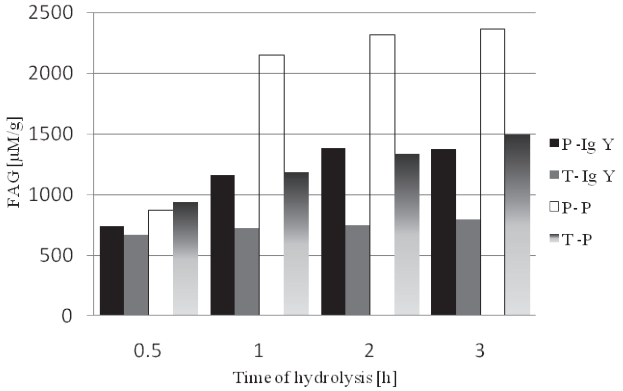

Fig. 2. Changes if free amino groups contents [FAG] in phosvitin and IgY hydrolysate preparations [T-P, T-IgY, P-P, P-IgY – see Fig. 1]

The greatest increase in free amino-group concentration was also observed for pepsin. The level of free amino groups determined in the phosvitin hydrolysate [2 365.3 µM Gly · g^{-1}] was 1.7 times higher than in the Ig Y hydrolysate. The final concentrations of free amino groups in tryptic hydrolysates of phosvitin and Ig Y reached 1 492.1 and 791.6 µM Gly · g^{-1} protein.

Our results confirm the observation of other authors that pepsin and trypsin are efficient enzymes in dephosphorylated phosvitin degradation [Jiang and Mine 2000]. Potentially, trypsin hydrolyzes peptide bonds involving the carboxyl group of L- arginine and L-lysine. There are 26 arginine and lysine residues in a mole of native phosvitin. In fully hydrolyzed phosvitin the chain length is 8 [Jiang and Mine 2000]. Jiang and Mine [2000] obtained tryptic hydrolysates of phosvitin with average peptide chain length ranged from 9.6 to 22.8.

RP-HPLC of the final [three-hour] hydrolysates was performed (Fig. 3). The peptide profiles of the degraded phosvitin and Ig Y confirmed the different cleavage patterns of the investigated proteolytic enzymes.

Many studies [Sakanaka et al. 2006, 2004, Xu et al. 2007, Park et al. 2001] showed that enzymatic hydrolysis of egg-yolk proteins with different proteolytic enzymes produces hydrolysates that exhibit antioxidant activity. The antioxidative capacities of obtained hydrolysates were expressed as free-radical scavenging activity with a stable DPPH radical (Fig. 4), ferric reducing activity [FRAP] (Fig. 5) and chelating activity on iron [II] (Fig. 6).

The phosvitin hydrolysate obtained with trypsin after one hour reaction was the most effective as DPPH free radical scavenger [0.7 µM Trolox · mg^{-1}]. Enzymatic hydrolysis of Ig Y produced peptides exhibiting DPPH scavenging activity in a range from 0.3 to 0.5 µM Trolox · mg^{-1} and from 0.3 to 0.4 µM Trolox · mg^{-1} for pepsin and trypsin respectively.

Our results are very similar to Zambrowicz and Trziszka [2011] in that the degradation of tryptic 24-hour hydrolysates of phosvitin preparation exhibited antioxidant activity of 0.27 µM Trolox · mg^{-1}. In our previous study [Graszkiewicz et al. 2007, 2010] we also showed that the degradation of egg-white proteins with trypsin generated peptides exhibiting stronger free-radical scavenging activity than hydrolysates obtained with another proteolytic enzymes. After 7-hours of hydrolysis of egg-white protein precipitate with trypsin, the peptides exhibited antioxidant activity of 0.38 µM Trolox · mg^{-1}.

Different levels of antioxidative activity of peptides obtained from tryptic hydrolysate of phosvitin were observed by Xu et al. [2010]. The residual radical percentage reached 82.5% after 2.5 hour reaction.

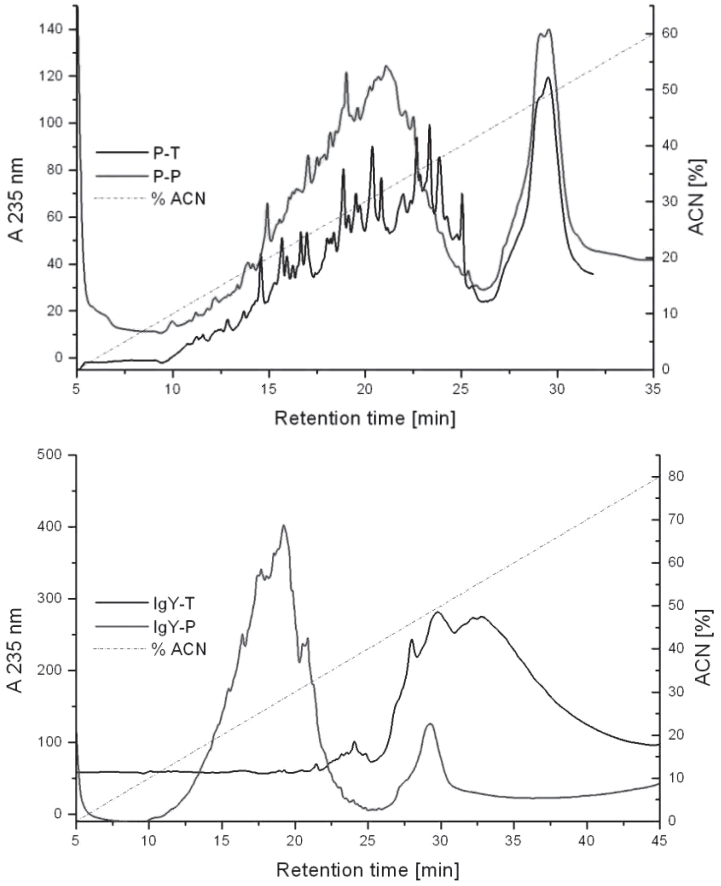

Fig. 3. RP-HPLC peptide profiles of phosvitin [A] and IgY [B] hydrolysate preparations [P-T-phosvitin-trypsin, P-P- phosvitin- pepsin, IgY-T- imminoglobulin Y- trypsin, IgY-P- imminoglobulin Y- pepsin, % ACN- acetonityle gradient]

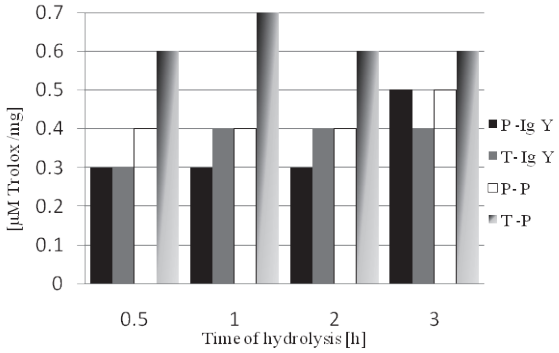

Fig. 4. Free radical [DPPH] scavenging activity of phosvitin and IgY hydrolysate preparations with trypsin and pepsin [T-P, T-IgY, P-P, P-IgY – see Fig. 1]

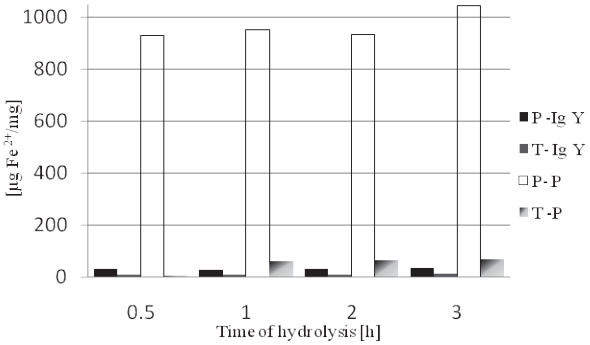

Fig. 5. The ferric reducing ability [FRAP] of phosvitin and IgY hydrolysate preparations with trypsin and pepsin [T-P, T- IgY, P-P, P- IgY – see Fig. 1]

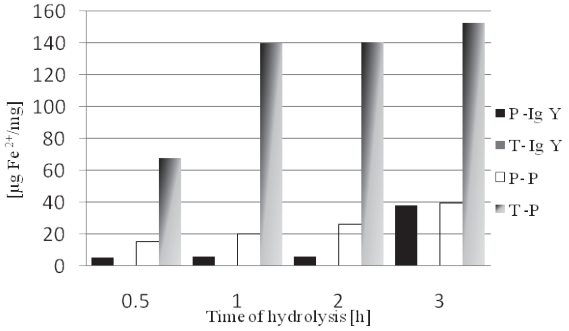

Figure 6. Chelating activity on iron [II] of phosvitin and IgY hydrolysate preparations with trypsin and pepsin [T-P, T- IgY, P-P, P- IgY – see Fig. 1]

Pepsin hydrolysates of phosvitin exhibited strong ferric reducing activity. The activity value significantly increased during hydrolysis finally reaching 1044.4 µg $Fe^{2+} \cdot mg^{-1}$. The trypsin and pepsin hydrolysates of immunoglobulin showed only weak ferric reducing activity: 34.9 and 13.2 µg $Fe^{2+} \cdot mg^{-1}$, respectively. Similar levels of antioxidative activity have been reported for phosvitin hydrolysate with protease from *A. melleus* (31.3 µg $Fe^{2+} \cdot mg^{-1}$) [Zambrowicz and Trziszka 2010]. The hydrolysis products obtained from phosvitin exhibited stronger chelating activity than immunoglobulin hydrolysates. In the our study the same levels of chelating activity of peptic hydrolysates were observed [38.1 and 39.9 µg $Fe^{2+} \cdot mg^{-1}$ for Ig Y and phosvitin hydrolysates, respectively]. However, tryptic hydrolysate of phosvitin possessed very strong chelating activity (152.9 µg $Fe^{2+} \cdot mg^{-1}$). While tryptic hydrolysates of Ig Y didn't exhibit chelating activity on Fe^{2+}, Xu et al. [2010] also demonstrated the chelating activity of pure peptides isolated from tryptic hydrolysate of phosvitin. These results indicate that phosvitin peptides exhibited weaker chelating capability on iron [II] compared with intact phosvitin.

Ig Y has a high resistance to the activity of digestive enzymes. It is possible that this is associated with its biological function. Ig Y activity is directed against bacteria and viruses, particularly against pathogens that attack the digestive tract. The protein effectively inhibits the adhesion of *Streptococcus mutant* on the surface of epithelial cells both *in vitro* and *in vivo*.

Based on tests conducted on animals, it was found that Ig Y prevents tooth decay [Mine and Kovacs-Nolan 2006]. It has not been shown that Ig Y hydrolysates have an antioxidant activity.

Conclusions

Functional bioactive hydrolysates of phosvitin and Ig Y exhibited significant radical scavenging activity on DPPH. Pepsin hydrolysates of phosvitin possess strong ferric reducing activity. The present study indicates that enzymatic hydrolysates of phosvitin and Ig Y could be used as food additives.

Acknowledgements

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12

SUGAR BEET AND RAW JUICE AS RAW MATERIALS FOR BIOETHANOL PRODUCTION

Introduction

Application of ethanol as a biofuel decreases the dependence on crude oil import, increases the energy independence of the country and reduces green house gas emissions to the atmosphere [Oliveira Dias De 2008].

Bioethanol can be produced from all feedstocks that contain mono-, oligo- and polysaccharides (like starch and cellulose) [Lin and Tanaka 2006]. From an economic point of view and in comparison with cereals, sugar beet and intermediates from beet processing are very good raw materials for ethanol production due to their content of fermentable sugars. Sucrose contained in sugar beet as well as in intermediate products of its processing and molasses do not require enzymatic or chemical hydrolysis of present polysaccharides [Leiper et al. 2006]. This is readily slit into glucose and fructose in the initial stage of fermentation by the enzyme invertase, located in the periplasmic space between the yeast cell wall and cell membrane [Dodic et al. 2009].

Economic profitability of sugar beet breeding makes this crop an attractive raw material for bioethanol production. Another aspect is the reform of sucrose market in UE countries resulting in transformation of superfluous sugar factories into bioethanol distilleries by sugar companies [Kaliszewski 2001, Rogulska and Gemeniuk 2006]. Production of bioethanol from intermediates of sugar beet processing enables the undisturbed operation of sugar factories (not only superfluous ones) that is constrained by limitation of sucrose production in EU countries.

Disadvantage of direct sugar beet pulp fermentation is a slow release of sucrose into the fermenting solution. However, the long-term storage of sugar beets results in substantial sucrose losses [Hinkova and Bubnik 2001] and therefore they should be converted to stable intermediates [Dodic et al. 2009]. For improvement of storability and to limit the decomposition by the microorganisms action, raw juice should be concentrated. Obtained product contain high sugar concentration and can be stored in tanks for later processing, e.g. to bioethanol.

This study aimed at evaluation of sugar beet and raw juice (intermediate product of sugar beet processing) as potential raw materials for ethanol production. Effect of composition of worts prepared from sugar beet (after pretreatment by pressure cooking) and method of raw juice-based worts pretreatment and their pH on fermentation dynamics and ethanol yield was determined.

Materials and Methods

Raw materials and supplements

Sugar beet and raw juice were obtained from the sugar factory in Dobrzelin (Poland). Fermentations were carried out by using dried distillery yeast preparations:

– strain As-4, purchased from the yeast factory in Maszewo Lęborskie (Poland), added in the dose of 2.0 $g \cdot dm^{-3}$, determined as optimal in previous study [Balcerek and Pielech-Przybylska 2008]
– Ethanol-Red (Lesaffre, France), added in the dose of 0.5 $g \cdot dm^{-3}$, recommended by the manufacturer.

$(NH_4)_2HPO_4$ (0.3 $g \cdot dm^{-3}$) was used as nutrient for yeast. Selected samples of sugar beet-based worts were supplemented with enzymatic preparation Cellustar (cellulase) (0.1 $cm^{-3} \cdot dm^{-3}$) (Novozymes, Denmark).

Preparing of fermentation worts

Washed sugar beet roots have been pre-chopped into cubes and then subjected to pressure cooking at 140ºC (0.3 MPa) for 30 minutes. Obtained pulp was cooled to the temperature ca. 28–30ºC, its pH was adjusted to 4.8 by using sulfuric acid solution (30% $w \cdot w^{-1}$) and used to prepare fermenting worts in the following variants:

– without addition of $(NH_4)_2HPO_4$; yeast As-4
– with addition of $(NH_4)_2HPO_4$; yeast As-4
– with addition of $(NH_4)_2HPO_4$ and preparation Cellustar; yeast As-4
– without addition of $(NH_4)_2HPO_4$; yeast Ethanol-Red
– with addition of $(NH_4)_2HPO_4$; yeast Ethanol-Red
– with addition of $(NH_4)_2HPO_4$ and preparation Cellustar; yeast Ethanol-Red

Fermentation worts were prepared from undiluted raw juice. A part of the worts was acidified with 25% sulfuric acid to 4.8 and a part was used for fermentation without pH adjustment (pH = 5.95). Moreover the worts were subjected to the following treatments:

– pasteurization at 85ºC, 30 min
– autoclaving at 121ºC (0.1 MPa), 20 min
– ozonization (100 g $O_3 \cdot m^{-3}$, 20 min)

Control samples were worts from the raw juice without treatment (without and after adjusting pH). All the worts were supplemented with $(NH_4)_2HPO_4$ (0.3 $g \cdot dm^{-3}$) as mineral nitrogen sources and inoculated with yeast strain As-4 (2.0 $g \cdot dm^{-3}$) which were earlier re-hydrated.

Fermentation

Fermentation trials were carried out in 3 dm^{-3} glass flasks closed with stoppers equipped with fermentation pipes filled with glycerol and kept in a thermostated room at 28–30°C. Fermentation was controlled by measurements of a decrease in mass of the worts caused by CO_2 evolving. Collected data were plotted as curves showing the dynamics of fermentation.

Distillation

When fermentation was complete the whole ethanol was distilled from worts by using a distillation unit. Ethanol concentration in distillates was assayed by refractometric measurements. Raw spirits containing 20–26% $v \cdot v^{-1}$ ethanol were refined up to approximately 43% $v \cdot v^{-1}$ ethanol in a distillation apparatus equipped with a birectifier unit (dephlegmator according to Golodetz) and subjected to chemical analyses.

Analytical methods

Sugar beet and raw juice (intermediate product of sugar beet processing) were analyzed by methods recommended in sugar industry. Total extract (so-called apparent dry solids) was measured by using an areometer with scale in Balling degrees (ºBlg). It refers to the concentration of dissolved solids, mostly sugar, expressed as the weight percentage of saccharose. Total nitrogen was determined by Kjeldahl method and calculated as protein (N x 6.5). Volatile acids (expressed as acetic acid) were assayed by using steam distillation [AOAC 1995]. Reducing sugars and total sugars (after acid hydrolysis) were estimated by Schoorl and Regenbogen method [Schoorl and Regenbogen, 1917]. Both were expressed in g of invert sugar·100 g^{-1} of sugar beet pulp or raw juice. Sucrose concentration was calculated as a difference between total sugars and reducing sugars (taking into consideration the conversion coefficient of 0.95). Also ash content and pH were determined.

Worts were analyzed before and after fermentation by methods recommended in distilleries [Suchodolski 1955]. Total extract in worts before fermentation was measured by using an aerometer with scale in Balling degrees (ºBlg) (equivalent to Brix degrees). Assays of reducing sugars, total sugars and sucrose were carried out by the methods presented above.

On completion of fermentation, the worts were analyzed for the apparent extract (in the presence of ethanol) and real extract (after distillation of ethanol), both expressed in ºBlg. Ethanol concentration was measured by using an aerometer with scale in % v·v^{-1} of ethanol. Concentration of residual sugars was assayed in worts after distillation of ethanol by the Schoorl and Regenbogen method [Schoorl and Regenbogen, 1917].

Distillates were analyzed by using Agillent 6890N gas chromatograph (USA), equipped with a flame – ionization detector (FID), a split/splitless injector and a capillary column HP-Innowax (60 m x 32 mm x 0.5 μm). The temperature at the injector (split 1:45) and FID was kept at 250ºC. The temperature program was as follows: 40ºC (6 min), a rise to 83ºC (2ºC·min^{-1}) and then to 190ºC (5ºC·min^{-1}) (2 min). The flow rate of carrier gas (helium) through the column was 2 cm^3·min^{-1}

All assays were carried out in triplicate.

Results and Discussion

Chemical characteristics of sugar beet and raw juice

Chemical composition of raw materials i.e. sugar beet pulp and raw juice used in this study is shown in Table 1. Content of solid substance in sugar beet pulp was at the level of 23.45%, while extract of raw juice was 14.96 °*Bx*.

Total sugars concentrations ranged between 13.39 g·100 g^{-1} of raw juice and 19.38 g invert sugar·100 g^{-1} of sugar beet pulp. Sucrose was the main extract component of tested raw materials (17.42 g·100 g^{-1} of sugar beet and 11.78 g·100 g^{-1} of raw juice). The pH of sugar beet pulp was 6.50 while pH of raw juice was 5.95. Nitrogen contents in raw juice and in sugar beet pulp ranged between 0.28% w·w^{-1} and 0.45% w·w^{-1} while concentrations of volatile acids varied between 0.04% w·w^{-1} and 0.12 w·w^{-1}, respectively.

Our results are consistent with literature data [Rankovic et al. 2009] with an exception of nitrogen content in raw juice. The juice described by Ranković et al. [2009] contained twice less total nitrogen (0.13–0.14% w·w^{-1}) compared to the raw juice used in our study (0.28 w·w^{-1}).

Table 1

Compositions of raw materials

Parameters	Sugar beet	Raw juice
solid substance [% [1]] total extract [°Blg [2]]	23.45[1]	14.96[2]
pH	6.50	5.95
total sugars [g invert sugar·100 g^{-1}]	19.38	13.39
reducing sugars [g invert sugar·100 g^{-1}]	1.04	0.99
sucrose [g·100 g^{-1}]	17.42	11.78
total nitrogen [% w·w^{-1}]	0.45	0.28
volatile acids as acetic acid [% w·w^{-1}]	0.12	0.04

Chemical composition of the investigated raw materials makes them attractive for alcoholic fermentation. Because they are not subjected to saccharification like starch-based feedstocks and only inexpensive mineral nitrogen sources for yeasts could be added to worts, the overall costs of bioethanol synthesis are relatively low.

The effect of process conditions on fermentation results

In the first stage of the study the effect of distillery yeast (As-4 and Ethanol-Red), supplementation of worts with $(NH_4)_2HPO_4$ and treatment of sugar beet pulp with cellulase (EC 3.2.1.4) contained in Cellustar preparation on fermentation dynamics and ethanol yield were determined. Results obtained are presented in Figure 1–2 and Table 2.

The longer initial fermentation phase was observed for all the sugar beet pulp-based wort, fermented by the yeast AS-4, both in a case of wort with and without addition of mineral nitrogen. The course of fermentation of wort treated with Cellustar preparation was analogous.

The yeast Ethanol-Red fermented more dynamically, the initial phase was relatively short in all trials. However total duration of fermentations was almost the same (ca. 68–70 h) for the worts fermented by both yeast preparations (Fig. 1).

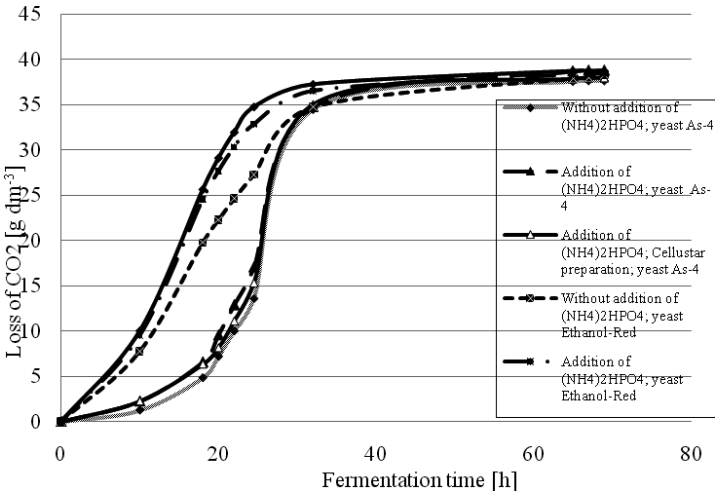

Fig. 1. Fermentation dynamics of sugar beet pulp-based worts

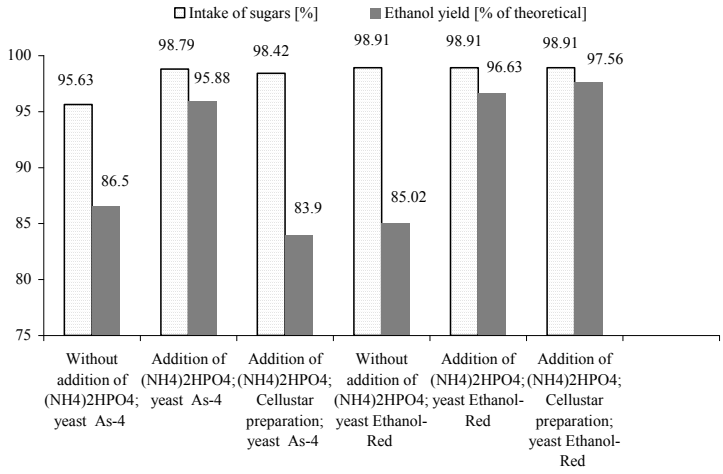

Fig. 2. Intake of sugars and ethanol yields in fermented sugar beet pulp-based worts

The analysis of worts after fermentation showed a bit lower values of residual sugars and extract (apparent and real) for trials fermented with yeast Ethanol –Red than for those fermented with strain As-4. Supplementation of sugar beet worts with $(NH_4)_2HPO_4$ was needed to keep their high fermentation activity and ethanol production. Moreover, treatment of mentioned worts with supportive enzymatic preparation containing cellulase had impact on ethanol biosynthesis. The highest ethanol concentration (5.21% $v \cdot v^{-1}$) was determined in the wort treated with Cellustar preparation and supplemented with $(NH_4)_2HPO_4$, fermented with yeast Ethanol-Red (Tab. 2).

The lowest intake of sugars (95.63%) was observed in the wort fermented by the yeast strain As-4, without supplementation with mineral nitrogen. In the remaining fermentation trials the degree of sugar assimilation varied between 98.42 and 98.91% irrespective of fermentation conditions. Also the lowest ethanol yields ranged from 85.02 to 86.5% of theoretical yield were observed in worts fermented both by the yeast As-4 and Ethanol-Red, without supplementation with nutrient for yeast. Whereas in wort fermented by the same yeast strains, but previously supplemented with $(NH_4)_2HPO_4$, ethanol yields were alike and ranged between 95.88 and 96.63% theoretical yield. Presented results proved that treatment of sugar beet pulp with supportive enzymatic preparation containing cellulase had significant impact on an increase of ethanol yield compared to reference wort (without addition of Cellustar preparation). When sugar beet pulp-based worts were supplemented with cellulase preparation, the highest ethanol yields (ca. 97.56% of theoretical) were obtained (Fig. 2).

In the second stage of our study the effect of method of raw juice-based worts pretreatment (pasteurization, autoclaving, ozonization) and their pH (5.95 and 4.80) on fermentation dynamics and ethanol yield was determined.

The course of fermentation of raw juice based-worts, irrespective of variants of fermentation, was analogous to the course of sugar beet pulp-based worts fermentations and the whole process time did not exceed 70–72 h.

Table 2

Fermentation results of sugar beet pulp-based wort

Wort before fermentation: pH 4.8; extract 16,02°Blg; reducing sugars 3,27 g invert sugar·100 cm^{-3}; total sugars 8,24 g invert sugar 100 cm^{-3}; sucrose 4.72 g 100 cm^{-3}

Fermentation condition	pH	Extract [°Blg]		Ethanol content in wort [% v·v^{-1}]	Reducing sugars [g invert sugar ·100 cm^{-3} wort]	Total sugars [g invert sugar 100 cm^{-3} wort]	Sucrose [g ·100 cm^{-3} wort]
		apparent	real				
without addition of $(NH_4)_2HPO_4$; yeast strain As-4	4.51	0.76	2.75	4.62	0.10	0.36	0.25
with addition of $(NH_4)_2HPO_4$; yeast strain As-4	4.42	0.50	2.61	5.12	0.08	0.10	0.02
with addition of $(NH_4)_2HPO_4$ and preparation Cellustar; yeast strain As-4	4.45	0.85	2.78	5.21	0.06	0.13	0.06
without addition of $(NH_4)_2HPO_4$; yeast strain Ethanol-Red	4.20	0.75	1.95	4.54	0.05	0.09	0.04
with addition of $(NH_4)_2HPO_4$; yeast strain Ethanol-Red	4.23	0.95	2.91	5.16	0.05	0.09	0.04
with addition of $(NH_4)_2HPO_4$ and preparation Cellustar; yeast strain Ethanol-Red	4.29	0.64	1.55	5.21	0.05	0.09	0.04

The pretreatment of raw juice-based worts with pasteurization, sterilization or ozonization had no significant effect on sugar intake, ethanol production and its yield in comparison with reference worts (without pretreatment). Advisable action was only correct pH of raw juice-based worts from ca 5.95 to 4.80 which ensured high productivity of ethanol. (Tab. 3, Fig. 3). Also Ogbonna et al. [2001] found that the raw sugar beet juice could be efficiently fermented to ethanol.

Table 3

Fermentation results of raw juice-based wort

Raw material	Fermentation condition (type of treatment)		Concentration [mg· dm^{-3} spirit 100% v·v^{-1}]							
			Acetaldehyde	Etyl acetate	Methanol	n-propanol	2-methyl-1-propanol	n-butanol	2-methyl-1-butanol	3-methyl-1-butanol
Sugar beet pulp	without addition of $(NH_4)_2HPO_4$; yeast strain As-4		308.44	130.30	4 236.67	561.94	1 449.56	8.38	509.08	1 618.21
	with addition of $(NH_4)_2HPO_4$; yeast strain As-4		330.59	123.47	4 459.77	630.42	1 411.24	9.66	495.46	1 610.86
	with addition of $(NH_4)_2HPO_4$ and preparation Cellustar, yeast strain As-4		327.65	141.69	5 021.90	649.42	1 456.81	9.17	508.24	1 820.86
	without addition of $(NH_4)_2HPO_4$; yeast strain Ethanol-Red		219.12	145.39	4 535.37	328.74	1 002.39	7.84	621.64	2 393.36
	with addition of$(NH_4)_2HPO_4$; yeast strain Ethanol-Red		142.94	96.73	3 743.65	307.96	865.09	6.30	524.08	1 940.60
	with addition of $(NH_4)_2HPO_4$ and preparation Cellustar, yeast strain Ethanol-Red		141.53	126.94	5 206.81	383.67	1 014.73	7.45	611.18	2 164.66
Raw juice	without treatment	pH=5.95	106.78	142.22	220.39	328.97	1 214.66	10.19	634.12	2 357.00
		pH=4.80	65.99	145.54	240.40	307.51	1 063.93	8.12	509.00	2 307.43
	pasteurization at 85ºC, 30 min	pH=5.95	48.96	173.77	195.39	312.98	1 089.13	7.91	569.59	2 176.65
		pH=4.80	44.90	192.14	207.77	321.57	1 247.30	9.21	684.92	2 618.18
	autoclaving at 121ºC (0.1 MPa), 20 min	pH=5.95	71.45	243.14	177.44	379.50	1 113.10	8.46	613.74	2 332.05
		pH=4.80	80.53	174.37	145.81	331.07	981.78	8.96	551.85	2 139.82
	ozonization (100 g $O_3 \cdot m^{-3}$, 20 min)	pH=4.39	204.20	149.62	306.14	354.99	1 296.60	8.99	262.83	1 315.80

* pH of wort after ozonization decreased from 5.95 to 4.39

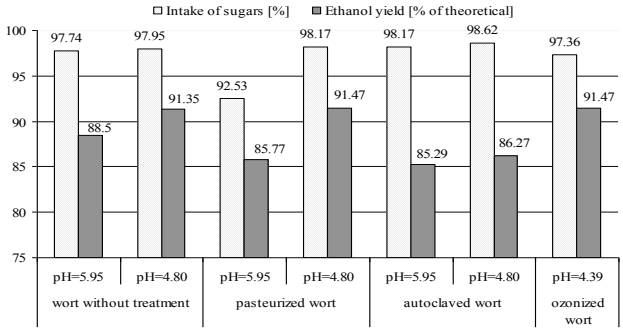

Fig. 3. Intake of sugars and ethanol yields in fermented raw juice-based wort

Analysis of chemical composition of distillates obtained

The chemical composition of distillates obtained by fermentation of sugar beet and raw juice is shown in Table 4.

Acetaldehyde dominated aliphatic carbonyl compounds present in tested samples of spirits. Majority of raw spirits obtained from fermented raw juice contained less acetaldehyde (between 44.90 mg and 106.78 mg·dm^{-3} spirit 100% v·v^{-1}) compared to distillates obtained from fermented sugar beet pulp (between 141.53 and 330.59 mg/l spirit 100% v/v). The exception was distillate produced by the fermentation of raw juice-based wort after ozonization which contained 204.20 mg acetaldehyde·dm^{-3} spirit 100% v·v^{-1}

Ethyl acetate was the most abundant among esters that were quantified in the distillates. Its concentration in spirits derived from the sugar beet pulp and raw juice varied between 96.73 and 141.69 mg·dm^{-3} spirit 100% v·v^{-1}, and between 142.22 and 243.14 mg·dm^{-3} spirit 100% v·v^{-1}, respectively.

Table 4

Chemical composition of distillates obtained by fermentation of sugar beet and raw juice

Type of treatment of raw juice-based wort	pH of wort		Extract [°Blg]		Ethanol in wort [% v·v^{-1}]	Reducing sugars [g invert sugar ·100 cm^{-3} wort]	Total sugars [g invert sugar·100 cm^{-3}wort]	Sucrose [g ·100 cm^{-3} wort]
	initial	final	apparent	real				
without treatment	5.95	5.43	0.0	2.47	7.67	0.24	0.30	0.054
	4.80	4.68	0.2	2.92	7.92	0.21	0.28	0.063
pasteurization at 85ºC, 30 min	5.95	5.50	0.1	2.45	7.44	0.18	0.24	0.054
	4.80	4.65	0.1	2.31	7.93	0.21	0.25	0.036
autoclaving at 121ºC (0.1 MPa), 20 min	5.95	5.20	0.0	2.45	7.39	0.19	0.25	0.054
	4.80	4.35	0.0	2.05	7.48	0.19	0.24	0.045
ozonization (100 g O_3·m^{-3}, 20 min)	4.39	4.00	0.0	2.51	7.93	0.51	0.59	0.036

* pH of wort after ozonization decreased from 5.95 to 4.39

Methanol concentrations in distillates derived from the sugar beet pulp and raw juice varied between 3743.65 and 5206.81 mg·dm^{-3} spirit 100% v·v^{-1}, and between 145.81 and 306.14 mg·dm^{-3} spirit 100% v·v^{-1}, respectively. Concentration of methanol was lower considerably in spirits obtained from the raw juice-based wort. It is thought to result from removing of pectinesterases associated with sugar beet pectin during raw juice extraction.

All the distillates were rich in higher alcohols irrespective of fermentation variant. Concentrations of n-propanol in all distillates obtained from the raw juice-based worts were not significantly different and ranged between 307.51 and 379.50 mg·dm^{-3} spirit 100% v·v^{-1}. Much greater differences in this respect were observed between distillates from the sugar beet pulp-based worts. Concentration of n-propanol was the lowest when the sugar beet pulp was fermented without addition of $(NH_4)_2HPO_4$ by the yeast Ethanol-Red (307.96 mg·dm^{-3} spirit 100% v·v^{-1}) and the highest when the sugar beet pulp was fermented with addition of $(NH_4)_2HPO_4$ and Cellustar preparation, and this process was conducted by the strain As-4 (649.42 mg·dm^{-3} spirit 100% v·v^{-1})

The quantity of 2-methyl-1-propanol in distillates from the sugar beet pulp and raw juice-based worts varied between 865.09 and 1449.56 mg·dm^{-3} spirit 100% v·v^{-1}. Concentration of n-butanol in all the tested distillates was relatively small (6.30–10.19 mg·dm^{-3} spirit 100% v·v^{-1}). Contents of 2-methyl-1-butanol in the distillates obtained from the sugar beet pulp as well as from raw juice were alike and ranged between 495.46 and 684.92 mg·dm^{-3} spirit 100% v·v^{-1}. The exception was distillate produced by the fermentation of raw juice-based worts after ozonization, which contained the lowest amount of this alcohol (262.83 mg·dm^{-3} spirit 100% v·v^{-1}) 3-methyl-1-butanol was the most abundant of isoamyl alcohols detected in the distillates obtained from fermented both sugar beet pulp and raw juice-based worts (1610.86–2618.18 mg·dm^{-3} spirit 100% v·v^{-1}). The lowest concentration of this compound (1315.80 mg·dm^{-3} spirit 100% v·v^{-1}) was determined in spirit obtained from raw juice subjected to ozonization (Tab. 4).

Conclusions

Results of our study proved that sugar beet and raw juice are attractive raw materials for ethanol production without the need of additional technological operations increasing costs of biosynthesis. Sucrose is principal component of their extract and therefore the only necessary operations before alcoholic fermentation are pH regulation and possible addition of mineral nitrogen sources. Both yeast As-4 and Ethanol-Red ensured the high ethanol productivity and good process dynamics.

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AUTHORS

Augšpole Ingrida – Latvia University of Agriculture, Latvia

Balcerek Maria – Technical University of Łódź, Poland

Błażewicz Józef – Wrocław University of Environmental and Life Sciences, Poland

Borowski Sebastian – Technical University of Łódź, Poland

Chrzanowska Józefa – Wrocław University of Environmental and Life Sciences, Poland

Dąbrowska Anna – Wrocław University of Environmental and Life Sciences, Poland

Drozdowska Krystyna – Wrocław University of Environmental and Life Sciences, Poland

Dziekońska Urszula – Technical University of Łódź, Poland

Dziugan Piotr – Technical University of Łódź, Poland

Eckert Ewelina – Wrocław University of Environmental and Life Sciences, Poland

Gawrysiak-Witulska Marzena – Poznań University of Life Sciences, Poland

Karklina Daina – Latvia University of Agriculture, Latvia

Kordialik-Bogacka Edyta – Technical University of Łódź, Poland

Kozłowska Katarzyna – Wrocław University of Environmental and Life Sciences, Poland

Kruma Zanda – Latvia University of Agriculture, Latvia

Księżopolska Mirosława – Technical University of Łódź, Poland

Liszewski Marek – Wrocław University of Environmental and Life Sciences, Poland

Nogala-Kalucka Małgorzata – Poznań University of Life Sciences, Poland

Nowak Adriana – Technical University of Łódź, Poland

Patelski Piotr – Technical University of Łódź, Poland

Pielech-Przybylska Katarzyna – Technical University of Łódź, Poland

Pokora Marta – Wrocław University of Environmental and Life Sciences, Poland

Riekstina-Dolge Rita – Latvia University of Agriculture, Latvia

Seglina Dalija – Latvia State Institute of Fruit Growing, Latvia

Siepka Ewelina – Wrocław University of Environmental and Life Sciences, Poland

Siger Aleksander – Poznań University of Life Sciences, Poland