Agronomy Research

Established in 2003 by the Faculty of Agronomy, Estonian Agricultural University

Aims and Scope:

Agronomy Research is a peer-reviewed international Journal intended for publication of broad-spectrum original articles, reviews and short communications on actual problems of modern biosystems engineering incl. crop and animal science, genetics, economics, farm- and production engineering, environmental aspects, agro-ecology, renewable energy and bioenergy etc. in the temperate regions of the world.

Agronomy Research online:

Agronomy Research is available online at: https://agronomy.emu.ee/

Acknowledgement to Referees:

The Editors of *Agronomy Research* would like to thank the many scientists who gave so generously of their time and expertise to referee papers submitted to the Journal.

Abstracted and indexed:

SCOPUS, EBSCO, DOAJ, CABI Full Paper and Clarivate Analytics database: (Zoological Records, Biological Abstracts and Biosis Previews, AGRIS, ISPI, CAB Abstracts, AGRICOLA (NAL; USA), VINITI, INIST-PASCAL.)

Subscription information:

Institute of Technology, EMU
Fr.R. Kreutzwaldi 56,
51006 Tartu,
ESTONIA
e-mail: timo.kikas@emu.ee

ISSN 1406-894X

CONTENTS

Agronomy Research **19**(S3), 1227–1233, 2021
https://doi.org/10.15159/AR.21.077

Characterization of red raspberry (*Rubus idaeus* L.) for their physicochemical and morphological properties

I. Augšpole[1*], F. Dimiņš[1], I. Romanova[2] and A. Liniņa[2]

[1]Latvia University of Life Sciences and Technologies, Faculty of Food Technology, Rīgas iela 22, LV-3001 Jelgava
[2]Latvia Latvia University of Life Sciences and Technologies, Faculty of Agriculture, Lielā iela 2, LV-3001 Jelgava, Latvia
[*]Correspondence: ingrida.augspole@llu.lv

Received: January 30th, 2021; Accepted: May 9th, 2021; Published: May 13th, 2021

Abstract. Different raspberry cultivars are grown in Latvia suitable both for fresh market and for processing. Fresh local red raspberry is available for consumers from July to October. Information about the physical, chemical, and morphology properties of raspberry fruit is essential for understanding the behavior of the product during the postharvest operations such as harvesting, transporting, sorting, grading, packaging and storage. Knowledge of the physicochemical properties of red raspberries is essential because variations in the levels of these properties may exist between cultivars. New varieties of plants were used to describe and compare the fruit quality of red raspberry cultivars: 'Daiga', 'Shahrazada', 'Norna' and 'Polana' grown in Zemgales region of Latvia. The samples were collected from farm 'Pluģi' the full stage of ripening. The current research aimed to investigate and determine the chemical composition (total phenolic content (TPC), total flavonoid content (TFC), titratable acidity (TAc), total soluble solids content of raspberry fruits, the physical parameter pH, and color. Presented morphological parameters of fruit included their weight, receptacle length (Rl), receptacle width (Rw), fruit length (Fl), fruit width (Fw), weight of fruit (M), number of seeds. Research results showed significant differences in all chemical and physical characteristics as well as in morphology properties ($P < 0.05$) between cultivars.

Key words: raspberry, *Rubus idaeus* L, physicochemical, physical and morphological properties.

INTRODUCTION

Raspberries (*Rubus idaeus* L.) are a member of the *Rosaceae* family, grown as a perennial crop. Red raspberry contains numerous phenolic compounds with potential health benefits (Ilhami et al., 2011). Phenolic compounds are ubiquitous in plants which collectively synthesize several thousand different chemical structures characterized by hydroxylated aromatic ring(s). These compounds play several important functions in plants. They represent a striking example of metabolic plasticity enabling plants to adapt to changing biotic and abiotic environments and provide to plant products color, taste, technological properties and putative health promoting benefits (Augšpole et al., 2018b).

Fresh raspberries have a very short shelf life and are generally only readily available around summer. Most of the produced raspberries worldwide are processed, i.e., frozen and sold within different frozen fraction blocks or in jams and sauces. However, there has been an increasing demand for fresh raspberries out-of-season lately, and so many producers appear to be interested in growing primocane fruiting raspberry cultivars (Beekwilder et al., 2005; Atkinson et al., 2006). Today, raspberry fruit is being used in the production of bakery goods, jams, jellies, beverages, dairy products like ice cream and yogurt, fruit syrups, and many other specialty products such as fruited honey. Red raspberry juice is used in blended fruit drinks and other food products (Riaz & Bushway, 1996). The most significant health benefits of raspberry fruits are attributed to the phenolic compounds, such as flavonoids, phenolic acids, and tannins (Paredes-Lopez et al., 2010). It is believed that raspberry has a higher antioxidant capacity than most other fruits and vegetables (Alibabić et al., 2018). Polyphenols are a group of compounds synthesized exclusively by plants, especially for the protection against UV-radiation and the activity of pathogens. About 8,000 plant polyphenol compounds have been identified so far, whereas only some hundred occur in edible plants (Manach et al., 2004; Linina et al., 2020). Phenolic compounds of plants can be divided into two broad categories: 1) phenolic acids (oxometallic) and their derivatives, and 2) flavonoids (polyphenols). Phenolic acids and their derivatives, mainly esters, have more basic structures (Dimiņš & Augšpole, 2019). The flavonoid class of compounds has a more complex molecular structure, which is usually heterocyclic with an attracted phenolic ring(s).

Flavonoids include anthocyanidins (water-soluble pigments, which are oxidized flavonoles), catechins, isoflavones, and proanthocyanidins (David et al., 2008). In addition, flavonoids are known to inhibit lipid-peroxidation, platelet aggregation, capillary permeability and fragility, cyclooxygenase and lipoxygenase enzyme activities, flavonoids act as antioxidants, free radical scavengers or chelators of divalent cation (Augšpole et al., 2018b).

Organization (WHO) emphasized the importance of the antioxidant activity of phenolic components, especially from small colorful fruits, for prevention of the most important health problems namely cardiovascular diseases, diabetes, cancer, and obesity (The World Health Report, 2002; Stapleton et al., 2008). Raspberry is also an excellent source of vitamin C, a very powerful antioxidant with anticancer and immunomodulatory properties and known to prevent colds (Alibabić et al., 2018).

Therefore, the goal of raspberry production is to grow a cultivar with good productivity, large fruit, and excellent quality. This study aimed to explore the four most common cultivars of raspberry ‘Daiga’, ‘Shahrazada’, ‘Norna’ and ‘Polana’, which are grown in Zemgales region farm ‘Pluģi’ of Latvia. The study aimed to compare the chemical and morphological characteristics of different raspberry cultivars.

MATERIALS AND METHODS

Investigations were carried out at the Latvia University of Life Sciences and Technologies, Institute of Soil and Plant Sciences. In the experiment, the following red raspberry cultivars: ‘Daiga’, ‘Shahrazada’, ‘Norna’ and ‘Polana’ grown in the Zemgales region (GPS-coordinates: N56° 33' 29.5302", E23° 46' 26.04") of Latvia. The samples were collected from the farm ’Pluģi’ at fully stage of ripening.

Titrable acidity (TAc) was determined titrimetrically (Duma et al., 2019) with a solution of sodium hydroxide 2 ± 0.0001 g of raspberry was quantitatively transferred in 100 mL tubes, added 40 mL of distilled water (0.055 μS cm^{-1}) and mixed. After 30 minutes, the solutions were centrifuged for 10 min at 5,000 rpm. For determination, 10 mL of the supernatant was titrated with 0.1 M NaOH in the presence of indicator phenolphthalein, and results expressed as g of citric acid 100 g^{-1} raspberry sample fresh matter (FM).

The total soluble solids content (expressed as BRIX degree) was measured with a digital refractometer (A.KRÜSS Optronic Digital Handheld Refractometer DR301-95), calibrated at +20 °C ± 2 °C with distilled water (deviation of the measuring instrument face value ± 0.1%) by standard method ISO 2173:2003.

The total phenolics content (TPC) of raspberries was analysed spectrometrically according to the Folin-Ciocalteu method (Dewanto et al., 2002; Kaškoniene et al., 2009). Each sample of raspberries was diluted with distilled water and filtered. This solution was then mixed with 0.2 N Folin - Ciocalteu reagent for 5 min and then a solution of sodium carbonate was added. After incubation at room temperature for 2 h, the absorbance of the reaction mixture was measured at 760 nm against a methanol blank. Gallic acid was used as a standard to produce the calibration curve. The total phenolic content was expressed in mg of gallic acid equivalents (GAE) and 100 g^{-1} of raspberry fresh matter (FM).

The total flavonoid content (TFC) was expressed as quercetine equivalent mg QE 100 g^{-1} was determined using the Dowd method. 2% $AlCl_3$ solution in methanol was mixed with raspberries. Absorbance readings at 415 nm were taken after 10 min against a blank sample consisting of 5 mL coffee solution with 5 mL methanol without $AlCl_3$. The total flavonoid content was determined using a standard curve with quercetin as a standard. The total flavonoid content was expressed in mg of quercetin equivalents (QE) 100 g^{-1} of raspberry fresh matter (FM), described by (Meda et al., 2005; Xu & Chang, 2007; Augšpole et al., 2018a) with some modifications.

The colors of the raspberry samples were evaluated by measuring CIE L*, a*, b* parameters using "ColorTec–PCM/PSM" (ColorTec Associates, Clinton, USA). L*, a*, and b* indicate whiteness/darkness, redness/greenness, and blueness/yellowness values, respectively. The maximum color value for L* is 100, which would be a perfect reflecting diffuser. The minimum color for L* would be zero, which would be black. The value colors of the a* and b* axes have no specific numerical limits. Positive a* is red and negative a* is green. Positive b* is yellow and negative b* is blue (Augspole & Rakcejeva, 2013).

pH value was measured using Jenway 3510 pH meter, by the standard method LVS ISO 5542:2010.

Statistical analysis. The data of the research was analysed by the statistical and mathematical methods (standard deviation, mean). Data compared by the analysis of variance (ANOVA) and significance was defined at $P < 0.05$. For the data analysis the Microsoft Excel software of the version 2019 was used.

RESULTS AND DISCUSSION

The mean of total phenolic content (TPC), total flavonoid content (TFC), titrable acidity (TAc), total soluble solids (TSS) content, and pH in the raspberry cultivars

selected for the study are shown in Table 1. TPC significantly ranged between raspberry cultivars ($P < 0.05$). The highest TPC was detected for the 'Shahrazada' raspberry cultivars (148.87 ± 3.43 mg GAE 100 g^{-1} FM). The lowest TPC for the 'Polana' raspberry cultivars (111.96 ± 3.11 mg GAE 100 g^{-1} FM). In turn, Alibabić et al. (2018) from Bosnia and Herzegovina University of Bihać reported significantly higher and similar values - total phenols of different raspberry cultivars were 102.0–521.4 mg GAE 100 g^{-1}, (FM). Latvian researchers Ozola et al. (2019) reported that the total phenol content depends on the extracting solvent. In turn, Turkmen et al. (2006) mentioned that it is higher in ethanol extract than aqueous extract, but Shirin & Jamuna (2010) found the highest total polyphenol content in water extracts.

Flavonoids are phenolic compounds that are common in different plants. These compounds have a wide range of biological functions - they protect plants from biotic and abiotic stresses, and they actively participate in the interaction between plants and the environment (Amalesh et al., 2011). Flavonoid content is one of the important influencing plant nutrition quality. Flavonoids content affects the color, flavor, and fragrance of plants (Sergejeva et al., 2018). The total flavonoid content in analyzed raspberry cultivars ranged from 411.36 ± 2.99 to 475.00 ± 2.04 mg QE 100 g^{-1}, FM (Table 1). It can be explained by the fact that the total phenolic properties of the raspberry samples are determined not only by phenolic compounds, but also trace elements, vitamins, individual amino acids and enzymes, etc. (Dimiņš & Augšpole, 2019).

However, the relationship between total soluble solids (TSS) (°Brix) and pH may be observed (Table 1). That is, the higher the content of TSS, the greater the pH content. The content of soluble solids (°Brix) depended on raspberry cultivar. Slightly higher content was observed in cultivars 'Daiga' 10.58 ± 0.75 °Brix and 'Norna' 10.28 ± 0.32 °Brix. Raspberry cultivars 'Norna' and 'Daiga' distinguish themselves with the significantly lowest amount of TAc (1.81 ± 0.32 and 2.06 ± 1.81 ± 0.32 g 100 g^{-1}, FM). In turn researcher Sergejeva et al. (2018) reported that the soluble sugars are main product of photosynthesis and effects plant nutrition.

Table 1. Raspberry cultivars average total phenolics, total flavonoids, titrable acidity, and total soluble solids, and pH

Raspberry cultivars	TPC, mg GAE 100 g^{-1}, (FM)	TFC, mg QE 100 g^{-1}, (FM)	TAc, g 100 g^{-1}, (FM)	TSS (°Bix)	pH
'Daiga'	141.76 ± 2.53	475.00 ± 2.04	2.06 ± 0.21	10.58 ± 0.75	3.23 ± 0.12
'Shahrazada'	148.87 ± 3.43	456.06 ± 3.44	2.42 ± 0.11	9.78 ± 0.11	3.03 ± 0.32
'Norna'	142.35 ± 1.05	465.91 ± 2.11	1.81 ± 0.32	10.28 ± 0.32	3.21 ± 0.12
'Polana'	111.96 ± 3.11	411.36 ± 2.99	2.42 ± 0.11	8.33 ± 0.21	2.91 ± 0.41

TPC – total phenolic; TFC – total flavonoid; TAc – titrable acidity; TSS – total soluble solids.

It was found that the titratable acidity in fresh raspberry cultivar samples at harvest was in the range of 1.81 ± 0.32 g 100 g^{-1} (FM) till 2.42 ± 0.11 g 100 g^{-1}. Our findings are lower that the results noted by Riaz & Bushway (1996) who determined the titratable acidity was from 2.35 g 100 g^{-1} (FM) till 2.64 g 100 g^{-1} (FM) in fresh raspberry cultivar samples. Researchers Vinha et al. (2013) reported that high levels of acidity are responsible for the stability of vitamin C during storage of fruits and vegetables.

The results of pH level performed on the fresh raspberry cultivar samples formulation revealed that the pH ranged from 2.91 to 3.23 (Table 1), which indicates an acidic environment. The difference between the lowest and highest pH was not significant - 0.32 units ($P > 0.05$). The highest pH level was found in fresh raspberry cultivar samples in descending order: raspberry cultivar 'Daiga' 3.23 ± 0.12, 'Norna' 3.21 ± 0.12, 'Shahrazada' 3.03 ± 0.32 and 'Polana' 2.91 ± 0.41. The lowest pH value results were gained in Moore (2006) where the pH value in raspberries ranged from 2.41 to 2.76. The obtained results indicated that the selected red raspberry cultivar fruits can be estimated as a rich source of biological active compounds. This study demonstrated that different red raspberry cultivar fruits have high potential value for fruit growers as well as for food manufacturers because of their high polyphenolic contents.

The results of fruit dimensions (fruit length and width, seed number, receptacle length, and width) as well as fruit weight are shown in Table 2. Significant differences ($P < 0.05$) can be observed between the cultivar 'Shahrazada' and the other three examined cultivars. All morphological parameters were higher ($P < 0.05$) than the dimensions of the 'Daiga', 'Norna' and 'Polana' raspberry cultivars. In turn, the total seed number of 'Shahrazada' was significantly lower compared to the other four raspberry cultivars. The morphological characteristics of the fruit, including chemical and sensory characteristics, vary among cultivars and depend on many factors, such as environmental factors (temperature, rainfall, soil type), irrigation, yield efficiency, ripeness of harvested fruits, and agrotechniques (Alibabić et al., 2018).

Table 2. Raspberry fruit morphological characteristics

Raspberry cultivars	M, g	Lf, mm	Wf, mm	TSN	Rl, mm	Rw, mm
'Daiga'	2.78 ± 0.33	6.0 ± 1.3	49.83 ± 3.5	73 ± 9	49.83 ± 3.40	4.00 ± 0.50
'Shahrazada'	4.40 ± 0.81	11.0 ± 2.3	72.55 ± 4.2	66 ± 4	72.55 ± 4.10	4.75 ± 1.50
'Norna'	3.68 ± 0.21	7.25 ± 2.0	62.55 ± 2.1	57 ± 5	62.55 ± 2.63	4.13 ± 1.00
'Polana'	2.74 ± 0.41	5.25 ± 1.7	54.77 ± 3.0	49 ± 7	54.83 ± 3.11	4.50 ± 1.00

M – fruit weight; Lf – fruit length; Wf – fruit width;TSN – total seed number of fruit; Rl – receptacle length; Rw – receptacle width.

It is assumed that the greater intensity of the color of plants indicates its higher nutritive value (Shibghatallah & Suhandono, 2013). Significant differences were found between the values of the color components L*, a*, and b* of 'Norna', 'Daiga', 'Shahrazada', and 'Polana' raspberry cultivars (Table 2). The highest L* values, related to the lightness, were found for fresh 'Daiga', 'Shahrazada' and 'Polana' raspberry cultivars, respectively (29.13 ± 1.43), (27.09 ± 1.74) and (27.18 ± 1.84) (Table 3). The lowest value of this color parameter was determined for 'Norna' raspberry cultivar (23.69 ± 2.42) (showing a darker color intensity). For fresh raspberry cultivar samples 'Daiga' (17.32 ± 1.42) and 'Shahrazada' (16.29 ± 1.32) the

Table 3. Raspberry fruit color L*, a*, b*

Raspberry cultivars	L*	a*	b*
'Daiga'	29.13 ± 1.43	17.32 ± 1.42	7.77 ± 2.11
'Shahrazada'	27.09 ± 1.74	16.29 ± 1.32	13.56 ± 1.42
'Norna'	23.69 ± 2.42	13.56 ± 1.22	6.03 ± 0.94
'Polana'	27.18 ± 1.84	15.81 ± 2.03	6.26 ± 1.05

highest b* value was determined. This color component is related to the yellowness. In turn, the highest values of parameter a* were obtained for raspberry cultivar samples (13.56 ± 1.42). This color component is related to the redness. Our results are also a little lower than the data of Patrick P. Moore (2006) who analysed the raspberry fruit colors L*, a*, b*. That can be explained with the fact that there are many factors - raspberry cultivar, as well as environmental growth factors such as temperature, light, and soil properties, that affect raspberry fruit color. As well as the region and conditions for growing, the studied plants are essential.

CONCLUSIONS

1. This study generated valuable information on the biologically active compounds of different red raspberry cultivar species grown in Zemgales region farm 'Pluģi' and it highlights the crucial influence of cultivar on the physiochemical properties of raspberry fruits.
2. Raspberry represents a diverse source of potentially healthy antioxidants and as such can provide a useful component to our daily diet. The raspberry cultivar fruits are rich source of total phenolics, total flavonoids, titrable acidity, and total soluble sugar, demonstrating its potential use as a food additive.
3. Significant differences were determined between the color components L*a*b* of fresh raspberry cultivar samples.
4. Knowledge of raspberry composition will give the food processor the option to select the proper cultivar for a particular use. Information on raspberry composition at harvest will also help for the fresh market.

REFERENCES

Alibabić, V., Skender, A., Bajramović, M., Šertović, E. & Bajrić, E. 2018. Evaluation of morphological, chemical, and sensory characteristics of raspberry cultivars grown in Bosnia and Herzegovina. *Turkish Journal of Agriculture and Foresty* **42**, 67–74.

Amalesh, S., Gouranga, D. & Sanjoy, K.D. 2011. Roles of flavonoids in plants. *International Journal of Pharmacy and Pharmaceutical Sciences* **6**(1), 12–35.

Atkinson, C.J., Doddis, P.A.A., Ford, Y.Y., J.Le, Taylor, J.M. & Blake, P.S. 2006. Effects of cultivar, fruit number and reflected photosynthetically active radiation on Fragaria x ananasa productivity and fruit ellagic and ascorbic acid concentrations. *Annals of Botany*, **97**, 429–441.

Augšpole, I., Dūma, M. & Ozola, B. 2018a. Bioactive compounds in herbal infusions. *Agronomy Research* **16**(2), 1322–1330.

Augšpole, I., Dūma, M., Cinkmanis, I. & Ozola B. 2018b. Herbal teas as a rich source of phenolic compounds. *Chemija.* **29**(4), 257–262.

Augspole, I. & Rakcejeva, T. 2013. Effect of hydrogen peroxide on the quality parameters of shredded carrots. **In:** *Proceedings of Annual 19th International Scientific Conference Research for Rural Development,* Jelgava, Latvia, 91–97.

Beekwilder, J., David Hall, R. & De Vos, R. 2005. Identification and dietary relevance of antioxidants in raspberry. *BioFactors* **23**(4), 197–205.

David, W., Reische, Dorris, A., Lillard, Ronald, R. & Eiten, M. 2008. Antioxidants, in: Casimir C.Akoh and David B.Min (Eds.), Food Lipids. *Chemistry, Nutrition, and Biotechnology,* CRC Press (Taylor & Francis Group), New York, 409–430.

Dewanto, V., Wu, X., Adom, K.K. & Liu, R.H. 2002.Thermal processing enhances the nutritional value of tomatoes by increasing total antioxidant activity. *Journal of Agricultural and Food Chemistry* **50**(10), 3010–3014.

Dimiņš, F. & Augšpole, I. 2019. Total phenolic, antioxidant activities and flavonoid contents of herbal syrups. *Key Engineering Materials. Materials Science and Applied Chemistry II* **800**, 60–64.

Duma, M., Alsina, I., Dubova, L., Augspole, I. & Erdberga, I. 2019. Suggestions for consumers about suitability of differently coloured tomatoes in nutrition. **In:** *FoodBalt 20019: 13th Baltic conference on food science and technology "Food. Nutrition. Well-Being": conference proceedings.* Jelgava: LLU, 261–264.

Ilhami, G., Fevzi, T, Ramazan, C., mine, B., Ahmet, C.G. & Ummugulsum, E. 2011. Pomological Features, Nutritional Quality, Polyphenol Content Analysis, and Antioxidant Properties of Domesticated and 3 Wild Ecotype Forms of Raspberries (*Rubus idaeus* L.). *Journal of Food Science* **76**(4), 585–593.

Kaškoniene, V., Maruška, A., Kornyšova, O., Charczun, N., Ligor, M. & Buszewski, B. 2009. Quantitative and qualitative determination of phenolic compounds in honey, *Chemine Technologija* **3**(52), 74–80.

Linina, A., Augspole, I., Romanova, I. & Kuzel, S. 2020. Winter rye (*Secale cereale* L.), antioxidant capacity, total phenolic content and quality indices. *Agronomy Research* **18**(S3), 1751–1759.

Manach, C., Scalbert, A., Morand, Ch., Remesy, Ch. & Jimenez, L. 2004. Polyphenols: Food source and bioavailability. *The American Journal of Clinical Nutrition* **5**, 727–747.

Meda, A., Lamien, C.E., Romito, M., Millogo, J. & Nacouluma, O.G. 2005. Determination of the total phenolic, flavonoid and proline contents in Burkina Fasan honey, as well as their radical scavenging activity, *Food Chemistry* **91**, 571–577.

Moore, P.P. 2006. 'Cascade Dawn' Red Raspberry. *HortScience* **41**(3), 857–859.

Ozola, B., Augspole, I., Duma, M. & Kreicbergs, V. 2019. Bioactive compounds in fresh and dried ginger root (Zingiber officinale). **In:** *FoodBalt 2019: 13th Baltic conference on food science and technology „Food. Nutrition. Well-Being": conference proceedings*, Jelgava: LLU, 265–268.

Paredes-Loppez, O., Cervantes-Ceja, M.L., Vigna-Perez, M. & Hernandez-Perez, T. 2010. Berries: Improving Human Health and Healthy Aging, and Promoting Quality of Life-A Review. *Plant Foods for Human Nutrition* **65**(3), 299–308.

Riaz, M.N. & Bushway, A.A. 1996. Compositional analysis of red raspberry cultivars grown in Maine. *Journal of Food Quality* 19, 457–465.

Sergejeva, D., Alsina, I., Duma, M., Dubova, L., Augspole, I., Erdberga, I. & Berzina, K. 2018. Evalution of different lighting sources on the growth and chemical compootanysition of lettuce. *Agronomy Research* **16**(3), 892–899.

Shibghatallah, K. & Suhandono, V.K. 2013. Measuring leaf chlorophyll concentration from its color: A way of monitoring environment change in plantations. *Biological Physics.* **In:** *AIP Conference Proceedings* **1554**(1), 210–213.

Shirin, A.P.R. & Jamuna, P. 2010. Chemical composition and antioxidant properties of ginger root (*Zingiber officinale*). *Journal of Medicinal Plants Research* **4**(24), 2674–2679.

Stapleton, A.P, James, E.M, Goodwill, G.A. & Frisbee, J.C. 2008. Obesity and vascular dysfunction. *Pathophysiology* **15**, 79–89.

The World Health Report 2002. Reducing risks and promoting healthy life. Geneva, *World Health Organization*, 2002.

Turkmen, N., Sari, F. & Velioglu, Y. 2006. Effects of extraction solvents on the concentration and antioxidant activity of black and black mate tea polyphenols determined by ferrous tartrate and Folin-Ciocalteu methods. *Food Chemistry* **99(**4), 835–841.

Vinha, A.F., Barreira, S.V.P., Castro, A., Costa, A. & Oliveira, M.B.P.P. 2013. Influence of the Storage Conditions on the Physicochemical Properties, Antioxidant Activity, and Microbial Flora of Different Tomato (*Lycopersicon esculentum* L.) Cultivars. *Journal of Agricultural Science* **5**(2), 118–128.

Xu, B.J. & Chang, S.K.C. 2007. A comparative study on phenolic profiles and antioxidant activities of legumes as affected by extraction solvents. *Journal of Food Science* **72**(2), 159–166.

Agronomy Research **19**(S3), 1234–1248, 2021
https://doi.org/10.15159/AR.21.067

Effects of α-amylase, endo-xylanase and exoprotease combination on dough properties and bread quality

M. Kostyuchenko[1,*], V. Martirosyan[1], M. Nosova[1], G. Dremucheva[1], E. Nevskaya[1] and O. Savkina[2]

[1]Scientific Research Institute for the Baking Industry, Bolshaya Cherkizovskaya Street 26A, RU107553 Moscow, Russia
[2]St.Petersburg branch Scientific Research Institute for the Baking Industry, Podbelskogo highway 7, RU196608, St. Petersburg, Pushkin, Russian Federation
[*]Correspondence: kostyuchenkomn@yandex.ru

Received: January 31st, 2021; Accepted: May 2nd, 2021; Published: May 6th, 2021

Abstract. The enzymes composition is an actual alternative to chemicals to improve functional properties of flours and to generate changes in the structure of the dough and bread quality. The objective of this study was to analyze the individual and synergistic effects of enzymes preparation (α-amylase, endo-xylanase and exoprotease), newly produced in Russia, on dough properties and bread quality made from wheat flour with different amylolytic activity. Reofermentometric results revealed decreases in gas-forming capacity of dough by 10.0–13.9% when single α-amylase preparates were used. The α-amylase addition had significant effect on gas retention coefficient in flour possessed low amylolytic activity. The effect of endo-xylanase and exoprotease on hydration and amount of wheat gluten was established. The fractional composition of gluten proteins in the dough made with combination of endo-xylanase and exoprotease was established using Lowry method immediately after kneading and after fermentation. It was found that mainly water-soluble, alcohol-soluble and alkaline-soluble proteins were undergone by transformation. The bread with enzymes had a higher specific volume, porosity and aldehyde content and lower shape stability indicator than the control bread made without enzymes. Bread with enzymes was characterized by tenderer and not crumbly crumb with developed thin-walled uniform porosity compared to the control. The crusts were more brightly colored. The combined usage of α-amylase and endo-xylanase and exoprotease retarded bread staling during 5-day storage period. New enzyme composition may be a potentially strong candidate for future applications in the bread-making industry.

Key words: alpha-amylase, bread quality, dough, endo-xylanase, enzymes, exopeptidase.

INTRODUCTION

Functional properties and enzymatic activity of flours greatly depend on diverse factors such as wheat variety and growing conditions. Enzymes preparation usage is alternative to chemical compounds which are used in bread making as a way of adjusting the variations in flour properties. Chemical improvers have been a common way of flour quality compensation for many years (Cauvain & Young, 2007). But last decades many

of chemicals are associated with health hazards, for example, azodicarbonamide may cause allergic reactions (Arts, & Kimber, 2017), semicarbazide produced from azodicarbomide and potassium bromate may cause some forms of cancer (Kornbrust et al., 2012; Ibrahim et al., 2019; Shanmugavel et al., 2019). The replacement of chemical dough improvers by enzymes allows production of safety products. The enzymes composition also allows generating changes in the structure of the dough and improving bread quality, but they are generally recognized as safe (GRAS) and do not remain active in the final product after baking. Therefore, enzymes do not have to mark on the label, which is an additional commercial advantage.

Nowadays a wide range of enzymes is available for bread-making industry. A variety of aims may be pursued by enzyme addition. Enzymes provides improved dough handling and technological process tolerance, increased bread specific volume, finer crumb structure, softer crumb, extended shelf life (Codina & Leahu, 2009; Goesaert et al., 2009; Ahmad et al., 2013; Kornbrustet al., 2012; Sanz-Penella et al., 2014; Ait Kaki El-Hadef El-Okki et al., 2017).

Amylases have been used in bread-making as standardizing and anti-staling agents of flour (Goesaert et al., 2007; Lagrain et al., 2008; Goesaert et al., 2009; He et al., 2017). The use of α-amylase in bread formulations significantly improves the product quality, including bread volume, texture, firmness, shelf life and flavor (Lagrain et al., 2008; Goesaert et al., 2009; Sahnoun et al., 2013; Sanz-Penella et al., 2014). α-Amylase functions are related to the reduction of dough viscosity during starch gelatinization as a result of the action of the enzyme (Goesaert et al., 2009; Sahnoun et al., 2013; Grewal et al, 2015; Bueno et al., 2016). Amylase decreases the molecular weight of starch polymers and causes the formation of maltodextrins, which contribute to increased crumb softness, improved crumb elasticity and decreased staling rate (Rojas et al., 2001; Hug-Iten et al., 2003; Goesaert et al., 2009; Calvin, O., 2016). Fungal α-amylase could be used to restore loaf volume losses caused by low protein content in flour (Goesaert et al., 2009; Sahnoun et al., 2013). α-Amylase increases the level of fermentable sugars in the dough, thus promoting the fermentation of yeast (Cauvain & Young, 2007) and the formation of Maillard reaction products, which, in turn, intensify bread flavor and crust color (Goesaert et al., 2009; Ait Kaki El-Hadef El-Okki et al., 2017).

The application of xylanase has increased for the last few decades owing to its potential effectiveness in bread making. The xylanase may enhance dough rheological characteristics (extensibility, flexibility, stability), bread quality (crumb structure, texture, porosity), and shelf life (Driss et al., 2012; Cunha et al., 2018; Yegin et al., 2018; Sharma et al., 2020). Endo-xylanases increase dough viscosity and promote an improvement in the dough rheology by increasing the extensibility and malleability of the dough caused by hydrolysis of water-unextractable arabinoxylans (Ahmad et al., 2013). Xylanase acting on water-unextractable arabinoxylans provide more reduction in water absorption (Hardt, Boom & van der Goot, 2014; Bueno et al., 2016; Yegin et al., 2018). Xylanase can convert parts of insoluble polymers into soluble. It affects the water balance between components in the dough, therefore decreasing dough firmness, increasing volume and creating finer and more uniform crumbs. Enhanced water absorption is a desirable property as it leads to reduced dough stickiness and improved dough processing. Dough become more 'machine-friendly' and does not stick to the machinery parts (Autio 2009; Yegin et al., 2018; Both, 2020). Different xylanase preparations have different effects on arabinoxylans in terms of its scission point and

reaction products and therefore, they show different effects on bread making (Bueno et al., 2016; Yegin et al., 2018).

Proteases are used in the production of bread and other baked goods (Melim Miguel et al., 2013; Heredia-Sandoval et al., 2016). Proteases can be subdivided into two major groups according to their site of action: exopeptidases and endopeptidases. Exopeptidases are those proteases that cleave the peptide bond proximal to the amino or carboxy termini of the substrate (cleave N- or C-terminal peptide bonds of a polypeptide chain). Exopeptidase addition helps to achieve a partial gluten hydrolysis for improving rheological properties and machinability. These enzymes can be added to reduce mixing time, to decrease dough consistency, to assure dough uniformity, to regulate gluten strength in bread, to control bread texture and to improve flavor (Goesaert et al., 2009; Melim Miguel et al., 2013).

The wheat-milling and bread-making industries follow the dosages indicated by the enzyme suppliers. But obviously enzyme effects depend on the initial characteristics of the flour (Bueno et al., 2016; Kin & Yoo, 2020). For the best results, enzymes should be used at optimum levels, as the over or under dosage has adverse effects on the bread. Modern investigations have shown that it is a good strategy to use enzymes combination because of the synergistic effects which provide better results as compared to its sole use.

The objective of this study was to analyze the individual and synergistic effects of enzymes preparation (α-amylase, endo-xylanase and exo-protease), newly produced in Russia, on dough properties and quality of bread made from wheat flour with different amylolytic activity.

MATERIALS AND METHODS

Characteristic of ingredients

Wheat flour

Two wheat flour samples obtained from different milling companies located in Russia were used for this study. Flour had different amylolytic activit, i.e. different falling number indicator (FN). It is known, that flours with a high FN (more than 350 s) have low amylolytic activity and a reduced capacity to form fermentable sugars (Codina & Leahu, 2009; Struyf et al., 2016). In this study wheat flour with satisfactory amylolytic activity had FN 290 s (Every & Ross, 1996; Savkina et al., 2020) and wheat flour with low amylolytic activity had FN 440s (Codina & Leahu, 2009; Struyf et al., 2016).

The FN was determined according to ICC approved method 107/1 (1995). The wet gluten quantity and gluten quality were determined according to Russian Standard (State Standard of the Russian Federation, 2013), because this method commonly used all over the Russian Bread making Industry.

The amount of gluten was determined in the following way. Dough was mixed from 25 g of flour and 14 g ofwater, and then it was held for 20 min for hydration and the formation of intra- and intermolecular bonds in substances forming gluten (gliadin and glutenin). Crude gluten was washed by a working body of a mechanized device MOK-1M (Mototeh, Russia) using water to remove water-soluble substances, starch and brans from the dough. The resulting gluten was weighed and the percentage of crude gluten was calculated relative to the mass of the analyzed flour sample.

The gluten quality was determined as the deformation index of raw gluten under the influence of a load (120 g) for a 30 s on a special device `IDK-3M` (Plaun, Russian).

The results were expressed in units of the device IDK. According State Standard (State Standard of the Russian Federation GOST 27839-2013) the gluten quality is divided into the following groups: unsatisfactory strong (less than 32 units of the device), satisfactory strong (33–52 units of the device), good (53–77 units of the device), satisfactory weak (78–102 units of the device), unsatisfactory weak (more 103 units).

The basic quality parameters of wheat flour are presented in Table 1.

Table 1. Quality parameters of wheat flour

Indicators	Wheat flour	
	F1	F2
Moisture content, %	13.6 ± 0.3^{a}	14.8 ± 0.4^{b}
Falling number, s	290 ± 14^{a}	440 ± 18^{b}
Wet gluten,%	28.0 ± 0.9^{a}	24.2 ± 0.9^{b}
Gluten deformation index, units of device IDK	48 ± 3^{a}	51 ± 2^{a}

a–b = Means ± SD within the same row with different lowercase superscript letters are significantly different ($p \leq 0.05$).

Enzymes

The enzyme preparation 'Amiloryzin' (LLC 'Trading House 'Biopreparat', Moscow, Russia) was used in the experiments. Amilorizin is α-amylase from *Aspergillus oryzae* with amylolytic activity 2,500 FAU g^{-1}. The performance of the enzyme preparation from *Aspergillus oryzae* was tested in the bread-making process and compared to a currently commercialized α-amylase Fungamyl 2500 SG ® (Novozymes, Bagsvaerd, Denmark) from *Aspergillus oryzae.*

The enzyme preparation 'Protozym' (LLC 'Trading House 'Biopreparat', Moscow, Russia) was used in the experiment. Protozym is a complex enzyme preparation containing exopeptidase, endo-xylanase and β-glucanase, obtained from *Penicillium canescens*. The proteolytic activity of the preparation is 75,000–10,000 AU g^{-1}, xylanase - 800–1,000 FXU g^{-1}, β-glucanase - 250–270 AU g^{-1}.

Enzymes were used in optimum dosages. In preliminary analysis (data not shown), the best concentration of each enzyme was determined separately. The optimal dosages of Amyloryzin and Fungamyl were: 0.0002% for wheat flour with normal amylolytic activity (FN 290 s) and 0.0004% for wheat flour with low amylolytic activity (FN 440 s). The optimal dosages of Protozym were: 0.003% for wheat flour with normal amylolytic activity (FN 290 s) and 0.006% for wheat flour with low amylolytic activity.

Other ingredients

Potable grade water was used in the study, as well as edible sodium chloride (LLC 'Russol', Russia), pressed baker's yeast (Lesaffre, Russia), local sunflower oil (Bunge Ltd, Russia) and sugar (sucrose, RUSAGRO Group, Russia).

Individual effects of enzymes assessment

α-Amylase assessment

The influence of α-amylase preparation (Amylorysin and Fungamyl) on gas-forming capacity of the dough was determined using a Reofermentometer F3 (Chopin, France). Dough samples with α-amylase preparation were prepared in

accordance with the recipe in Table 2. Commercial enzyme Fungamyl (Novozyme) was used as positive control, and a trial with no enzyme was a negative control.

Dough samples weighing 315 g were placed on the bottom of the drum, preheated to 28.5 °C. Then it was installed on the dough piston and the system lid was tightly closed. The duration of the experiment was 300 minutes. The rise of the dough during the fermentation was estimated by the movement of the piston, which was mounted directly on the dough.

Assessment of enzyme preparations with endo-xylanase and exoprotease activities

The effect of enzyme preparations with combined endo-xylanase and exo-protease activities (Protozym) on hydration and amount of wet and dry gluten was studied in flour with normal amylolytic activity (FN 290 s). The gluten test method is described above. Dough formulation is in Table 2.

The conversion of gluten protein fractions was studied using Lowry method immediately after kneading the dough and after fermentation and proofing of the dough.

Table 2. Formulations of the dough with single enzyme preparation

Ingredients and parameters	Flour with FN 290 s			Flour with FN 440s	
	Control without enzymes	With α-amylase preparation (Amylorysin or Fungamyl)	With Protozym	Control without enzymes	With α-amylase preparation (Amylorysin or Fungamyl)
Wheat flour, g	100				
Pressed yeast, g	2.0				
Salt, g	1.5				
Enzyme, g	-	0.0002	0.003	-	0.0004
Water, g	56				
Process parameters					
Moisture content, %	41.5				

The fractional composition of flour and dough proteins was determined by successive dissolution of a sample in 0.05N NaCl, 70% ethanol and 0.05N NaOH (Puchkova, 2004). A weighed portion of a sample weighing 2–4 g was suspended in 20 cm^3 of 0.05 N NaCl for an hour on a mechanical shaker (PE-6410, Russia), and then left overnight in a refrigerator. Then the dispersion was centrifuged (centrifuge Hettich Rotofix 32A, German) for 15 min at 6,000 rpm and the supernatant was poured into a 100 cm^3 volumetric flask. The precipitate was again poured with the same amount of solvent, and then it was washed, centrifuged (centrifuge Hettich Rotofix 32A, German), and poured into the same volumetric flask. The solution was brought to the mark, and the precipitate was poured with another solvent, and all operations were repeated. The protein was determined in centrifugates according to Lowry (1951), the results were expressed as a percentage of the total content of alkali-soluble proteins in solution.

Bread preparation

The dough was prepared using one-step method. For research, formulation of bread 'Nareznoi' traditional and commonly produced in Russia, Ukraine and Belarus was used. Doughs formulations presented in Table 3. Control doughs were made without enzymes.

Table 3. Formulations of the dough

Ingredients and parameters	Flour with FN 290 s		Flour with FN 440s	
	Control	Amylorysin+ Protosym	Control	Amylorysin+ Protosym
Wheat flour, g	100			
Pressed yeast, g	3.0			
Salt, g	1.5			
Sugar, g	4.0			
Sunflower oil, g	3.0			
Enzyme, g	-	0.0002 + 0.003	-	0.0004 + 0.006
Water, g	53.0			
Process parameters				
Moisture content, %	41.5			
Fermentation time, h	5	5	2.5	5

All ingredients were mixed for 5 minutes and the dough was then fermented for 1 h at 30 ± 1 °C. After fermentation all dough samples were shaped into 450-g round-shaped loaves, placed at aluminium pans, and leavened at 30 °C until the volume was twice the initial volume. The leavened dough was baked in an oven Miwe ideal (German) at 20 °C for 25 min.

Assessment of baked bread

The quality of bread was evaluated by following parameters.

Porosity was determined as the ratio between pore volume and the total volume of products (Puchkova, 2004), specific volume - as the ratio between product volume and mass of whole bread (cm g^{-1}) (State Standard of the Russian Federation GOST 27669-88. 2007).

The diameter D and the height H of the round pan bread was measured in millimeters (Puchkova, 2004). For hearth (pan) bread, the minimum and maximum diameters were measured. The shape stability indicator was counted as the ratio between the height and the diameter -H: D (Puchkova, 2004).

Aldehydes content assessment

There is a large list of volatile compounds reported in wheat bread, including alcohols, aldehydes, esters, ethers, ketones, acids, hydrocarbons, pyrazines, pyrrolines, furans, lactones or sulphur compounds. Volatile aldehydes found in wheat bread (crumb and/or crust) reported in literature their typical odours (Pico et al., 2015).

Method for the determination of aromatic substances in bakery products is based on the binding of aldehydes and ketones with sodium bisulfite (Koryachkina et al., 2010). Aldehydes and reactive ketones can be successfully transformed into charged bisulfite adducts that can then be separated from other organic components of a mixture by the introduction of an immiscible organic layer. 10 g of bread crumb was ground in a mortar with 0.4% sodium bisulfite solution and transferred to a 100 cm^3 flask. Water was added until 100 cm^3 and then flask was shaken for 10 minutes. After that the flask was left for 10 min for sedimentation, after that the precipitate was separated by filtration. 10 $cm^3$of the sediment was taken. Excess sodium bisulfite was first titrated with 0.1 mol dm^{-3} iodine solution until a weak violet-blue color was obtained. If iodine was overdose than necessary, the excess was titrated with 0.01 mol dm^{-3} hyposulfite

solution. The amount of iodine solution spent on the oxidation of excess sodium bisulfite was not taken into account. To destroy the aldehyde sulfite compound, a saturated solution of sodium hydrocarbonate was poured into the reaction liquid until pH ≥ 8. The sodium bisulfite пgenerating due to the sodium hydrocarbonate addition was immediately titrated with 0.01 mol dm^{-3} iodine solution. The titration was considered complete if the violet-blue color wasn`t disappear in a 15 s after stirring. The content of aldehydes is conventionally expressed in cm^3 of 0.1 mol dm^{-3} iodine solution used for titration of bisulfite bound to carbonyl compounds, accounted per 100 g dry matter.

Crumb firmness assessment

Crumb firmness was determined on the same loaves according to method created in Scientific Research Institute for the Baking Industry using a texture analyser, model `Structurometer ST-1M` (Scientific Research Institute for the Baking Industry, Russia). The method is based on determining the compression force value of 36-mm-diamete indenter (with a rounded edge) when it is inserted into a piece of loaf 25 mm thick to a depth of 6.25 mm (with a movement speed of 1 $mm \cdot s^{-1}$ with an initial contact force of 5 g) and on the establishment of the final loading force on the indenter. Then its reverse movement is carried out up to an effort of 5 g. The method for assessing the degree of staleness of baked products allows determining the structural and mechanical properties of the crumb of bread, which can act as a measure of the quality and freshness of products. The index of the final loading force on the indenter can be taken as an index of the softness of the crumb. Baked bread loaves were bagged after cooling for 180 min and kept at room temperature for 5 day. The crust and first two slices of bread were discarded. Eight readings were done for each loaf of bread (Chernykh & Maksimov, 2004).

Assessment of sensory characteristics

A panel of 10 non-specialists was used to evaluate the sensory characteristics of the bread produced. Then, they were asked to evaluate separately crust (shape, surface, colour) and crumb (colour, odour, taste, taste, chewiness and porosity). The ranking scale ranged from 1 to 5 (5–like extremely, 4.5–like very much, 4–like moderately, 3.5–like slightly, 3–neither like nor dislike, 2.5–dislike slightly, 2–dislike moderately, 1.5–dislike very much, 1–dislike extremely).

Statistical analysis of the data

All of the experiments were carried out a total of five times. Statistical analysis was performed using Excell software. Comparison of the influence of factors was carried out by the method with significance tested at the 95% confidence level and differences among means were determined using the least significant difference and Duncan's test of two-factor analysis of variance with one repetition (ANOVA). The confidence intervals shown in the histograms and in the table reflect the accuracy of the used methods.

RESULTS AND DISCUSSION

Individual effects of enzymes on wheat flour proprieties

Before evaluating the combined effect of new enzymes on the dough, the effect of each enzyme on flour quality was studied.

Influence of α-amylase on wheat flour

The effect of α-amylase on the gas-forming ability of wheat flour with different amylolytic activity was studied. Reofermentometric characteristics of dough samples are presented in Table 4.

The effect on the gas-forming capacity of dough was studied (Table 4). The total volume of released CO_2 was higher by 10.0–13.9% when the enzymes were added. It may be due to the fact, that fungal amylase affects the maltose generation from the starch (Van der Maarel et al., 2002; Goesaert et al., 2009; Codina & Leahu, 2009; Struyf et al., 2016). Yeast produces carbon dioxide from maltose because it is fermentable sugar (Cauvain & Young2007).

The gas retention coefficient was comparable in samples with Amylorysin and positive control with Fungamyl. And it was lower compared the negative control by 16–19.8% for flour with FN 290 and by 3.2–5.8% for flour with FN 440s. The decrease of gas retention coefficient also may be due to a fact that α-amylase reduces dough extensibility and stability because of the gluten protein alteration due to the high amounts of amylase-reducing polysaccharides (Sahnoun et al., 2016). In flour with different amylolytic activity the degree of the process was different.

Table 4. Influence of enzymes on the rheological characteristics of wheat flour

		Total volume of released CO_2, cm^3	Gas retention coefficient, %
Flour with	Negative control	$1{,}819.0 \pm 88.0^{a}$	$85.2 \pm 4{,}2^{a}$
FN 290 s+0.0002%	Positive control (with Fungamyl)	$2{,}029.0 \pm 97.0^{b}$	$69.7 \pm 3{,}2^{b}$
of enzyme	Amylorysin	$1{,}987.0 \pm 55.0^{b}$	$68.3 \pm 3{,}2^{b}$
Flour with	Negative control	$1{,}606.0 \pm 71.0^{c}$	$80.7 \pm 3{,}4^{c}$
FN 440 s +0.0004%	Positive control (with Fungamyl)	$1{,}797.0 \pm 58.0^{d}$	78.1 ± 3.6^{d}
of enzyme	Amylorysin	$1{,}829.0 \pm 88.0^{d}$	76.0 ± 1.3^{d}

a–d = Means ± SD within the same row with different lowercase superscript letters are significantly different ($P \leq 0.05$).

Influence of Protozyme and Pentopan on gluten

The combined effect of endo-xylanase and exo-peptidase on the on the amount and property of gluten and the content of the protein fraction was studied (Table 5).

After kneading the dough, the amount of wet gluten in the dough made with enzymes usage was similarly to the control without enzymes.

After fermentation, content of raw gluten was higher in the samples with enzymes than in the control. The water absorption capacity in the control sample was worser compared to the initial. In the sample with Protozym, on the contrary, it became better. It can be assumed that it was due to the action of exo-protease, which is part of Protozym. An increase in gluten content and an improvement in its hydration capacity when using endo-xylanases and exo-peptidases confirm the data obtained by other authors (Yegin et al., 2018; Both et al., 2020). Endo-xylanases cause the hydrolysis of insoluble arabinoxylans to form water-soluble arabinoxylans. At the same time, the water absorbed by insoluble arabinoxylans is released and redistributed between the structural components of the dough, mainly between gluten proteins and pentosans. Thus, endo-xylanases contribute to the creation of a continuous structure and strengthening of gluten (Autio, 2009; Filipcev et al., 2014; Yegin et al., 2018).

Table 5. Influence of Protozym on wheat gluten in the dough

Indicators	Indicators of gluten in dough			
	Immediately after kneading		After 2.5 h fermentation	
	Control	Protozym	Control	Protozym
Wheat gluten, %	27.90 ± 0,56[a]	28.10 ± 0.80[a]	23.3 ± 0.26[b]	24.0 ± 0.31[c]
Gluten deformation index, units of device IDK	47.0 ± 2.0[a]	47.0 ± 1.0[a]	30.0 ± 1.0[b]	24.0 ± 2.0[b]
Water absorption capacity, %	166.7 ± 0.8[a]	167.8 ± 1.3[a]	153.4 ± 1.2[b]	174.5 ± 1.2[c]
Dry gluten, %	8.8 ± 0.2[a]	9.0 ± 0.3[a]	7.2 ± 0.2[b]	7.7 ± 0.2[c]

a–c = Means ± SD within the same row with different lowercase superscript letters are significantly different ($P \leq 0.05$).

The Protozym affects the amount of protein fractions in the dough after kneading in different degrees (Table 6). After kneading, the amount of water-soluble, salt-soluble and alcohol-soluble proteins decreased in all samples in comparison with their amount in flour. The amount of alkali-soluble proteins, on the contrary, increased. The results obtained indicate that when the dough is kneaded, a part of water-soluble, salt-soluble, alcohol-soluble proteins aggregates with the alkali-soluble proteins formation. It confirms data that xylanases limit the aggregation of glutenin polymers as a result of pentosan breakdown which in turn strengthens the gluten network (Filipcev et al., 2014; Yegin et al., 2018; Both et al., 2020)

During the fermentation of the dough and proofing of the dough pieces, the amount of water-soluble proteins in the control sample was higher than in the experimental ones with enzymes. The content of salt-soluble proteins at all stages of dough preparation changed insignificantly (Table 6).

Table 6. Influence of enzymes on protein fraction of wheat flour

Indicators	Protein fractions, $mg \cdot g^{-1}$			
	Water-soluble	Salt-soluble (0.5 m NaCI)	Alcohol soluble (70% ethanol)	Alkali-soluble (0.05H NaOH)
Wheat flour	25.0 ± 1.0[a]	23.0 ± 1.0[a]	18.0 ± 1.0[a]	33.0 ± 1.0[a]
The control dough				
After kneading	19.5 ± 1.0[b]	11.5 ± 1.0[b]	8.3 ± 1.0[b]	51.7 ± 1.0[b]
After fermentation	21.2 ± 0.6[c]	11.0 ± 0.8[c]	9.2 ± 1.1[c]	49.0 ± 1.0[c]
After proofing the dough pieces	21.7 ± 1.0[c] x	11.2 ± 1.0[c] x	11.0 ± 1.0[d] x	46.0 ± 1.0[d] x
The dough with Protozym				
After kneading	16.8 ± 1.0[d]	11.7 ± 1.0[b]	10.5 ± 0.9[f]	53.3 ± 1.0[f]
After fermentation	17.6 ± 0.6[f]	11.7 ± 1.0[b]	8.8 ± 1.0[c]	52.7 ± 1.1[g]
After proofing the dough pieces	18.5 ± 1.2[g] y	12.0 ± 0.9[d] y	8.0 ± 0.9[b] y	50.1 ± 1.0[h] y

a–h = Means ± SD within the same column with different lowercase superscript letters denote significantly different among dough types ($P \leq 0.05$) while letters 'x-y' denote significantly different values among type of flours (Tukey's test, $p < 0.05$).

The amount of alcohol-soluble proteins gradually increased during the dough preparation process in the control samples, while in experimental samples with enzymes it decreased. An increase in the proportion of alcohol-soluble proteins during dough

preparation is consistent with the known data that during dough fermentation, protein macromolecules are depolymerized due to hydrolysis of peptide bonds under the action of flour proteolytic enzymes (Cauvain & Young, 2007). With the addition of endopeptidases and endo-xylanases, the degree of depolymerization is obviously less than the degree of aggregation of protein molecules. This conclusion is confirmed not only by an increase in the amount of alcohol-soluble and alkali-soluble proteins, but also by a decrease in the proportion of salt-soluble proteins in the dough preparation process, since the effect of baker's yeast on the assimilation of soluble peptides is present in all dough samples.

In all samples, during the dough preparation process, the amount of alcohol-soluble and alkali-soluble proteins decreased, which is consistent with the known fact of a decrease in the amount of gluten during dough fermentation (Cauvain & Young, 2007).

The strengthening of the gluten in the dough with enzymes compared to the control (noticed in Table 5) after fermentation and proofing may be associated with the content of the alcohol-soluble and alkaline-soluble fraction of gluten. In the dough with enzymes (Table 6) alkali-soluble fraction was higher by 8–9% than in control after fermentation and proofing. Alcohol-soluble fraction was comparable to the control after fermentation and was lower than in control by 37% after proofing. It is known that the higher alkali-soluble fraction (glutenin) levels make the dough more elastic thus giving dough its property of resistance to extension while higher alcohol-soluble fraction (gliadin) content increases the extensibility of the dough (Barak et al., 2013; Dhaka & Khatkar 2015).

Effects of α-amylase, endo-xylanase and exo-protease combination on bread quality

The performance of the combined action of new enzyme preparations (Amilorizin with alpha-amylase activity and Protozym with endo-xylanase and exo-peptidase activity) was investigated (Table 7).

Table 7. Bread quality indicators

Wheat flour	Bread formulation	Porosity, %	Specific volume, $cm^3\ g^{-1}$	Shape stability indicator, H/D	Aldehyde amount, mL 0.1 n iodine solution per 100 g dry matter
Flour (FN 290 s)	Control	84 ± 1^a	4.1 ± 0.3^a	0.31 ± 0.04^a	12.3 ± 0.3^a
	Amylorysin+Protosym	87 ± 2^b x	4.7 ± 0.3^b x	0.30 ± 0.04^b x	13.5 ± 0.5^b x
Flour (FN 440 s)	Control	78 ± 1^c	3.5 ± 0.3^c	0.39 ± 0.06^c	12.0 ± 0.4^a
	Amylorysin+Protosym	83 ± 1^d y	$4,2 \pm 0.3^d$ y	0.35 ± 0.04^d y	13.4 ± 0.5^b x

a–d = Means ± SD within the same column with different lowercase superscript letters denote significantly different among bread samples ($P \leq 0.05$) while letters 'x-y' denote significantly different values among type of flours (Tukey's test, $p < 0.05$).

The bread with enzymes had a higher specific volume, porosity and aldehyde content than the control made without enzymes. In comparison with the control, addition of enzymes improved bread specific volume significantly. It confirmed data obtained by other researchers (Hemalatha et al., 2010; Baratto et al., 2015; Kim & Yoo, 2020).

Shape stability indicator was lower for bread with enzymes. It is presumably related to the reduction of the dough viscosity during starch gelatinization as a result of the action of the enzyme (Goesaert et al., 2009; Fuentes et al., 2016) and reduction of dough extensibility and stability because of the gluten protein (Sahnoun et al., 2016).

According to the organoleptic assessment (Table 8), the experimental bread samples were characterized by tenderer and not crumbly crumb with developed thin-walled uniform porosity compared to the control. The crusts were more brightly colored (from yellow to light brown). It confirmed data obtained by other researchers that combination of amylases and xylanase improve sensory characteristics (Hemalatha et al., 2010; Barrato et al., 2015; Kim & Yoo, 2020). Better porosity may be due to the fact that xylanase improves the bread texture and volume (Moers et al., 2005). The pale or greyish crust colour of control samples, especially made using flour with low amylolytic activity, indicated a lack of residual sugars that might have resulted from a lean fermentation without added enzymes. Crumb colour became brighter when enzymes were used. This may be due to the α-amylase action (Sahnoun et al., 2016). The taste and smell were more pronounced. This is confirmed by the higher content of aldehydes in the samples with enzymes (Table 7). It consistent with data, obtained by other researcher, that α-amylase increases the amount of fermentable sugar and therefore enhances the yeast fermentation and the Maillard reaction products, which, in turn, strengthen the flavor and colour of bread (Sahnoun et al., 2013; Sahnoun et al., 2016). Other reason for taste and flour improvement is that the exopeptidases catalyze hydrolysis of peptide bonds and the free amino acids or small peptides formed. They may function in food as pleasant-tasting flavor compounds or as flavor precursors (Raksakulthai & Haard, 2003).

Table 8. Sensory characteristics of bread

Indicators	Flour (FN 290 s)		Flour (FN 440 s)	
	Bread formulation			
	Control	Amylorysin+ Protosym	Control	Amylorysin+ Protosym
Crust				
Shape	3.80 ± 0.18^{ax}	4.80 ± 0.13^{bx}	3.40 ± 0.11^{cy}	3.90 ± 0.13^{ay}
Surface	3.80 ± 0.18^{ax}	4.79 ± 0.13^{bx}	3.30 ± 0.12^{cy}	3.91 ± 0.11^{ay}
Colour	3.20 ± 0.16^{ax}	4.31 ± 0.19^{bx}	3.00 ± 0.15^{cy}	3.82 ± 0.14^{dy}
Crumb				
Colour	3.04 ± 0.10^{ax}	3.49 ± 0.38^{bx}	2.78 ± 0.12^{cy}	3.19 ± 0.18^{dy}
Odour	3.18 ± 0.18^{ax}	3.82 ± 0.28^{bx}	3.01 ± 0.14^{cy}	3.42 ± 0.12^{dy}
Taste	3.45 ± 0.08^{ax}	4.32 ± 0.18^{bx}	3.25 ± 0.14^{cy}	3.92 ± 0.11^{dy}
Chewiness	3.04 ± 0.09^{ax}	3.25 ± 0.15^{bx}	2.49 ± 0.14^{cy}	3.15 ± 0.17^{dy}
Porosity	3.59 ± 0.23^{ax}	4.63 ± 0.31^{bx}	3.59 ± 0.23^{ax}	4.13 ± 0.31^{cy}

a–d = Means ± SD within the same line with different lowercase superscript letters denote significantly different among bread samples ($P \leq 0.05$) while letters 'x-y' denote significantly different values among type of flours (Tukey's test, $p < 0.05$).

Studies have shown that the new enzyme preparations improve the properties of the dough, physico-chemical and organoleptic indicators of the quality of bread made from wheat flour with normal and low autolytic activity.

Effects of α-amylase, endo-xylanase and exoprotease combination on bread staling

It was found (Fig. 1) that the compression force value after 24 h of storage of samples with enzymes was less compared to the control. Evaluation of indicators of the bread crumb staleness have showed that the crumb of bread with enzymes had a lower rate of staling after 120 h of storage compared to the control. The combined use of α-amylase and endo-xylanase and exo-peptidase retarded bread staling synergistically after a 5-day storage period. The findings confirm the findings of other researchers that alpha-amylases and endo-xylanase decreased crumb hardness and slow down staling (Barrato et al., 2015; Kin&Yoo, 2020).

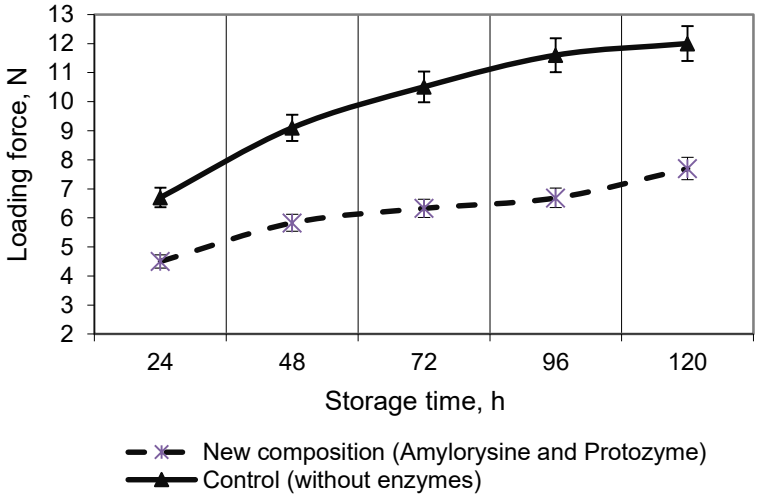

Figure 1. Dynamics of loading force indicators in the analysis of bread crumb.

CONCLUSIONS

Single addition of α-amylases preparation in the amount of 0.002 and 0.004% by weight of flour (0.5 and 1.0 units of AC / g flour) increased the gas-forming capacity of flour and reduced the gas retention coefficient. The technological properties of Russian α-amylase Amilorizin were no worse than those of the commercial preparation Fungamil 2500. It was found that single addition of new enzyme preparation with endo-xylanase and exoprotease activity (Protozym) increases the degree of hydration of gluten proteins and contributes to the strengthening of its structure. Endo-xylanase indirectly reduces the degree of peptization of gluten proteins during dough fermentation, which were confirmed by the higher amount of dry gluten in the experimental samples compared to the control. When used endo-xylanase and exoprotease, there was no significant change in the amount of salt-soluble proteins after kneading the dough, but water-soluble, alcohol-soluble and alkaline-soluble proteins was undergone transformation.

As a result of the addition of the formulated new composition of α-amylase, xylanase and exoprotease allowed the improvement of physico-chemical and organoleptic quality indicators and increasing shelf life of bakery products. New enzyme composition may be a potentially strong candidate for future applications in the bread-making industry.

REFERENCES

Ahmad, Z., Butt, M.S., Ahmed, A. & Khalid, N. 2013. Xylanolytic modification in wheat flour and its effect on dough rheological characteristics and bread quality attributes. *Journal of the Korean Society for Applied Biological Chemistry* **56**(6), 723–729.

Ait Kaki El-Hadef El-Okki, A., Gagaoua, M., Bourekoua, H., Hafid, K., Bennamoun, L., Djekrif-Dakhmouche, S., El-Hadef El-Okki, M. & Meraihi, Z. 2017. Improving Bread Quality with the Application of a Newly Purified Thermostable α-Amylase from Rhizopusoryzae FSIS4. *Foods* **6**(1), 1. doi: 10.3390/foods6010001

Arts, J. & Kimber, I. 2017. Azodicarbonamide (ADCA): A reconsideration of classification as a respiratory sensitiser. *Regulatory Toxicology and Pharm*acology **89**, 268–278. doi: 10.1016/j.yrtph.2017.07.018

Autio, K. 2009. Effects of cell wall components on the functionality of wheat gluten. *Biotechnol Adv* **24**, 633–635. https://doi.org/10.1016/j.biotechadv.2006.07.002

Barak, S., Mudgil, D. & Khatkar, B.S. 2013. Relationship of gliadin and glutenin proteins with dough rheology, flour pasting and bread making performance of wheat varieties. *LWT - Food Science and Technology* **51**(1), 211–217. doi: 10.1016/j.lwt.2012.09.011

Baratto, C.M., Becker, N.B., Gelinski, J.M.L.N. & Silveira, M.S. 2015. Influence of enzymes and ascorbic acid on dough rheology and wheat bread quality. *African Journal of Biotechnology* **15**(3), 55–61. doi:10.5897/ajb2015.14931

Both, J., Biduski, B., Gómez, M., Bertolin, T.E., Friedrich, M.T. & Gutkoski, L.C. 2020. Micronized whole wheat flour and xylanase application: dough properties and bread quality. *Journal of Food Science and Technology.* doi:10.1007/s13197-020-04851-2

Bueno, M.M., Thys, R.C.S. & Rodrigues, R.C. 2016. Microbial Enzymes as Substitutes of Chemical Additives in Baking Wheat Flour–Part I: Individual Effects of Nine Enzymes on Flour Dough Rheology. *Food and Bioprocess Technology* **9**(12), 2012–2023. doi:10.1007/s11947-016-1780-4

Calvin, O. 2016. Starch and modified starch in bread making: A review. *African Journal of Food Science* **10**(12), 344–351. doi:10.5897/ajfs2016.1481

Cauvain, S.P. & Young, L.S. 2007. Technology of Breadmaking, second ed. Springer, BakeTran, UK., 397 pp.

Chernykh, V. & Maksimov, A. 2004. *Laboratory workshop on the rheology of raw materials, semi-finished and finished products of bakery and confectionery production*. MGUPP, Moscow, 163 pp. (in Russia)

Codina, G.G. & Leahu, A. 2009. The improvement of the quality of wheat flour with a lower content of alpha-amylase trough the addition of different enzymatic products. *LucrariStiintifice Ser. Agron.* **52**, 629–635.

Cunha, C.C.D.Q.B, Gama, A.R., Cintra, L.C., Bataus, L.A.M. & Ulhoa, C.J. 2018. Improvement of bread making quality by supplementation with a recombinant xylanase produced by Pichia pastoris. *PLOS ONE* **13**(2), e0192996. doi: 10.1371/journal.pone.0192996

Dhaka, V. & Khatkar, B.S. 2015. Effects of Gliadin/Glutenin and HMW-GS/LMW-GS Ratio on Dough Rheological Properties and Bread-Making Potential of Wheat Varieties. *Journal of Food Quality* **38**(2), 71–82. doi: 10.1111/jfq.12122

Driss, D., Bhiri, F., Siela, M., Bessess, S., Chaabouni, S. & Ghorbel, R. 2012. Improvement of Breadmaking Quality by Xylanase GH11 from Penicillium occitanis Pol6. *Journal of Texture Studies* **44**(1), 75–84. doi: 10.1111/j.1745-4603.2012.00367.x

Every, D. & Ross, M. 1996. The role of dextrins in the stickiness of bread crumb made from pre-harvest sprouted wheat or flour containing exogenous alpha amylase. *J. Cereal Sci.***23**, 247–256.

Filipcev, B., Simurina, O. & Bodroza-Solarov, M. 2014. Combined effect of xylanase, ascorbic and citric acid in regulating the quality of bread made from organically grown spelt cultivars. *Journal of Food Quality* **37**, 185–195.

Fuentes, C., Zielke, C., Prakash, M., Kumar, P., Peñarrieta, J.M., Eliasson, A.C. & Nilsson, L. 2016. The effect of baking and enzymatic treatment on the structural properties of wheat starch. *Food Chemistry* **213,** 768–774. doi: 10.1016/j.foodchem.2016.07.045

Grewal, N., Faubion, J., Feng, g., Kaufman, R.C., Wilson, J.D. & Shi, Y.C. 2015. Structure of Waxy Maize Starch Hydrolyzed by Maltogenicα-Amylase in Relation to Its Retrogradation. *Journal of Agricultural and Food Chemistry* **63(**16), 4196–4201. doi:10.1021/jf506215s

Goesaert, H., Gebruers, K., Courtin, C.M., Brijs, K. & Delcour, J.A. 2007. Enzymes in Breadmaking. *In Bakery Products; Blackwell Publishing: Oxford, UK*, 337–364.

Goesaert, H., Leman, P., Bijttebier, A. & Delcour, J.A. 2009. Antifirming Effects of Starch Degrading Enzymes in Bread Crumb. *Journal of Agricultural and Food Chemistry* **57**(6), 2346–2355. doi:10.1021/jf803058v

Hardt, N.A., Boom, R.M. & van der Goot, A.J. 2014. Wheat dough rheology at low water contents and the influence of xylanases. *Food Research International* **66**, 478–484.

He, L., Mao, Y., Zhang, L., Wang, H., Alias, S.A., Gao, B. & Wei, D. 2017. Functional expression of a novel α-amylase from Antarctic psychrotolerant fungus for baking industry and its magnetic immobilization. *BMC Biotechnology* **17**(1). doi:10.1186/s12896-017-0343-8

Hemalatha, M.S., Prasada Rao, U.J.S., Leelavathi, K. & Salimath, P.V. 2010. Influence of amylases and xylanase on chemical, sensory, amylograph properties and microstructure of chapati. *LWT - Food Science and Technology* **43**(9), 1394–1402. doi: 10.1016/j.lwt.2010.04.020

Heredia-Sandoval, N., Valencia-Tapia, M., Calderón de la Barca, A. & Islas-Rubio, A. 2016. Microbial Proteases in Baked Goods: Modification of Gluten and Effects on Immunogenicity and Product Quality. *Foods* **5**(3), 59. doi:10.3390/foods5030059

Hug-Iten, S., Escher, F., & Conde-Petit, B. 2003. Staling of Bread: Role of Amylose and Amylopectin and Influence of Starch-Degrading Enzymes. *Cereal Chemistry Journal* **80**(6), 654–661. doi:10.1094/cchem.2003.80.6.654

Ibrahim, A.A., Osman, A., Nasser, M. & Alshathly, M.R. 2019. Mitigating and anti-apoptotic effect of turmeric on semicarbazide-induced damage in testicular tissue of juvenile male rats. *Journal of Complementary Medicine Research* **10**(3), 103–111.

ICC–standard methods: 107/1 (1995) Determination of the `falling number` according to Hagberg - as a measure of the degree of alpha-amylase activity in grain and flour

Kim, H.J. & Yoo, S.H. 2020. Effects of Combined α-Amylase and Endo-Xylanase Treatments on the Properties of Fresh and Frozen Doughs and Final Breads. *Polymers* **12**(6), 1349. doi:10.3390/polym12061349

Kornbrust, B.A., Forman, T. & Matveeva, I. 2012. Applications of enzymes in breadmaking. *Breadmaking. Woodhead Publishing, Cambrige*, 470–498. doi:10.1533/9780857095695.2.470

Koryachkina, S., Berezina, N. & Khmeleva, E. 2010. Quality research methods for Bakery products. *Russia, Oryol: Orel State Technical University*, 86–88 (in Russian).

Lagrain, B., Leman, P., Goesaert, H. & Delcour, J.A. 2008. Impact of thermostable amylases during breadmaking on wheat bread crumb structure and texture. *FoodRes. Int.* **41**, 819–827.

Lowry, O.H., Rosebrough, N., Farr, A. & Randall, R. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem* **193**(1), 265–275.

Melim Miguel, A.S., Souza, T., Costa Figueiredo, E.V., Paulo Lobo, B.W. & Maria, g. 2013. Enzymes in Bakery: Current and Future Trends. *Food Industry.* doi:10.5772/53168

Moers, K., Celus, I., Brijs, K., Courtin, C.M. & Delcour, J.A. 2005. Endoxylanase substrate selectivity determines degradation of wheat water-extractable and water-unextractablearabinoxylan. *Carbohydrate Research* **340**(7), 1319–1327.

Pico, J., Bernal, J. & Gómez, M. 2015. Wheat bread aroma compounds in crumb and crust: A review. *Food Research International* **75**, 200–215. doi: 10.1016/j.foodres.2015.05.051

Puchkova, L. 2004. *Laboratory Workshop on Bakery Technology*.Moscow, pp. 264 (in Russian).

Raksakulthai, R. & Haard, N.F. 2003. Exopeptidases and Their Application to Reduce Bitterness in Food: A Review. *Critical Reviews in Food Science and Nutritin* **43**(4), 401–445. doi:10.1080/10408690390826572

Rosell, C.M., Rojas, J.A. & Benedito de Barber, C. 2001. Combined Effect of Different Antistaling Agents on the Pasting Properties of Wheat Flour. *European Food Research and Technology* **212**(4), 473–476. doi:10.1007/s002170000282

Sanz-Penella, J.M., Laparra, J.M. & Haros, M. 2014. Impact of α-Amylase DuringBreadmaking on In Vitro Kinetics of Starch Hydrolysis and Glycaemic Index of Enriched Bread with Bran. *Plant Foods for Human Nutrition* **69**(3), 216–221. doi:10.1007/s11130-014-0436-7

Sahnoun, M., Naili, B., Elgharbi, F., Kammoun, R., Gabsi, K. & Bejar, S. 2013. Effect of Aspergillus oryzae CBS 819.72 α-amylase on rheological dough properties and bread quality. *Biologia* **68**(5). doi:10.2478/s11756-013-0233-z

Sahnoun, M., Kriaa, M., Besbes, S., Jardak, M., Bejar, S. & Kammoun, R. 2016. Optimization of Aspergillus oryzae S2 α-amylase, ascorbic acid, and glucose oxidase combination for improved French and composite Ukrainian wheat dough properties and bread quality using a mixture design approach. *Food Science and Biotechnology* **25**(5), 1291–1298. doi:10.1007/s10068-016-0203-7

Savkina, O., Kuznetsova, L., Burykina, M., Kostyuchenko, M. & Parakhina, O. 2020. The influence of the flour amylolytic enzymes activity, dosage of ingredients and bread making method on the sugar content and the bread quality. *Agronomy Research* **18**(3), 1873–1887. https://doi.org/10.15159/ar.20.047

Shanmugavel, V., Komala Santhi, K., Kurup, A.H., Kumar Kalakandan, S., Anandharaj, A. & Rawson, A. 2019. Potassium bromate: effects on bread components, health, environment and method of analysis: a review. *Food Chemistry, 125964.* doi: 10.1016/j.foodchem.2019.125964

Sharma, S., Sharma, V., Parushi, N. & Bajaj, B.K. 2020. Bioprocess development for production of a process-apt xylanase with multifaceted application potential. *SN Applied Sciences* **2**(4) doi: 10.1007/s42452-020-2541-6

State Standard of the Russian Federation GOST 27839-2013. 2013. Wheat flour. Methods for determining the quantity and quality of gluten, 18 pp. (in Russian).

State Standard of the Russian Federation GOST 27669-88. 2007. Wheat bread flour. Method for experimental laboratory breadmaking, 9 pp. (in Russian).

Struyf, N., Verspreet, J. &Courtin, C.M. 2016. The effect of amylolytic activity and substrate availability on sugar release in non-yeasted dough. *Journal of Cereal Science* **69**, 111–118. doi: 10.1016/j.jcs.2016.02.016

Van der Maarel, M.J.E.C., Van der Veen, B., Uitdehaag, J.C.M., Leemhuis, H. & Dijkhuizen, L. 2002. Properties and applications of starch-converting enzymes of the alpha-amylase family. *J. Biotechnol.* **94**, 137–155.

Yegin, S., Altinel, B. &Tuluk, K. 2018. A novel extremophilic xylanase produced on wheat bran from Aureobasidium pullulans NRRL Y-2311-1: Effects on dough rheology and bread quality. *Food Hydrocolloids* **81**, 389–397. doi: 10.1016/j.foodhyd.2018.03.012

Agronomy Research **19**(S3), 1249–1259, 2021
https://doi.org/10.15159/AR.21.096

Obtention of omega-3-fatty acids cryoconcentrated fish oil from by-products of preserves industry

E. Kuprina*, V. Filipov, A. Yakkola, A. Manuilov, V. Abramzon, M. Kremenevskaya, M. Zashikhin, A. Kuznetsova, A. Kopylov and A. Maksimenkov

ITMO University, Faculty of Biotechnologies Lomonosov street 9, RU191002 Saint-Petersburg, Russia
*Correspondence: elkuprina@yandex.ru

Received: February 24th, 2021; Accepted: May 2nd, 2021; Published: May 19th, 2021

Abstract. The technology for obtaining and cryoconcentration of high-quality fish oil from collagen-containing wastes of slightly salted herring under gentle conditions using electrochemically obtained catholytes has been developed. Physicochemical analysis of raw materials was carried out and the yields of products from raw materials at all stages of processing were determined. Concentration of omega-3 fatty acids in oil was carried out using the cryo method. The main phase transitions in oil with decreasing temperature have been determined. The mass yields were determined and the biochemical composition of the cryoconcentrated fish oil fractions was investigated. It was defined the temperature of -14° C at which a phase transition is observed, providing an increase in the concentration of omega-3 fatty acids in oil by 3 times. The usage of cryoconcentrated fish oil allows to produce biologically active food supplement or raw materials for a functional food.

Key words: cryoconcentration, omega-3 fatty acids, by-products, fish oil, salted herring preserves, biologically active substances, hydrolyzate, functional food, extraction.

INTRODUCTION

It is known that unsaturated fatty acids are an indispensable factor in nutrition, affect many biochemical processes, ensure normal growth, development and determine the physical state of the body as a whole (Freitas et al., 2017; Calder et al., 2020).

Omega-3 fatty acids are the most important nutrient, a structural element of cell membranes, providing vascular permeability, a regulator of nervous, endocrine, and metabolic processes in the body (Vorslov, 2017; Sacca et al., 2018; Radzikowska et al., 2019). Currently, there is a deficiency of omega-3unsaturated fatty acids in the daily human diet, since their main food sources, such as sea fish, seafood (Mohanty et al., 2019) are not consumed in the required amount, which is due to the nutritional traditions of the population of many European countries (Sioen et al., 2017).

Omega-3 deficiency is experienced by about 80% of the inhabitants of all developed countries, which might provoke cardiovascular diseases, leading to heart attacks or strokes, which occupy a leading place in mortality. Also, their lack in the diet causes a decrease in mental activity, a violation of mental states, etc. (Jahangard et al., 2018).

The richest sources of omega-3 polyunsaturated fatty acids (PUFA) are seafood, in particular oils from marine fish and other aquatic organisms (Durmuş, 2018). Also, these valuable components are found in large quantities in the waste from their cutting (Repnikov, 2017; Suresh et al., 2018). A particularly valuable source of omega-3 acids are fatty fish of the Herring family, the capture of which accounts for more than a third of the world catch of aquatic organisms (Kurkotilo & Vasilyeva, 2017; Bazarnova et al., 2020; Egerton et al., 2020,).

The most popular product of these fish in the northwest region of Europe is lightly salted herring preserves (Neuimin, 2017). In the production of preserves, more than 30% of secondary waste is generated, among which the ratio of skin during skinning of fillets accounts for about 10% (Kazakova & Zemlyakova, 2020). Therefore, investigation on the production of essential fatty acids and fish oil obtained from collagen-containing waste from the production of preserves of slightly salted herring is very promising.

It is known that wastes from cutting Atlantic herring, like fish itself, are characterized by a valuable chemical composition and high fat content (Table 1), which indicates the expediency of using them as a raw material source for obtaining biologically active substances (BAS) of lipoid nature (Kosman et al., 2016; Alfio, et al., 2021). Collagen-containing waste from lightly salted herring is one of the most promising sources of fish oil and protein hydrolysates, since due to the popularity of this type of product, the amount of this waste is large and they are not used in agriculture due to the presence of salt in them. Also, the promising use of these wastes is due to the partial hydrolysis of raw materials during the salting process, which facilitates the processes of extracting nutrients from the protein matrix (Mayta-Apaza et al., 2021). If in oil isolated from the carcass of fresh fish, amine nitrogen is almost completely absent, then in the oil isolated from the carcasses of salted fish, amine nitrogen is found in a rather significant amount. Its accumulation proceeds in proportion to the time, which suggests the formation of compounds from the breakdown products of proteins and oil and their dissolution in oil. Control over ripening (loss of raw taste and smell by fish, acquisition of the appropriate taste and aroma – 'bouquet') can be carried out by observing the distribution of oil in fish flesh (Porotikova et al., 2015). In fresh and salted immature fish, oil is either in the cells of muscle tissue, or in the subcutaneous tissue in the form of isolated drops; in fish, in which the processes of protein breakdown and the structure of muscle tissue have changed, oil permeates its entire mass - a solid film of fat is visible on the cut. This indicator is very characteristic of ripened salted fish.

The most widespread technologies are the enzymatic and chemical hydrolysis of wastes (Mukatova et al., 2018, Abuine et al., 2019, Varygina & Davydova, 2021, Zakharchuk et al., 2021), but they are expensive and do not always ensure the quality of the lipids obtained. The method of electrochemical hydrolysis is devoid of these drawbacks (Zakharchuk et al., 1995), which makes it possible to obtain hydrolysates with a given degree of hydrolysis at low energy consumption and does not require the use of acids and alkalis.

In the process of electrochemical treatment, proteins, polypeptides, lipids go into solution in the form of an emulsion, and the mineral precipitate deposits (Chikisheva et al., 2020).

The chosen method has several advantages:

– 95–97% of lipids from raw materials go into solution;

– the released lipids are of high quality due to the gentle conditions and processing modes;

– when extracting oil, it is refined, which is especially important when processing herring skin, which, as it's known, undergoes unfavorable oxidative changes during salting and fermentation.

It is known that in fish of the Herring family, most of the oil is located in the subcutaneous layer and goes to the waste at the skinning stage (Baydalinova et al., 2011; Kosman et al., 2016). Obviously, it is advisable to use skin to obtain fish oil enriched with unsaturated fatty acids, which can be used either as biologically active food additives (BAA) or as part of functional food products (FFP) (Kuprina et al., 2019).

Research aim. Developing a technology for obtaining and cryoconcentration of fish oil from skin of slightly salted herring, investigating biochemical, physicochemical and fatty acid composition of cryoconcentrated fractions of fish oil and developing recommendations for its use.

MATERIALS AND METHODS

Raw materials

The main industrial semi-finished product was used - Atlantic herring fillet on the skin. Producer - Faroe Islands, plant FO229, catch area FAO227, size range 4–7 pcs kg^{-1}, catch period - October 2020.

Sampling

Research objects are wastes of skin obtained by cutting slightly salted herring in the process of producing frozen preserves from Atlantic herring were selected. The herring salting was carried out in the traditional way - wet salting in accordance with the technological scheme (Fig. 1).

The collection of herring skin was carried out mechanically by means of a vacuum system. To the skinning machine is mounted a branch pipe of the vacuum pipeline, into which the removed skin enters and then moves to the place of waste collection - plastic vat containers.

Protein hydrolysate obtention

The processing of waste from the production of preserves from herring fillets was carried out electrochemically, followed by the release of lipids from protein solutions (Kuprina et al., 2019. For complete protein dissolution, the optimal parameters of the raw material processing were selected: voltage, current, hydronic modulus, time and temperature of suspension heating in a stirred reactor.

Fermented herring skin was dispersed to a particle size of 5×10^{-3} m, mixed with catholyte in stirred laboratory reactor LR 1000 (CZL, Russia). The catholyte was a weak saline solution obtained in the cathode chamber of a diaphragm electrolyzer, pH not less than 12.2, Eh less than - 860 mV in a ratio of 1: 3. The mixture was thermostated in the

stirred reactor for 40 minutes at 85 ± 5 °C. After holding in the reactor, the mixture was centrifuged at 4,000 rpm for 15 minutes to obtain oil.

To obtain protein-lipid hydrolyzate from herring skin wastes remaining in the production of preserves, with the subsequent isolation of oil, the raw material was electro-chemically hydrolyzed in catholyte medium, obtained electrochemically and possessing alkaline and reducing properties (pH not less than 12.0, Eh not more than -860 mV. The catholyte was obtained in industrial electrolyzers on STEL-20 installation (Rostekhnologiya, Russia).

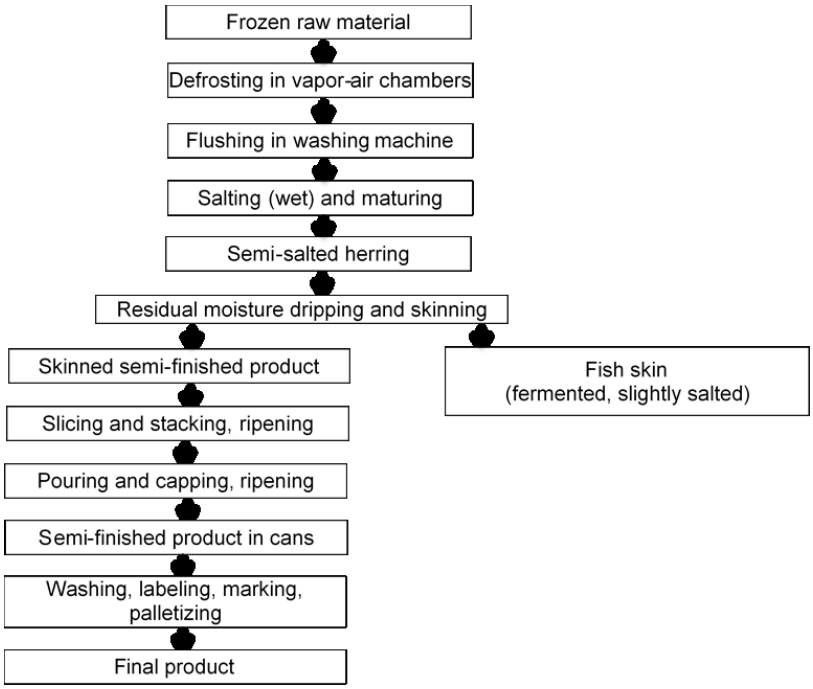

Figure 1. General technological scheme for the production of preserves from herring fillets.

Physico-chemical analysis

The content of protein, fat, ash of raw materials, as well as the iodine and acid value of lipids were determined in accordance with GOST 7636-85 (Fish, marine mammals, invertebrates and products of their processing. Methods of analysis).

Determination of the Fatty acid composition of lipids

The oil was treated with a KOH solution in methanol. As a result, methyl esters of fatty acids were formed, which were extracted with hexane. The hexane solution was used for gas chromatography-mass spectrometric analysis.

The analysis of the fatty acid composition of the obtained fish oil samples was carried out on gas chromatograph (GCMC-TQ 8040 Shimadzu, Japan) in the full ion current mode and in the scanning mode for individual ions (SIM). The temperature of the ion source was 200 °C, the interface was 250 °C, and the mass scanning was in the range $m\,z^{-1} = 45–500$. In the analysis of fatty acids, was used their precolumn derivatization with KOH solution in methanol to obtain methyl esters of carboxylic

acids. Separation of the resulting derivatives was carried out on Rxi-5SiMs capillary chromatographic column (30 m × 0.25 mm × 0.25 μm). The carrier gas was helium, the gas flow rate was 1.03 mL min^{-1}. Split / splitless mode was used (splitless 1 min, then split 10:1). Injector temperature was 220 °C. Temperature programming mode: the initial isothermal section was 50 °C for 1 min, then the column temperature rises to 250 °C (10 °C min^{-1}), the final isothermal section was 250 °C for 10 minutes. The total chromatography time was 35 min.

Fractional division of lipids by cryoconcentration

In order to concentrate unsaturated fatty acids in fish oil, lipid processing was carried out by the cryoconcentration method. 4 glass tubes (2×14 cm) with fish oil with a volume of 50 mL were placed in a container with a 28% aqueous solution of calcium chloride cooled by low-temperature refrigeration unit. The temperature was measured inside the sample and in a cooling medium using electronic thermometers 'Vapan' (TZ 003 X VP, Russia) with a standard deviation of 0.14. Phase transitions in oil were recorded at temperatures of 4, -7, -14 and -37 °C. Then the oil was mixed with acetone in a ratio of 1: 8 (v v^{-1}) and the cooling was repeated from 20 to - 40 °C. The average speed of cooling and freezing is 0.3 °C min^{-1} (Kuprina et al., 2019). After each phase transition, the oil was separated from the liquid fraction by filtration. For this purpose, synthetic filter with a pore diameter in the range of 0.3–0.5 mm was used. Filtration was carried out in a cooling unit.

The necessary and sufficient level of data reliability ($p < 0.05$) was obtained by repeating the experiment three times. Statistical data processing was performed using standard methods for evaluating test results for small samples using Microsoft Excel 2010.

RESULTS AND DISCUSSION

We studied the material balance of the skinning process of herring after salting and determined the chemical composition of Atlantic herring before and after salting.

It was found that the oil content of fresh Atlantic herring is higher than that of medium-salted Atlantic herring by 4%, and the moisture content is 1.7% lower. The protein content in herring before and after salting is approximately the same (Table 1).

Table 1. Chemical composition of fresh and slightly salted Atlantic herring

Sample	Moisture, %	Fat, %	Acid value of fat, mg KOH g^{-1}	Protein, %	Ash, %	Energy value, kcal
Fresh Atlantic herring	61.3 ± 2.0	19.5 ± 0.3	1.8	17.7 ± 0.2	1.5 ± 0.1	248
Atlantic herring, medium salted	63.0 ± 2.0	16.3 ± 0.3	2.6	17.0 ± 0.2	11.5 ± 1.0	215

It is also known that during the salting process, partial lipid hydrolysis occurs, leading to an increase in the acid number (Table 1). This process involves the tissue's own enzymes - lipases, while the quality of the fatty acid composition deteriorates. In addition, when interacting with atmospheric oxygen, lipids begin to oxidize (Ghnimi et al., 2017; Hematyar et al., 2019). However, since the process of obtaining lightly salted

herring under production conditions is optimized and reduced to seven days, these undesirable processes are not implemented.

The final output of the dispersed skin waste part is on average 6.5% of the mass of fillets skin-on taken for processing. In terms of physicochemical parameters, the semi-finished product of the waste part is close to the indicators of a similar initial semi-finished product - herring fillet on the skin:

Salt content - 3.8–4.2%;

Fat content - 27–28%

Acidity - 0.17–0.23% (according to vinegar essence70%);

pH = 5.8–6.1%.

The dispersed waste was processed in stirred reactor with in the environment of the catholyte obtained by the electrochemical method.

Unlike the previously developed electrochemical method for obtaining fish oil (Chikisheva et al., 2020), the dispersed raw material is not processed in the interelectrode space of the electrolyzer, which eliminates the need for frequent equipment washing.

At the same time, a complete dissolution of tissues was achieved, including bone tissues (fins), this effect was obtained in gentle conditions without processing the raw material in an electric field inside the electrolyzer space and a significantly lower hydromodule 1: 3, and not 1: 6, as was done earlier (Kuprina et al., 2019). This effect was probably achieved due to partial hydrolysis of tissues during salting.

Even if we assume the possibility of some oxidative deterioration of oil during salting, due to the presence of reducing properties in the electrochemically obtained catholyte, restoration of the quality of the original oil is ensured (Kuprina et al., 2019, Kuprina et al., 2020).

The mass yield of oil from the hydrolyzate of the skin of slightly salted herring after centrifugation was significant - 22–25% of the mass of the sample skin.

It was revealed that fish oil, obtained electrochemically from wastes of fermented salted herring (Table 2, Fig. 2) has an acid number of ≤ 0.15 mg KOH g^{-1}, contains more than 20% omega-3 polyunsaturated fatty acids from the total fatty acids, but this amount is not enough to meet the daily human needs (according to methodological recommendations MP 2.3.1.2432–08) (Rospotrebnadzor). Thus, the development of a technology for the concentration of omega-3 polyunsaturated fatty acids is relevant.

Table 2. Fatty acid composition of salted herring skin oil

No.	Acid name	Content, mg g^{-1}	Content, %
1	Lauric $C_{12}H_{24}O_2$	0.7	0.07
2	9-tetradecene $C_{14}H_{28}O$	0.6	0.06
3	Myristic $C_{14}H_{28}O_2$	52	5.2
4	Pentadecane $C_{15}H_{32}$	3	0.3
5	9-hexadecene $C_{16}H_{30}O$	41	4.1
6	Hexadecanoic (palmitic) $C_{16}H_{32}O_2$	85	8.5
7	Linoleic $C_{18}H_{32}O_2$	31	3.1
8	Linolenic $C_{18}H_{30}O_2$	4	4
9	9-octadecene (oleic) $C_{16}H_{36}$	53	5.3
10	Stearic $C_{18}H_{36}O_2$	5	0.5
11	Cis-5,8,11,14,17-Eicosaenoic $C_{21}H_{32}O_2$	43	4.3
12	Cis-4,7,10,13,16,19-Docosahexaenoic $C_{22}H_{32}O_2$	33	3.3
13	Docosanoic $C_{22}H_{44}O_2$	47	4.7

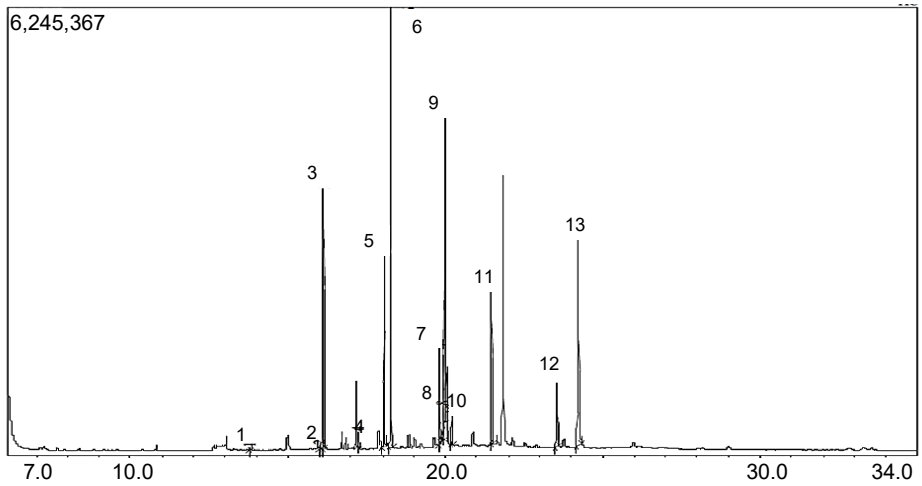

Figure 2. Chromatogram of the fatty acid composition of slightly salted herring lipids before cryoconcentration.

It was found that phase transitions in oil occur at temperatures: 4 °C, -7 °C, -14 °C and -37 °C. Phase transitions are accompanied by the precipitation of lipid fractions less saturated with double bonds. After filtration the contents of the tubes on a nylon filter with a pore diameter of ≤ 03 mm in the cooling unit, the lipid supernatant fraction enriched in unsaturated fatty acids was further cooled (Table 3).

Table 3. Fatty acid composition of oil from the skin of slightly salted herring after cryoconcentration at a temperature up to ─14 °C (Sample 'Raw Material')

No.	Acid name	Content, mg g^{-1}	Content, %
1	Lauric $C_{12}H_{24}O_2$	0,5	0,05
2	9-tetradecene $C_{14}H_{28}O$	0,6	0,06
3	Myristic $C_{14}H_{28}O_2$	35	3,5
4	Pentadecane $C_{15}H_{32}$	2	0,2
5	9-hexadecene $C_{16}H_{30}O$	38	3,8
6	Hexadecanoic (palmitic) $C_{16}H_{32}O_2$	54	5,4
7	Linoleic $C_{18}H_{32}O_2$	35	3,5
8	Linolenic $C_{18}H_{30}O_2$	9	0,9
9	9-octadecene (oleic) $C_{16}H_{36}$	41	4,1
10	Stearic $C_{18}H_{36}O_2$	3	0,3
11	Cis-5,8,11,14,17-Eicosaenoic $C_{21}H_{32}O_2$	135	13,5
12	Cis-4,7,10,13,16,19-Docosahexaenoic $C_{22}H_{32}O_2$	106	10,6
13	Docosanoic $C_{22}H_{44}O_2$	46	4,6

In the course of the experiment of cooling the oil obtained from the skin of slightly salted herring, a temperature versus time curve was obtained (Fig. 3) with areas of phase transitions and fractionation, which corresponds to the data of patent (Zakharchuk et al., 1995).

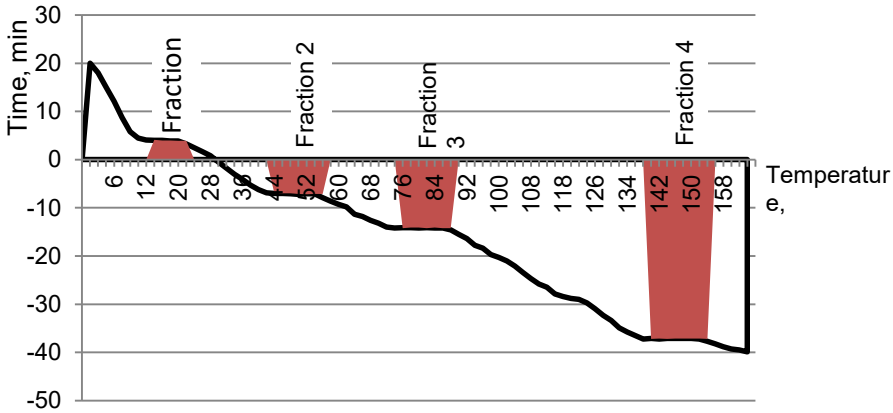

Figure 3. Dependence of phase transitions of fish oil on temperature and time of cooling and freezing.

Fractional analysis results are presented in Table 3, 4 and Fig. 4.

Table 4. Dependence of the yield of solid fraction of lipids from the skin of Atlantic herring on temperature

Temperature, °C		+4	-7	-14	-37
Separated fraction, %	Experiment 1	29.6	38.4	51.3	55.2
	Experiment 2	27.8	38.2	51.8	56

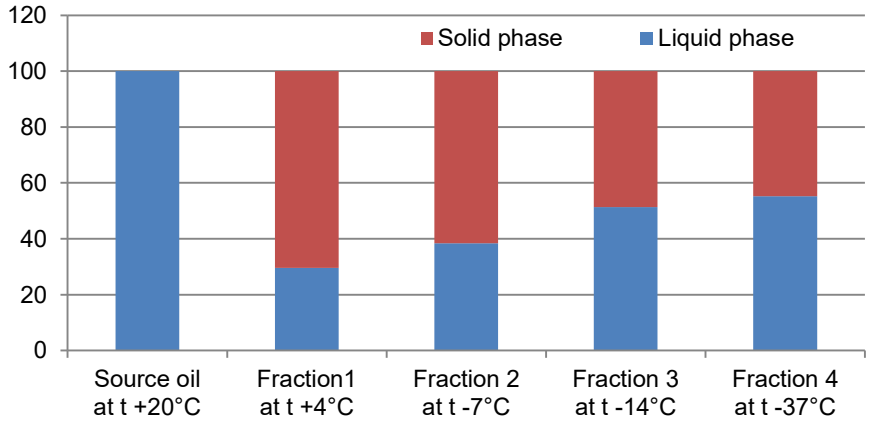

Figure 4. The phase composition of lipids, obtained from skin wastes from slightly salted herring.

With a decrease in temperature from 20 to -14 °C, an increase in the concentration of omega-3 fatty acids in oil is observed by 3 times. At the same time, the ratio of eicosapentaenoic and docosopentaenoic acids remains constant. When the oil temperature reached -14 °C, the concentration of omega-3 fatty acids increased to 24.1%, which was sufficient for the use of the obtained fish oil as a dietary supplement for food or as part of the FFP. Further temperature reduction is impractical, since the

next fraction is released at the temperature of -37 °C (Kuprina et al., 2019) and its achievement is not economically viable in industrial conditions, since it requires high energy consumption.

Table 5 presents biochemical properties and mass yields of omega-3 fatty acids of fish oil before and after cryoconcentration at different stages of phase transition.

Table 5. Biochemical properties and mass yields of cryoconcentrated lipid fractions isolated from skin hydrolyzate of Atlantic herring fillets

Indicator	Oil before cryoconcentration at t +20 °C	Solid fraction of oil released at t +4 °C (Fraction 1)	Solid fraction of oil released at t -7°C (Fraction 2)	Solid fraction of oil released at t -14 °C (Fraction 3)	Liquid fraction of oil released at t -14 °C (Fraction 4)
Acid value, mg KOH*g^{-1}	1.2	1.3	1.2	1.3	1.9
Iodinenumber, g 100 g^{-1}	96.03	101.05	104.6	103.29	201.52
Eicosapentaenoic acid, % in oil	4.3	1.3	1.4	3.6	13.5
Docosahexaenoic acid, % in oil	3.3	0.9	1.2	2.6	10.6
EPA:DHA ratio	1.3	1.4	1.2	1.4	1.3
Total content of ω-3,% in the skin oil, %	7.6	2.2	2.6	4.4	24.1
Mass of cryoconcentrated oil fractions, g	8	2.37	2.16	1.78	1.69
Yield of fractions obtained from the skin oil, %	100	29.6	27	22.3	21.1

CONCLUSIONS

1. Fish oil obtained by the electrochemical method from fish skin was characterized by satisfactory quality characteristics due to the presence of reducing properties in the extractant: Eh not more than 860 mV, which made it possible to reduce the acid number of fat contained in the initial raw material - slightly salted herring from 2.6 mg KOH g^{-1} to 0.5 mg KOH g^{-1}.

2. The technology of cryoconcentration of oil from hydrolysates of fish skin made it possible to enrich it with omega-3 fatty acids.

3. Chemical analysis of raw materials was carried out and the yields of the raw material product were determined at all stages of processing.

4. The composition of the fractions of cryoconcentrated oil was investigated and the expediency of lowering the temperature to -14 °C was established, at which a phase transition was observed, providing a significant concentration of omega-3 fatty acids. Their concentration increased from 7.6 to 24% in oil.

ACKNOWLEDGEMENTS. This work was financially supported by ITMO University Grant 620142 from 01/09/2020.

REFERENCES

Abuine, R., Rathnayake, A.U. & Byun, H.G. 2019. Biological activity of peptides purified from fish skin hydrolysates. *Fisheries and Aquatic Sciences* **22**(1), 1–14. doi: 10.1186/s41240-019-0125-4

Alfio, V.G., Manzo, C. & Micillo, R. 2021. From Fish Waste to Value: An Overview of the Sustainable Recovery of Omega-3 for Food Supplements. *Molecules* **26**(4), 1002. doi: 10.3390/molecules26041002

Baydalinova, L.S & Yarzhombek, A.A. 2011. Biochemistry of raw materials of water origin. *Morkniga* **510**.

Bazarnova, J., Korableva, A.N., Ozerova, O. & Moskvicheva, E. 2020. Biochemical composition and quality of herring preserves with addition of bio-protective cultures. *Agronomy research* **18**(3), 1629–1639. doi:10.15159/AR.20.098

Calder, P., Carr, A., Gombart, A. & Eggersdorfer, M. 2020. Optimal nutritional status for a well-functioning immune system is an important factor to protect against viral infections. *Nutrients* **12**(4), 1181. doi: 10.3390/nu12041181

Chikisheva, M.E., Kuprina, E.E., Manuilov, A.N., Yakkola, A.N., Naumov, I.A. & Kabysh, O. 2020. Development of electro-biochemical technology for processing secondary fish raw materials and new types of products based on minced fish. *E3S Web of Conferences* **161**, 6–5. doi: 10.1051/e3sconf/202016101095

Durmus, M.2018. Fish oil for human health: omega-3 fatty acid profiles of marine seafood species. *Ciência e Tecnologia de Alimentos* **39**(3–4). doi: 10.1590/fst.21318

Freitas, H.R., Ferreira, G., Trevenzoli, I., Oliveira, K. & Reis, R. 2017. Fatty acids, antioxidants and physical activity in brain aging. *Nutrients* **9**(11), 12639. doi: 10.3390/nu9111263

Ghnimi, S., Budilarto, E. & Kamal-Eldin, A. 2017. The new paradigm for lipid oxidation and insights to microencapsulation of Omega-3 fatty acids. *Comprehensive Reviews in Food Science and Food Safety* **16**(6), 1206–1218. doi: 10.1111/1541-4337.12300

GOST 7636–85. *Fish, marine mammals, invertebrates and products of their processing. Methods of analysis.* Interstate Standard for the countries of the Eurasian Economic Union (in Russian).

Hematyar, N., Rustad, T., Sampels, S. & Dalsgaard, T.K. 2019. Relationship between lipid and protein oxidation in fish . *Aquaculture Research* **50**(5), 1393–1403. doi: 10.1111/are.14012

Jahangard, L., Sadeghi, A., Ahmadpanah, M., Sadeghi-Bahmani, D., Holsboer-Trachsler, E. & Brand, S. 2018. Influence of adjuvant omega-3-polyunsaturated fatty acids on depression, sleep, and emotion regulation among outpatients with major depressive disorders – Results from a double-blind, randomized and placebo-controlled clinical trial. *Journal of psychiatric research* **107**, 48–56. doi: 10.1016/j.jpsychires.2018.09.016

Joint FAO / WHO Codex Alimentarius Commission, World Health Organization. Organically Produced Foods. - Food & Agriculture Org., 2007. During the Acid Whey and Fish Waste Fermentation. *Microorganisms* 2021(9), 100.

Kazakova, V.S. & Zemlyakova, E.S. 2020. HIGH-TEMPERATURE HYDROLYSIS OF FISH COVER TISSUE OF THE KALININGRAD REGION. *Izvestiya KSTU* **59**, 141–150. doi: 10.46845/1997-3071-2020-59-141-150

Kosman, V.M., Demchenko, D., Pozharitskaya, O.N., Shikov, A.N. & Makarov, V.G. 2016. Comparative analysis of the requirements for the quality of fish oil for food and medical use. *Nutrition issues* **85**(6), 110–117.

Kurkotilo, V.N & Vasilyeva, Zh.V. 2017. Resource-saving technology for processing lipid-containing waste from the fishing industry. *Bulletin of the Murmansk State Technical University* **20**(1). doi: 10.21443/1560-9278-2017-20-3-609-618

Kuprina, E., Filipov, V., Malova, A., Abramzon, V., Lepeshkin, A. & Chikisheva, M.2019. Technology development of obtaining essential fatty acids from hydrobionts hydrolyzates. *AgronomyResearch* **17**(6), 2317–2326. doi: 10.15159/AR.19.205

Kuprina, E., Yakkola, A., Kopylov, A., Zashikhin, M. & Kuznetsova, A. 2020. Development of functional product enriched with collagen hydrolysate from fish processing waste. *E3S Web of Conference* **164**(3), 06026. doi: 10.1051/e3sconf/202016406026

Mayta-Apaza, A.C., García-Cano, I., Dabrowski, K. & Jiménez-Flores, R.2021. Bacterial Diversity Analysis and Evaluation Proteins Hydrolysis During the Acid Whey and Fish Waste Fermentation. *Microorganisms* **9**(1), 100. doi: 10.3390/microorganisms9010100

Mohanty, B.P., Mahanty, A., Ganguly, S. & Mitra, T. 2019. Nutritional composition of food fishes and their importance in providing food and nutritional security. *Food chemistry* **293**(9), 561–570. doi: 10.1016/j.foodchem.2017.11.039

Mukatova, M.D., Kirichko, N.A., Moiseenko, M.S. & Skolkov, S.A. 2018. Development of a technology for obtaining fat from fat-containing wastes of commercial fish processing in the Volga-Caspian basin. *Izvestiya TINRO (Pacific Research Fisheries Center* **193**, 211–222. doi: 10.26428/1606-9919-2018-193-211-222

Neuimin, D.S. 2017. Current state and features of the development of the fish and fish products market. *Technologies for the food and processing industry of the agro-industrial complex - healthy food products* **1**(15).

Porotikova, E.Yu., Nehamkin, B.L. & Andreev, M.P. 2015. Influence of packaging material permeability on the quality and safety of salted Atlantic herring packaged in a modified gas environment. *III Baltic Maritime Forum 'Innovations in the technology of healthy food*', 124–130.

Radzikowska, U., Rinaldi, A., Sözener, Z., Karaguzel, D., Wojcik, M., Cypryk, K., Akdis, M., Akdis, C. & Sokolowska, M. 2019. The influence of dietary fatty acids on immune responses. *Nutrients* **11**(12), 2990. doi: 10.3390/nu11122990

Repnikov, B. 2017. Commodity Science and Biochemistry of Fish Products. Litres.

Ross, R.P., Stanton, S., Egerton, C., Whooley, J., Sarah, C.C. & Mannion, D. 2020. The proximate composition of three marine pelagic fish: blue whiting (Micromesistius poutassou), boarfish (Capros aper) and Atlantic herring (Clupea harengus). *Irish Journal of Agricultural and Food Research* **59**(1). doi: 10.15212/ijafr-2020-0112

Rospotrebnadzor. 2009. Norms of physiological needs for energy and nutrients for various groups of the population of the Russian Federation. Guidelines. Moscow, Federal Center for Hygiene and Epidemiology of Rospotrebnadzor /Роспотребнадзор. Нормы физиологической потенции в энергетике и физиологических вещах для различных групп населения Российской Федерации. Методические рекомендации/. Moscow, (in Russian). https://www.rospotrebnadzor.ru/documents/details.php?ELEMENT_ID=4583

Sacca, S.C., Cutolo, C.A., Ferrari, D. & Corazza, P. 2018. The eye, oxidative damage and polyunsaturated fatty acids. *Nutrients* **10**(6), 668. doi: 10.3390/nu10060668

Sioen, I., Lieshout, L., Eilander, A. & Fleith, M. 2017. Systematic review on n-3 and n-6 polyunsaturated fatty acid intake in European countries in light of the current recommendations – focus on specific population groups. *Annals of nutrition and metabolism* **70**(1), 39–50. doi: 10.1159/000456723

Suresh, P.V., Kudre, T.G & ohny, L.S. 2018. Sustainable valorization of seafood processing by-product / discard. *Waste to Wealth. Springer, Singapore* **111**, 139 pp. doi: 10.1007/978-981-10-7431-8_7

Varygina, V.P & Davydova, A.I. 2021. Processing wastes of fish production. *Complex research in the fishing industry*, pp. 192–197.

Vorslov, L.O. 2017. Omega-3-polyunsaturated fatty acids as a source of longevity. *Questions of dietology* **7**(1), 36–41. doi: 10.20953/2224-5448-2017-1-36-41

Zakharchuk, A.V., Lobova, E.I., Dubnitskaya, G.M., Munin, A.A. & Levachev, M.M. Method for obtaining fish oil. [Patent RUS No. 2031923 / 27.03.95. Byul. No. 9. Sposob poluchenija ryb'ego zhira. (In Russ).]. Available at: https://www.fips.ru/registers-doc-view/fips_servlet?DB=RUPAT&rn=783&DocNumber=2031923&TypeFile=html. Link active on 02/10/2021.

Agronomy Research **19**(S3), 1260–1272, 2021
https://doi.org/10.15159/AR.21.087

Evaluation of selected lactic acid bacteria as starter cultures for gluten-free sourdough bread production

O. Parakhina, M. Lokachuk, L. Kuznetsova, O. Savkina*, E. Pavlovskaya and T. Gavrilova

St. Petersburg branch Scientific Research Institute for the Baking Industry, Podbelskogo highway 7, RU196608 St. Petersburg, Pushkin, Russian Federation
*Correspondence: 1103savkina@mail.ru

Received: January 30th, 2021; Accepted: April 8th, 2021; Published: May 19th, 2021

Abstract. Sourdough is one of the most promising technologies for gluten-free bread. The selection of appropriate starter cultures for the production of gluten-free sourdoughs is of a great importance, since not all microorganisms can adapt equally to the same raw material. The aim was to create a new starter microbial composition for gluten-free sourdough preparation, allowing improving the quality and the microbiological safety of gluten-free bread. Screening was conducted on 8 strains of lactic acid bacteria (LAB) and 5 strains of yeast previously isolated from spontaneously fermenting rice and buckwheat sourdoughs. The strain *S. cerevisiae* Y205 had the highest fermentative activity and alcohols content. The lactic acid bacteria *L. brevis* E139 and *L. plantarum* E138 were also experimentally selected for new gluten-free sourdoughs on the basis of acidity and volatile acids production and antagonistic activity. Two types of microbial composition were created and its influence on sourdough biotechnological indicators was studied. Sourdough with *L. plantarum* E138 had in 1.2 times lower titratable acidity, in 3.4 times lower volatile acids content compared to sourdough with *L. brevis* E139. Alcohol content was the same in both sourdoughs similarly to yeast cells amount. Sourdough dough proofing time increased in 1.2–1.3 times compared to the control. Sourdough did not affect the specific volume, porosity and compressibility of gluten-free bread, but its sensory characteristics were improved. Bread made with sourdoughs had more pronounced taste and flavor, brighter crust color and better texture compared bread without sourdough. The microbiological safety of sourdough gluten-free bread was also increased, especially when *L. brevis* E139 was used.

Key words: celiac disease, biotechnology, sourdough, lactic acid bacteria, yeasts, gluten-free bread.

INTRODUCTION

In recent decades, researchers have paid increasing attention to the development of gluten-free bread technologies. New ingredients and different techniques are being used to improve the quality of gluten-free products (Houben et al., 2012; Ziobro et al., 2012; Masure et al., 2016, Bender & Schönlechner, 2019; Azizi et al., 2020; Cappelli et al, 2020; Nissen et al., 2020; Puerta et al., 2021). With the growing interest of consumers in healthy lifestyle, the demand for clean label products is growing. This leads to increased research in the field of safe technologies without chemical additives.

Sourdough fermentation and new baking technologies have come into interest as they offer textural and sensory advantages without chemical compounds (Mariotti et al., 2017; Cappelli et al, 2020; Nissen et al., 2020; Puerta et al., 2021). Sourdough allows generating changes in the dough properties and bread quality (Arendt et al., 2011; Wolter et al., 2014; Cappa et al., 2016; Bender et al., 2018; Olojede et al., 2019; Cappelli et al, 2020).

Research shows that sourdough is a promising way for gluten-free bread making as it improves sensorial, nutritional and textural bread properties (Arendt et al., 2011; Nionelli & Rizzello, 2016; Olojede et al., 2019; Ren et al., 2020). It is well known that lactic acid bacteria (LAB) strains are able to improve the shelf life of bread since the acids formed during fermentation process lower the pH thus inhibiting the growth of spoilage organisms ((Zacharof & Lovitt, 2012; Khandakar et al., 2014; Axel et al., 2015; Hassan et al., 2015; Bender et al., 2018; Bartkiene et al, 2019). During sourdough fermentation organic acids and free amino acids, depending on the selected starter culture, are responsible for the increase in taste intensity (Houben et al., 2012; Wolter et al., 2014).

The application of sourdough offers improved textural characteristics and improved viscosity and elasticity in gluten-free batters, and effects are dependent on the amount of added sourdough and on the LAB strain used for fermentation (Arendt et al., 2007; Gobbettii et al., 2008; Houben et al., 2010; Jekle et al., 2010; Masure et al., 2016).

Gluten-free sorghum sourdough fermented with L. *plantarum* and *L. casei* or *L. fermentum* and *L. reuteri* enhanced its the nutritional value by inducing the degradation of polyphenols (Svensson et al., 2010).

Wolter et al. (2014) have studied sourdoughs made with different gluten-free flours and wheat flour, fermented by *Lactobacillus plantarum FST 1.7*. It was found that dough made with sourdough had a lower strength and was softer. At the same time, the crumb porosity in baked bread was significantly higher. The studies have shown that the sourdough usage did not affect the shelf life and smell of gluten-free bread.

Mariotti et al (2017) obtained, in general, the promising results of adopting the sourdough technology in gluten-free bread making. Sourdough bread had a more uniform texture compared to yeast bread. However, sourdough bread had a lower specific volume and a denser crumb. So authors have recommended compressed yeasts and sourdough combination in order to obtain the best performances.

Bender et al (2018) have studied the effect of different strains of the genus *Lactobacillus on* the quality of the sourdoughs made from buckwheat and millet flour. Research has shown that the examined trains have a different effect on the quality of gluten-free bread. This shows the importance of the strains selection that allows improving the gluten-free bread quality.

The usage of LAB strains previously isolated from spontaneously fermenting wheat and rye sourdoughs in gluten-free sourdough technology ensured high quality of gluten-free bread when used together with the addition of rowan powder (Dubrovskaya et al., 2018).

Olojede et al. (2019) obteined good results when used *Pediococcus pentosaceus* SA8, 78 *Weissella confusa* SD8*, P. pentosaceus* LD7 and *Saccharomyces cerevisiae* YC1 previously isolated from spontaneously fermenting sorghum sponge (Ogunsakin et al., 2017; Olojede et al., 2019). The strains were selected on the basis of their functional properties. Sourdough from sorghum with these strains led to the development of bread

with improved rheology, texture and nutritional properties, thus confirming the functionality of the employed starter cultures.

All studies have shown the importance of selecting appropriate starter cultures for the production of gluten-free sourdoughs (Ogunsakin et al., 2017; Maidana et al., 2019; Olojede et al., 2019), since not all microorganisms can adapt equally to the same raw material. Microbial growth can be influenced by availability of carbohydrates, nitrogen sources, lipids and free fatty acids content, as well as the enzymatic activity, buffer capacity and the presence of growth factors (i.e. vitamins and minerals) in the substrate (Moroni et al., 2011; De Vuyst et al., 2014; Gänzle, 2014; Bender et al., 2018). However, the nature and quality of the raw material are not the only factors determining the dominant sourdough microbiota. Process parameters including temperature, dough yield, fermentation time and number of refreshment steps or interactions involving starter cultures, autochthonous strains and contamination microflora can influence the final composition of the sourdough (Arendt et al., 2007; De Vuyst et al., 2014; Gänzle, 2014; Zhang et al., 2019). These conditions contribute to the persistence of the dominating strains and can ensure a reproducible and controlled composition of the sourdough microbiota and ensure a constant bread quality.

Thus sourdough fermentation effectiveness in improving the quality of gluten-free bread depends on the strains of LAB and yeasts. The selection of appropriate starter cultures carefully chosen for specific gluten-free raw materials is of crucial importance. There is no substrate and activity limitation for the lactic acid bacteria in gluten-free flours, but its fermentation can differ between the strains used. There is always a need for a test of starter strain (Houben et al., 2012)

That is why the aim of this study was to investigate different strains of lactic acid bacteria, to create a new starter composition, and to develop gluten-free sourdough bread technology which would improve the quality and microbial safety of bread.

MATERIALS AND METHODS

Characteristics of ingredients

The study of lactic acid bacteria strains

The starter cultures of lactic acid bacteria used in this study were composed of: *Lactobacillus diolivorans* E133*, L. plantarum* E134*, L. plantarum* E135*, L. brevis* E136*, Pediococcus pentosaceus* E137*, L. plantarum* E138*, L. brevis* E139*, L. brevis* E140 previously isolated from spontaneously fermenting rice and buckwheat sourdoughs. The species of bacteria were previously determined based on the study of morphological, cultural and biochemical characteristics and by the method of 16S rRNA sequencing (Parakhina et al., 2020).

Acidifying activity. LAB were cultivated in MRS liquid selective culture medium (BioMerieux, France). 1 mL of lactic acid bacteria liquid culture was inoculated into 100 g mixture of water and rice flour (moisture content 60%) and was kept at 24 ± 1 °C for 24 h. The titratable acidity was calculated according to Di Renzo et al. (2018). The content of volatile acids was determined by neutralizing the evaporated volatile acid using a 0.1 M solution of NaOH (Afanasjeva, 2003).

Antagonistic activity. The antagonistic activity of lactic acid bacteria against pathogens of ropy bread disease was determined by the method of agar slab method (Polak-Berecka et al., 2009; Dec et al., 2016). Test culture of lactic acid bacteria was

inoculated in a deep way on the MRS (de Man, Rogosa and Sharp) agar (BioMerieux, France) in the Petri dishes and was incubated at the optimum temperature of 30 °C for 3 days for the formation and accumulation of inhibitory compounds in agar. Then, the agar slab with a grown culture of lactic acid bacteria was cut with a sterile cork borer (diameter 7 mm) and transferred to another Petri dish on the surface of the meat-peptone agar, freshly inoculated with the *Bacillus subtilis* test strain from the collections of the St. Petersburg branch of Scientific Research Institute for the Baking Industry.

B. subtilis test strain was grown on meat-peptone medium with agar and a suspension containing 10^8 cells mL^{-1} was prepared using a densitometer DEN-1 (BioSan, Latvia - England).

The plates were kept for 3 hours in a refrigerator at a temperature of 4 °C (in order to avoid premature growth of the test strain) to diffuse antibiotic substances from the slab into the agar with the test strain, and then incubated at a temperature favorable for the development of the B. subtilis test strain (37 °C). The degree of antagonistic activity of the test culture of lactic acid bacteria was judged by the size of the zone of growth inhibition of the *B. subtilis* test strain around the agar slab.

The study of yeasts

Five strains of yeasts previously isolated from spontaneously fermenting rice and buckwheat sourdoughs were used in this study: *Kazachstania bulderi* Y202, *Candida humilis* Y 203 and Y 204, *S. cerevisiae* Y 205 and Y 206. The species of yeast were previously determined based on the study of morphological, cultural and biochemical characteristics and by the method of ITS sequencing (Parakhina et al., 2020).

The fermentation activity of yeasts in a mixture of water and rice flour (moisture content 60%) was studied.

Yeast fermentation activity was determined by the amount of released carbon dioxide. 1 mL of yeast liquid culture containing 10^8 cells mL^{-1} was added to 100 g of rice flour and water mixture (moisture content 60%). Cells number was determined using a densitometer DEN-1 (BioSan, Latvia - England).

The flasks were tightly capped with a container filled with 96% sulfuric acid (special glass device - Muller valve). Sulfuric acid prevents the evaporation of water. Only CO_2 is removed from the flask. Flasks were left to ferment for 24 hours at 24 ± 1 °C, measuring the amount of CO_2 released. From the difference in mass, before and after fermentation, the fermentation activity of each yeast strain was judged (Kurtzman & Fell, 1998).

Sourdough preparation

To prepare the starter culture, lactic acid bacteria were grown on MRS agar. Then an aqueous suspension with a cell titer of 10^9 CFU mL^{-1} was prepared using a densitometer DEN-1 (BioSan, Latvia - England). Yeast was grown on malt agar and then aqueous suspension with a cell titer of 10^8 CFU mL^{-1} was prepared. 2% LAB suspension and 4% yeast suspension were introduced into a mixture of water and rice flour with a moisture content of 57%. The dosages of pure starter cultures were established experimentally based on previous studies (data not shown). The initial content of examined LAB cell in sourdough was $2 \cdot 10^7$ CFU g^{-1} and the yeast cell content was $4 \cdot 10^6$ CFU g^{-1}.

Sourdough were fermented at a temperature of 28–30 °C for 18–20 h, and then the acidity, the content of LAB and yeast were determined.

The formulation of a gluten-free sourdough is presented in Table 1.

Then the obtained gluten-free sourdoughs were propagated by feeding with rice flour and water in a ratio of 1:1:1 every 16–18 h and fermentation at a temperature of 22–24 °C for two weeks. Biochemical indicators were evaluated at 12th backslopping.

Table 1. Formulations used to prepare sourdough

Ingredients, g	Sourdough I	Sourdough II
Suspension of *L. plantarum* E138, mL	2.0	-
Suspension of *L. brevis* E139, mL	-	2.0
Suspension of *S. cerevisiae* Y205, mL	4.0	4.0
Rice flour, g	50.0	50.0
Water, g	44.0	44.0
Total	100.0	100.0

Bread-making procedure

Gluten-free mixture including corn starch, extrusion corn starch, soy protein isolate, and rice flour were used as flour. Dough humidity was 52%. The compressed yeasts and sourdough combination was used in order to obtain the best performance (Mariotti et al., 2017; Dubrovskaya et al., 2018). The formulation of bread is presented in Table 2.

The dough was mixed in a mixer Kitchen Aid KSM45 (USA) at 120 rev min^{-1} for 7 min. 250 g dough pieces were placed in baking moulds and kept at 30 °C until the doubled in volume, then baked for 18 min in oven Sveba Dahlen (Sweden) at 210 °C with steam for 5 s at the beginning of baking.

Table 2. Bread formulation

Ingredients, g	Control bread	Sourdough bread	
	Dough	Sourdough	Dough
Corn starch	60.0	-	60.0
Rice flour	20.0	10	5.0
Extruded corn starch	10.0	-	10.0
Soy protein isolate	10.0	-	10.0
Vegetable oil	3.8	-	3.8
Yeast	2.5	-	2.5
Sugar	2.0	-	2.0
Salt	0.8	-	0.8
Sourdough	-	10	30.0
Water	93.4	10	77.2

Sourdough and dough assessments

Sourdoughs and the doughs samples were evaluated for different parameters. Acidity was evaluated by titration with 0.1 N sodium hydroxide solution using phenolphthalein as indicator (Puchkova, 2004). The content of volatile acids was determined by neutralizing the evaporated volatile acid using a 0.1 M solution of NaOH (Puchkova, 2004). The alcohol content was determined by using the iodometric method, which is based on the quantity of sodium thiosulfate spent in titration (Puchkova, 2004). Viable plate counts of lactic acid bacteria and yeasts in sourdough and the ratio between lactic acid bacteria and yeasts were studied. 10 g of sourdough were homogenized with 90 mL of sterile chloride (0.9% wt·wol^{-1}) solution. Serial dilutions were plated on MRS agar for determination of LAB. Plates were incubated at 30 °C under anaerobic conditions. An AnaeroGen System (Oxoid, UK) was used to maintain an anaerobic environment. The number of yeast in sourdough was estimated by using malt agar at 30 °C for 72 h. (Afanasjeva, 2003).

Assessment of baked bread

Assessment of quality. The quality of bread was evaluated the following parameters. Titratable Acidity (TTA) was determined by titration of crumb sample with 0.1 M sodium hydroxide solution using phenolphthalein as indicator (State Standard of the Russian Federation GOST 5670-96, 1996). Cells volume was calculated as the ratio of cells volume to the total bread volume. Bread specific volume was determined by a rapeseed displacement and calculated as the ratio of volume to 100 g of bread (Puchkova, 2004). The automatic penetrometer (Labor, Hungary) was used to estimate crumb compressibility (Puchkova, 2004). The volatile acids amount was estimated by determining the amount 0.1 M sodium hydroxide solution used for neutralization of the evaporated volatile acid. The alcohol content was determined by using the iodometric method, which is based on the quantity of sodium thiosulfate spent in titration.

Ropy disease and moulds spoilage assessment

To estimate sourdough performance on bread microbial resistance, the bread was infected with the spore-forming bacteria *B. subtilis* and moulds *Penicillium chrysogenum*. Both methods were described in details in previous study (Dubrovskaya et al., 2018).

Assessment of organoleptic characteristics. Ten-member expert panel was used to evaluate the bread sensory (organoleptic) characteristics. Experts evaluated taste and smell, texture of crumb (chewiness), color of crust and porosity. Each trait was rated on a scale from 1 to 5: 1– poor; 2 – dislike; 3 – slightly dislike; 4 – moderately; 5 – very good).

Statistical analysis

Comparison of the influence of factors was carried out by the method with significance tested at the 95% confidence level and differences between means were determined using the least significant difference and Duncan's test of two-factor analysis of variance with one repetition (ANOVA). Each factor was analyzed at least in triplicate. Statistical analysis was performed using Excell software.

RESULTS AND DISCUSSION

Studies of the lactic acid bacteria antagonistic activity have shown that all strains inhibited the growth of *B. subtilis*. It may be stated that the size of inhibition zones depended on sensitivity of the target strain. The highest mean value of inhibition zones (14 ± 1 mm) were in case of *L. brevis* E139 and *L. plantarum* E138 (Fig. 1). Results evidenced that the antibacterial abilities of LAB were depended on the LAB

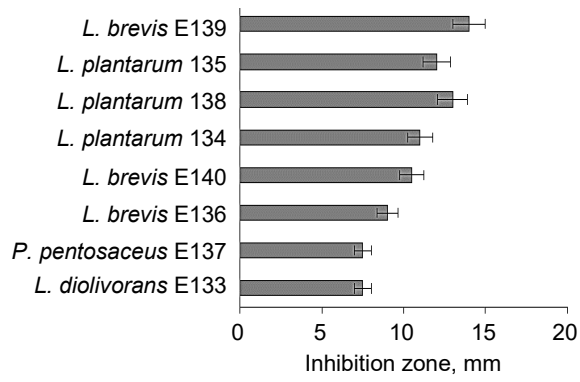

Figure 1. Antagonistic activity of lactic acid bacteria against *B. subtilis*.

species and strain (Khandakar et al., 2014; Dubrovskaya et al., 2018; Miadana et al., 2019; Bartkiene et al., 2020).

According to the lowest pathogen inhibition, *L. diolivorans* E133, *L. brevis* E136 and *P. pentosaceus* E137 were not used in further research. The acidifying activity of the chosen *Lactobacilli* was evaluated (Table 3).

The strains L. *plantarum* E138 and *L. plantarum* E135 had the highest acidity. The strain *L. brevis* E139 produced highest amount of volatile acids, most of which may be be acetic acid (Zalán et al., 2010). The antagonism activity could be due to the presence of organic acids, although it is possible to assume the production of bacteriocins (Zalán et al., 2010; Zacharof & Lovitt, 2012; Sadiq et al, 2019; Bartkiene et al., 2020).

Table 3. Acid-forming activity of lactic acid bacteria

Lactobacilli strains	Acidity, degrees N	Volatile acids, degrees N
L. brevis E139	6.2 ± 0.3^{a}	1.45 ± 0.13^{a}
L. brevis E140	7.2 ± 0.3^{b}	0.80 ± 0.11^{b}
L. plantarum 134	6.8 ± 0.2^{c}	0.95 ± 0.13^{c}
L. plantarum E138	8.1 ± 0.3^{d}	0.83 ± 0.11^{b}
L. plantarum E135	7.8 ± 0.2^{d}	0.90 ± 0.11^{c}

Mean values ± standard deviation (SD) within the same column with different letters are significantly different ($P \leq 0.05$).

Based on the data obtained, two strains (*L. brevis* E139 and L. *plantarum* E138) were selected for further research.

Yeast strains had different fermentative activity and produced different amount of alcohols (Figs 2, 3). Results confirm that the yeasts have different enzymatic activity (Okunowo et al., 2005; Joshi et al., 2009; Sharma et al., 2011) which may be due to the different ability of yeast strain to respond over various stress conditions subjected during fermentation, such as pH, high ethanol concentration, osmotic pressure, nutrient availability (Sharma et al., 2011; Bauer & Pretorius, 2000).

The strain *S. cerevisiae* Y205 had the highest fermentative activity and produced highest amount of alcohols after 24 hours of fermentation (Figs 2, 3). Therefore, this strain was selected for further research.

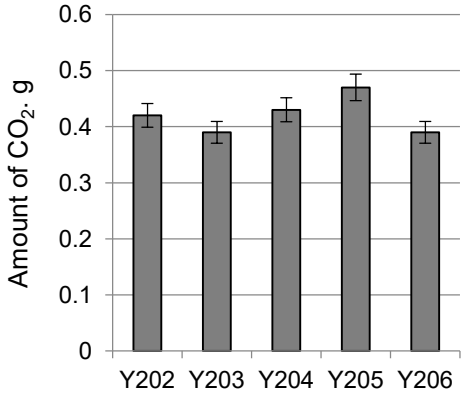

Figure 2. Fermenting activity of yeast.

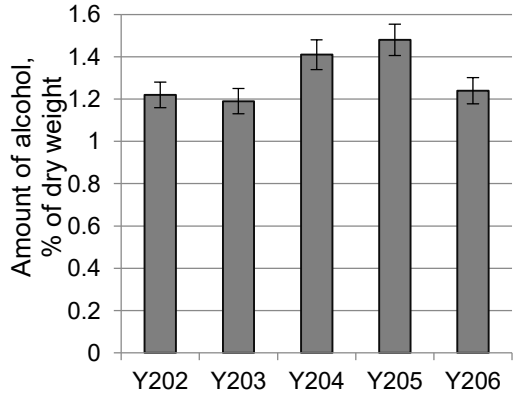

Figure 3. Alcohols production by yeast.

Two types of starter microbial composition were created: *L. plantarum* E138 and *S. cerevisiae* Y205 (starter I) and *L. brevis* E139 and *S. cerevisiae* Y205 (starter II).

The influence of two starter compositions on sourdough biotechnological indicators was studied (Table 4). When preparing the sourdough using pure cultures of LAB and yeast it was found that sourdough II had a lower acidity after fermentation, but the number of lactic acid bacteria was 3.6 times higher and yeast was 1.8 times less than in sourdough I. This may be due to the metabolic interaction of yeasts and LAB (De Vuyst et al., 2014; Carbonetto et al., 2020; Rogalski et al., 2020) Data obtained confirmed that the level of microorganisms competitiveness is strain specific (Carbonetto et al., 2020; Rogalski, et al., 2020).

The ability of LAB and yeast to grow together is very crucial in sourdough fermentation (Arendt et al., 2007, Wakil & Daodu, 2011; Arendt et al., 2011; Ogunsakin et al., 2017). Thus, the preferred yeast and LAB combination as metabolic partners should be studied during long-time propagation of sourdoughs.

Table 4. Biotechnological indicators of prepared sourdoughs after first fermentation

Indicators	Sourdough I	Sourdough II
Acidity, deg:	$7.3 \pm 0{,}3^{a}$	6.7 ± 0.2^{b}
LAB, CFU·g^{-1}	475 ± 21^{a}	1708 ± 52^{b}
Yeast, CFU·g^{-1}	63 ± 2^{a}	35 ± 2^{b}
Yeast/LAB ratio	1:7.5	1:48.8

Mean values ± SD within the same line with different letters are significantly different ($P \leq 0.05$).

Biotechnological indicators of sourdoughs were evaluated during multiple propagations. It was found (Table 5) that in 12th backslopping sourdough I made with *L. plantarum* 138 had lower titratable acidity, less volatile acids compared to sourdough II made with *L. brevis* E139. Alcohol content was the same in both sourdoughs similarly to yeast cells amount.

The LAB population was higher than the yeasts in both sourdoughs. This may be as a result of the fermentation conditions that tend to favour the growth of LAB than yeasts. This is in agreement with the report that LAB tend to dominate sourdough fermentations by the production of acid in the fermenting dough (De Vuyst et al., 2014; Ogunsakin et al., 2017; Go Carbonetto et al., 2020; Rogalski et al., 2020). The data showed that with long-term propagation, the cells amount of *L. brevis* E139 decreased compared to the amount after first fermentation of sourdough with pure cultures and remained approximately at the same level during multiple propagations. This indicates the stabilization of microflora.

Table 5. Biotechnological indicators for two types of sourdough during long-time propagation

Indicators	Sourdough I	Sourdough II
Acidity, degrees	7.5 ± 1.5^{a}	8.9 ± 1.5^{b}
Volatile acids acidity, degrees	0.85 ± 0.19^{a}	2.85 ± 0.37^{b}
Alcohol, % of dry weight	2.4 ± 0.4^{a}	2.4 ± 0.3^{a}
LAB, CFU·g^{-1}	770 ± 23^{a}	630 ± 20^{b}
Yeast, CFU·g^{-1}	43 ± 2^{a}	45 ± 3^{a}
Yeast/LAB ratio	1:77.9	1:14

Mean values ± SD within the same line with different letters are significantly different ($P \leq 0.05$).

The effect of the sourdough on the dough quality was studied. It was established (Table 6) that dough

Table 6. Dough indicators and parameters

Indicators and parameters	Control bread	Sourdough bread I	Sourdough bread II
Acidity, degrees	2.6 ± 0.3^{a}	3.9 ± 0.5^{b}	4.2 ± 0.6^{b}
Proofing time, min	38 ± 2^{a}	46 ± 2^{b}	48 ± 3^{b}

Mean values ± SD within the same line with different letters are significantly different ($P \leq 0.05$).

acidity was 1.5–1.6 times higher in samples with sourdough. Proofing time before baking increased compared to the control when the sourdoughs were used, which is confirmed by other studies (Cappa, 2016). This may be due to the increase in the acidity of the dough, and as a consequence, with the suppression of the development of yeast cells, which, in turn, leads to an increase in the proofing duration (Afanasjeva, 2003).

Table 7. Indicators of gluten-free bread quality

Indicators	Control	Sourdough bread I	Sourdough bread II
Acidity, degrees	0.5 ± 0.1^a	1.3 ± 0.2^b	1.6 ± 0.2^c
Volatile acids acidity, degrees	0.31 ± 0.09^a	1.28 ± 0.19^b	1.83 ± 0.19^c
Alcohols, % of dry weight	1.1 ± 0.2^a	2.2 ± 0.2^b	2.2 ± 0.2^b
Cells volume, %	58 ± 2^a	58 ± 2^a	59 ± 2^b
Specific volume, $cm^3 \cdot g^{-1}$	1.3 ± 0.2^a	1.2 ± 0.2^a	1.2 ± 0.2^a
Compressibility, equipment units	15 ± 2^a	14 ± 2^a	15 ± 2^a

Mean values ± SD within the same line with different letters are significantly different ($P \leq 0.05$).

Estimation of bread quality has shown that sourdough bread had higher acidity and content of volatile acids than the control (Table 7). The high amount of volatile acids has a positive effect on taste and smell (Afanasjeva, 2003). Alcohol content was higher in sourdough bread. Cells volume, crumb compressibility, specific volume was the same in all samples. Thus, an improvement in the physicochemical properties of gluten-free bread was noted only in an increase in acidity. Other studies confirmed that gluten-free sourdough microbiota determines the bread properties in terms of acidification (Bender & Schonlechner, 2020).

Sensory characteristics of bread is presented in Fig. 4. Sample I and sample II, made with sourdough, had better taste and smell than control sample. The smell was more intense, pleasant. Experimental bread also had a brighter crust color with a brownish shade and better texture and chewiness of crumbs: crumbs were softer, more elastic and less crumbly compared the control. The facts that sourdough creates a stronger flavor and taste and gluten-free sourdough bread had softer and stales more slowly were observed by other researchers (Houben et al., 2012; Wolter et al., 2014; Cappa et al., 2016; Olojede et al., 2019).

Bread made with sourdough II had more pronounced taste and flavor may be due to the fact that *L. brevise* produced higher amount of volatile acids (Jekle et al., 2010; Khandakar et al., 2014; Wolter et al., 2014).

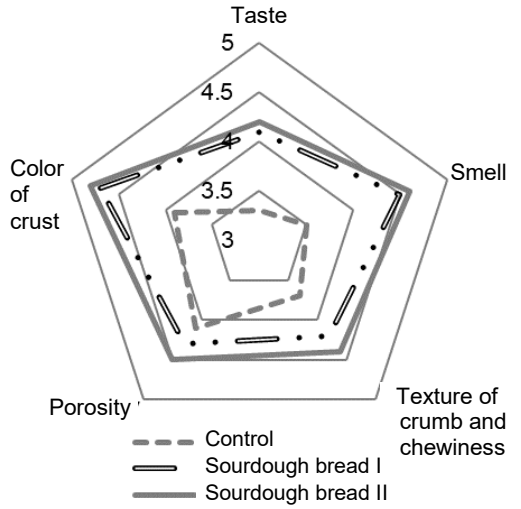

Figure 4. Sensory characteristics of gluten-free bread.

The effect of new sourdoughs on the microbial spoilage of gluten-free bread was studied (Fig. 5). The mould colonies of *Penicillium chrysogenum* on bread slices made with sourdough I and sourdough II appeared 6 and 12h respectively later than on control slices. The ropy-disease was not found in samples II during storage period (72 h). Samples I became ropy 24 hours later than the control. The inhibition of rope disease and mold developmentcan be attributed to the presence of organic acids and antimicrobial compounds of sourdough-resident microorganisms. A large number of published papers attribute the antifungal activity of LAB to organic acids (lactic, acetic, etc.) produced during fermentation leading to increased acidity which later inhibit mold growth (Hassan et al., 2014; Khandakar et al., 2014; Wolter et al., 2014; Axel et al., 2015; Bartkiene et al., 2019).

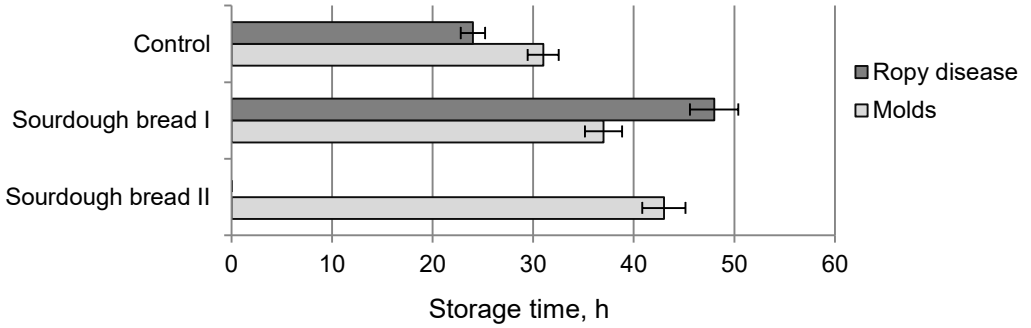

Figure 5. Microbial spoilage of gluten-free bread.

CONCLUSIONS

Based on their antagonistic and acidifying activities two LAB strains *L. brevis* E139 and *L. plantarum* E138 were selected to create new gluten-free sourdoughs as well as a yeast strain *S. cerevisiae* Y205 possessed highest fermentative activity. The influence of the LAB and yeast combination on the quality of gluten-free sourdough was studied. The starter microbial composition affected the sourdough and dough biochemical indicators in terms of cells content, leavening, acidification, volatile acids and alcohol content. New starter microbial composition usage contribute to the introduction of the dominating strains in the sourdough which allows ensuring a reproducible and controlled composition of the sourdough microbiota and ensure a constant bread quality.

The use of starter composition for sourdough preparation makes it possible to obtain bread with desired properties, taste and aroma characteristics. Sourdough did not affect the specific volume, porosity and compressibility of gluten-free bread, but its sensory characteristics were improved. Bread made with sourdoughs had more pronounced taste and flavor, brighter crust color and better texture compared bread without sourdough. Better results in flavor and antimicrobial activity of bread with *L. brevis* 139 compared to the bread with *L. plantarum* 138 may be due the higher production of volatile acids by *L. brevis* 139. Both of new starter composition may be recommended for applications in the gluten-free bread-making.

REFERENCES

Afanasjeva, O. 2003. *Microbiology of bakery production*. Beresta, Saint-Peterburg, 217 pp. (in Russian).

Arendt, E.K., Ryan, L.A.M. & Dal Bello, F. 2007. Impact of sourdough on the texture of bread. *Food Microbiology* **24**(2), 165–174. doi: 10.1016/j.fm.2006.07.011

Arendt, E.K., Moroni, A. & Zannini, E. 2011. Medical nutrition therapy: use of sourdough lactic acid bacteria as a cell factory for delivering functional biomolecules and food ingredients in gluten free bread. *Microbial Cell Factories* **10**(Suppl 1), S15. doi: 10.1186/1475-2859-10-s1-s15

Axel, C., Röcker, B., Brosnan, B., Zannini, E., Furey, A., Coffey, A. & Arendt, E.K. 2015. Application of Lactobacillus amylovorus DSM19280 in gluten-free sourdough bread to improve the microbial shelf life. Food Microbiology 47, 36–44. doi:10.1016/j.fm.2014.10.005

Azizi, S., Azizi, M.H., Moogouei, R. & Rajaei, P. 2020. The effect of Quinoa flour and enzymes on the quality of gluten-free bread. Food Science & Nutrition 00, 1–10. doi: 10.1002/fsn3.1527

Bartkiene, E., Bartkevics, V., Lele, V., Pugajeva, I., Zavistanaviciute, P., Zadeike, D. & Juodeikiene, G. 2019. Application of antifungal lactobacilli in combination with coatings based on apple processing by-products as a bio-preservative in wheat bread production. *Journal of Food Science and Technology* **56**(6), 2989–3000 doi: 10.1007/s13197-019-03775-w

Bartkiene, E., Lele, V, Ruzauskas, M., Domig, K., Starkute, V., Zavistanaviciute, P., Bartkevics, V., Pugajeva, I., Klupsaite, D., Juodeikien, G., Mickiene, R. & Rocha, M. 2020. Lactic Acid Bacteria Isolation from Spontaneous Sourdough and Their Characterization Including Antimicrobial and Antifungal Properties Evaluation. *Microorganisms* **8**(1), 64. doi: 10.3390/microorganisms8010064

Bauer, F. & Pretorius, I. 2000. Yeast stress response and fermentation efficiency: how to survive the making of wine. *South African Journal for Enology and Viticulture* **21**, 27

Bender, D., Fraberger, V., Szepasvári, P., D'Amico, S., Tömösközi, S., Cavazzi, G., Jäger H. & Domig, K.J. 2018. Effects of selected lactobacilli on the functional properties and stability of gluten-free sourdough bread *European Food Research and Technology* **244**, 1037–1046.

Bender, D. & Schönlechner, R. 2019. Innovative approaches towards improved gluten-free bread properties. *Journal of Cereal Science* **91**, 102904. doi: 10.1016/j.jcs.2019.102904

Cappa, C., Lucisano, M., Raineri, A., Fongaro, L., Foschino, R. & Mariotti, M. 2016. Gluten-Free Bread: Influence of Sourdough and Compressed Yeast on Proofing and Baking Properties. *Foods* **5**(4), 69. doi: 10.3390/foods5040069

Cappelli, A., Oliva, N. & Cini, E. 2020. A Systematic Review of Gluten-Free Dough and Bread: Dough Rheology, Bread Characteristics, and Improvement Strategies. *Applied Sciences* **10**(18), 6559. doi: 10.3390/app10186559

Carbonetto, B., Nidelet, T., Guezenec, S., Perez, M., Segond, D. & Sicard, D. 2020. Interactions between *Kazachstania humilis* yeast species and lactic acid bacteria in sourdough. *Microorganisms* **8**(2) 240. https://doi.org/10.3390/microorganisms8020240

Dec, M., Puchalski, A., Nowaczek, A. & Wernicki, A. 2016 Antimicrobial activity of Lactobacillus strains of chicken origin against bacterial pathogens. *International Microbiology* **19**(1), 57–67.

De Vuyst, L., Van Kerrebroeck, S., Harth, H., Huys, G., Daniel, H. -M., Weckx, S., 2014. Microbial ecology of sourdough fermentations: Diverse or uniform? *Food Microbiol.* **37,** 11–29. https://doi.org/10.1016/j.fm.2013.06.002

Dubrovskaya, N., Savkina, O., Kuznetsova, L. & Parakhina, O. 2018. The development of gluten-free sourdough bread technology with rowan powder. *Agronomy Research* **16**(S2), 1360–1372.

Gänzle, M.G. 2014. Enzymatic and bacterial conversions during sourdough fermentation. *Food Microbiol.* **37**, 2–10. https://doi.org/10.1016/j.fm.2013.04.007

Gobbetti, M., Angelis, M., Cagno, R & Rizzello, C. 2008. Sourdough/Lactic acid bacteria, In E.K. Elke and F.D. Bello, (eds.). Gluten-Free Cereal Products and Beverages. Academic Press. Burlington, MA, USA, 267–283.

Hassan, Y. I., Zhou, T. & Bullerman, L. B. 2015. Sourdough lactic acid bacteria as antifungal and mycotoxin-controlling agents. *Food Science and Technology International* **22**(1), 79–90. doi:10.1177/1082013214565722

Houben, A., Götz, H., Mitzscherling, M. & Becker, T. 2010. Modification of the rheological behavior of amaranth (Amaranthus hypochondriacus) dough. *Journal of Cereal Science* **51**(3), 350–356. doi: 10.1016/j.jcs.2010.02.003

Houben, A., Höchstötter, A. & Becker, T. 2012. Possibilities to increase the quality in gluten-free bread production: an overview. *European Food Research and Technology* **235**(2), 195–208. doi: 10.1007/s00217-012-1720-0

Jekle, M., Houben, A., Mitzscherling, M. & Becker, T. 2010. Effects of selected lactic acid bacteria on the characteristics of amaranth sourdough. *Journal of the Science of Food and Agriculture* **90**(13), 2326–2332. doi: 10.1002/jsfa.4091

Joshi, V., Sharma, S. & Devi, M.P. 2009. Influence of different yeast strains on fermentation behavior, physio-chemical and sensory qualities of plum wine. *Indian Journal of Natural Products and Resources* **8**(4), 445–451.

Khandakar, M., Kabir R., Islam, R. & Sabir, M. 2014. Antagonism of Lactic Acid Bacteria Against Selected Pathogenic Bacteria and Spoilage Fungi. *American-Eurasian J. Agric. & Environ. Sci.* **14**(3), 193–198. doi: 10.5829/idosi.aejaes.2014.14.03.83282

Kurtzman, C. & Fell, J.W. 1998. *The Yeasts, A Taxonomic Study*, 4th Edition. Elsevier Science B.V., Amsterdam, 1076 pp.

Mariotti, M., Cappa, C., Picozzi, C., Tedesco, B., Fongaro, L. & Lucisano, M. 2017. Compressed Yeast and Type I Gluten-Free Sourdough in Gluten-Free Breadmaking. *Food and Bioprocess Technolog*y **10**(5), 62–97.

Masure, H.G., Fierens, E. & Delcour, J.A. 2016. Current and forward looking experimental approaches in gluten-free bread making research. *Journal of Cereal Science* **67**, 92–111. doi: 10.1016/j.jcs.2015.09.009

Maidana, S.D., Ficoseco, C.A., Bassi, D., Cocconcelli, P.S., Puglisi, E., Savoy, G., Vignolo, G. & Fontana, C. 2019. Biodiversity and technological-functional potential of lactic acid bacteria isolated from spontaneously fermented chia sourdough. *International Journal of Food Microbiology*, 108425. doi: 10.1016/j.ijfoodmicro.2019.108425

Moroni, A.V., Arendt, E.K. & Bello, F.D. 2011. Biodiversity of lactic acid bacteria and yeasts in spontaneously-fermented buckwheat and teff sourdoughs. *Food Microbiology* **28**(3), 497–502. doi: 10.1016/j.fm.2010.10.016

Nionelli, L. & Rizzello, C. 2016. Sourdough-Based Biotechnologies for the Production of Gluten-Free Foods. *Foods* **5**(3), 65.

Nissen, L., Samaei, S.P., Babini, E. & Gianotti, A. 2020. Gluten free sourdough bread enriched with Cricket flour for protein fortification: Antioxidant improvement and Volatilome characterization. *Food Chemistry*, 127410. doi: 10.1016/j.foodchem.2020.127410

Okunowo, W., Okotore, R. & Osuntoki, A. 2005. The alcoholic fermentative efficiency of indigenous yeast strains of different origin on orange juice. *Journal of Biotechnology* **4**(11), 1290–1296.

Olojede, A.O., Sanni, A. I. & Banwo, K. 2019. Rheological, textural and nutritional properties of gluten-free sourdough made with functionally important lactic acid bacteria and yeast from Nigerian sorghum. *LWT- Food Science and Technology*, 108875. doi: 10.1016/j.lwt.2019.108875

Ogunsakin, A.O., Vanajakshi, V., Anu-Appaiah, K.A., Vijayendra, S.V.N., Walde, S.G., Banwo, K., Sanni, A.I. & Prabhasankar, P. 2017. Evaluation of functionally important lactic acid bacteria and yeasts from Nigerian sorghum as starter cultures for gluten-free sourdough preparation. *LWT- Food Science and Technology* **82**, 326–334.

Parakhina, O., Lokachuk, M., Kuznetsova, L., Pavlovskaya, E., Savkina, O. & Gavrilova, T. 2020. Development of biotechnology of gluten-free bakery products based on sourdough culture with a new microbial composition. *Bread products* **12**, 59–63 (in Russian).

Polak-Berecka, M., Waśko, A. & Koston, D. 2009. Comparison of different methods for detection of antimicrobial activity of probiotic strains of Lactobacillus rhamnosus against some food spoilage microorganisms. *Annales UMCS, Biologia* **64**(1), 15–24.

Puerta, P., Garzón, R., Rosell, C.M., Fiszman, S., Laguna, L. & Tárrega, A. 2021. Modifying gluten-free bread's structure using different baking conditions: Impact on oral processing and texture perception. *LWT* **140**, 110718. doi: 10.1016/j.lwt.2020.110718

Puchkova, L.I. 2004. *Laboratory research methods for bread technology*. GIORD, St. Petersburg, Russia, 259 pp. (in Russian).

Ren, Y., Linter, B.R., Linforth, R. & Foster, T.J. 2020. A comprehensive investigation of gluten free bread dough rheology, proving and baking performance and bread qualities by response surface design and principal component analysis. *Food & Function*. doi:10.1039/d0fo00115e

Rogalski, E., Ehrmann, M.A. & Vogel, R.F. 2020. Role of Kazachstania humilis and Saccharomyces cerevisiae in the strain-specific assertiveness of Fructilactobacillus sanfranciscensis strains in rye sourdough. *European Food Research and Technology*. doi: 10.1007/s00217-020-03535-7

Sadiq, F.A., Yan, B., Tian, F., Zhao, J., Zhang, H. & Chen, W. 2019. Lactic Acid Bacteria as Antifungal and Anti-Mycotoxigenic Agents: A Comprehensive Review. Comprehensive Reviews in Food Science and Food Safety. doi: 10.1111/1541-4337.12481

Sharma, A.K., Singh, P.N., & Sawant, S.D. 2011. Evaluation of Fermentation Efficiency of Yeast Strains and their Effect on Quality of Young Wines. *Indian Journal of Microbiology* **52**(3), 495–499. doi: 10.1007/s12088-011-0226-y

State Standard of the Russian Federation GOST 5670-96. 1996. Bread, rolls and buns. Methods for determination of acidity, 5 pp. (in Russian).

Svensson, L., Sekwati-Monang, B., Lutz, D.L., Schieber, A. & Gänzle, M.G. 2010. Phenolic Acids and Flavonoids in Nonfermented and Fermented Red Sorghum (Sorghum bicolor (L.) Moench). *Journal of Agricultural and Food Chemistry* **58**(16), 9214–9220. doi: 10.1021/jf101504v

Wakil, S.M. & Daodu, A.A. 2011. Physiological Properties of a Microbial Community in Spontaneous Fermentation of Maize (Zea mays) for Ogi Production. *International Research Journal of Microbiology* **2**(3),109–115.

Wolter, A., Hager, A.-S., Zannini, E., Czerny, M. & Arendt, E.K. 2014. Impact of sourdough fermented with Lactobacillus plantarum FST 1.7 on baking and sensory properties of gluten-free breads. *European Food Research and Technology* **239**(1), 1–12. doi: 10.1007/s00217-014-2184-1

Zacharof, M.P. & Lovitt, R.W. 2012. Bacteriocins Produced by lactic acid bacteria a review article. *APCBEE Procedia* **2**, 50–56. doi: 10.1016/j.apcbee.2012.06.010

Zalán, Z., Hudáček, J., Štětina, J., Chumchalová, J. & Halász, A. 2010. Production of organic acids by Lactobacillus strains in three different media. *European Food Research and Technology* **230**(3), 395–404. doi: 10.1007/s00217-009-1179-9

Ziobro, R., Korus, J., Witczak, M. & Juszczak, L. 2012. Influence of modified starches on properties of gluten-free dough and bread. Part II: Quality and staling of gluten-free bread. *Food Hydrocolloids* **29**, 68–74.

Zhang, G., Tu, J., Sadiq, F.A., Zhang, W. & Wang, W. 2019. Prevalence, genetic diversity, and technological functions of the Lactobacillus sanfranciscensis in sourdough: A review. *Comprehensive Reviews in Food Science and Food Safety*. doi: 10.1111/1541-4337.12459

Agronomy Research **19**(S3), 1273–1284, 2021
https://doi.org/10.15159/AR.21.078

A perspective of the Portuguese consumer awareness, beliefs and preferences towards piglet castration methods and its implications on the meat quality

R. Pereira Pinto[1,2,3*] and M. Vaz-Velho[1,2]

[1]Escola Superior de Tecnologia e Gestão, Instituto Politécnico de Viana do Castelo, Rua Escola Industrial e Comercial de Nun'Álvares, PT4900-347 Viana do Castelo, Portugal
[2] CISAS - Centre for Research and Development in Agrifood Systems and Sustainability, Instituto Politécnico de Viana do Castelo, Rua Escola Industrial e Comercial de Nun'Álvares, PT4900-347 Viana do Castelo, Portugal
[3]EDIUS – International PhD School of the USC, University of Santiago de Compostela Av. das Ciencias, 6, ES15705 Santiago de Compostela, Spain
[*]Correspondence: rpinto@ipvc.pt

Received: January 31st, 2021; Accepted: May 8th, 2021; Published: May 13th, 2021

Abstract. Neutering male piglets by surgical procedures without anaesthesia, with analgesia and/or anaesthesia and, recently, immunological-chemical castration are practices to avoid unwanted or aggressive sexual behaviour, and to prevent the development of meat boar taint. This exploratory study aims to investigate Portuguese consumer's awareness, beliefs and attitudes in issues like boar taint, piglet's castration and pork meat quality, observing possible demographic trends. It is also intended to identify clusters of consumers with similar attitudes, crossing them with demographic data to verify the existence of patterns in Portugal related to these issues. To attain this objective, a consumer's survey was performed through an online questionnaire open for 30 days. A total of 158 respondents completed the survey. Almost a half (46%) of respondents stated their unknowledge about boar taint. Surgical castration and its effects are topics with which older consumers with a rural background are more familiar with, while immunological-chemical castration is still unknown to most consumers: 65% of consumers said they were not aware of this method, and 75% did not know whether it is an effective method for eliminating boar taint. Hierarchical clustering followed by K-means analysis segmented consumers into three clusters characterized according to their opinions, mainly divided by ethical and chemical-free orientations and by a more conservative meat quality and flavour-oriented attitudes, generally independent of prevailing demographics. In general, there were no defined opinions about the subjects under study, due mainly to the lack of information or knowledge. Nevertheless, cluster classification revealed differences in consumer's opinions, especially regarding the reasons for castration and the pain inflicted, about meat quality and the willingness to buy pork from entire males or to pay more for this type of product.

Key words: boar taint, consumer perception, entire male pigs, immunocastration, surgical castration.

INTRODUCTION

Castration of male piglets is a procedure to prevent the development of boar taint in the meat of entire male pigs, and has been a traditional practice for ages and is still common in most countries (Bonneau & Weiler, 2019). This practice also makes it easier to handle the pigs, as castrates exhibit less aggressive and sexual behaviour than entire males (Font-i-Furnols et al., 2019).

Boar taint is an unpleasant odour often described as a sharp 'animal', 'urine', 'faecal' or 'sweat' like, which can be perceived when pork is cooked or eaten warm (Mathur et al., 2012), being an off-flavour that is mainly caused by the accumulation of two compounds: androstenone and skatole. Androstenone (5α androst-16-en-3-one) is a steroid produced in the testis, sensorially associated with the smell of urine, and skatole (3-methylindol), which has a stable-like smell, is a metabolite derived from the amino acid tryptophan produced in the lower gut by intestinal microbiota (Claus et al., 1994). Due to the lipophilic characteristics of these compounds, redistribution from blood to fat tissue is easily occurring with prolonged accumulation in fat tissues (Aldal et al., 2005; Wauters et al., 2016). Levels of skatole are lower in castrates and gilts than in entire male pigs and, although the mechanism has not been completely elucidated (Aldal et al., 2005), it is known that androstenone interferes with skatole clearance by the liver (Doran et al., 2002; Whittington et al., 2004).

Scientific research on animal welfare began because of ethical concerns over the quality of life of animals. Animal welfare concept can be defined by three ethical concerns: the quality of life of animals (animals should lead natural lives through the development and use of their natural adaptations and capabilities), its emotional state (that animals should feel well by being free from prolonged and intense fear, pain, and other negative states, and by experiencing normal pleasures), and its ability to express their normal behaviours (Fraser et al., 1997). In recent years, surgical castration of male piglets has become a welfare concern due to the pain and stress associated with this procedure. Until the 1980s it was wrongly assumed that new-borns could feel almost no pain, and this long-prevailing scientific view is the main reason why castration of male piglets is still carried out without pain treatment or anaesthesia (von Borell et al., 2020).

In European Union, the pork production chain and welfare-concerned citizens have demanded a ban on surgical castration without pain relief, and a number of European stakeholders committed themselves to stop surgical castration by 2018 through the European Declaration on Alternatives to Pig Castration (EC, 2010), if satisfactory solutions were found to the various challenges associated with the abandonment of this practice. However, currently none of the available alternatives are fully satisfactory: the deadline ended officially on 1 January 2018, and the European pork chain is still resisting the change, with some stakeholders considering that castration of male piglets without pain relief is not an issue (Bonneau & Weiler, 2019). There have been no new commitments on the part of the EU institutions to renew the declaration. The opinions on this issue and its practicability are widely dispersed: although countries using analgesia/anaesthesia routinely found this method practical and effective, only few countries seem to aim at meeting the deadline to phase out surgical castration completely (De Briyne et al., 2016).

Many farmers still consider methods to replace surgical castration without pain control unnecessary, ineffective, costly, or impractical. In some countries, the use of pharmacological tools requires the involvement of a veterinarian, which acts as a barrier

to the adoption of pain mitigation (Hötzel et al., 2020). There are currently three possible alternatives with practical relevance: surgical castration with anaesthesia and or analgesia, raising entire males, and immunocastration (Weiler & Bonneau, 2019). Also, genetic advances on boar taint reduction have shown that it is possible to reduce boar taint in few generations (Duarte et al., 2021). Other options, such as sperm sexing to produce only females, the injection of chemical compounds into the testes to destroy the tissue or the administration of exogenous hormones to inhibit the hypothalamic pituitary-gonadal axis cannot be realistically considered (Bonneau & Weiler, 2019).

The use of anaesthesia or analgesia during surgical castration is one of the current alternatives, having the advantage of the prevention of quality problems due to boar taint or changes fatty acid composition with the carcasses being suitable for all traditional pork products, but the downside is the risk of post-surgical wound infections and higher feed consumption compared with boars (Weiler & Bonneau, 2019). Application of analgesia is demanded by several quality assurance programs in Belgium, Germany and France, and some countries have a longer tradition of using anaesthesia during castration, such as Norway, Sweden and Switzerland, while this practice is recent in Denmark and will be introduced in Germany by 2021 (Aluwe et al., 2020).

Raising entire male pigs presents some economic advantages as boars have generally leaner carcasses and less feed is needed in order to achieve the same final weight when compared to castrates, whereby the environmental impact is lower (Morlein et al., 2015; Wauters et al., 2017; Bonneau et al., 2018). Furthermore the lower lipid content and the higher content of unsaturated fatty acids in adipose tissues of entire males (Škrlep et al., 2020) may be regarded as favourable from the human dietetic point of view (EFSA, 2004). The disadvantages of raising entire males include the risk of boar taint, the difficulties in managing the restless entire males, the lower meat quality in relation to the reduced intramuscular fat content and increased fat unsaturation, which is detrimental for processing dry-cured products (Bonneau & Weiler, 2019). Production of boars is common in Spain, Portugal, Ireland, and United Kingdom, and since 2010, introduced in countries like the Netherlands, Belgium, and France (De Briyne et al., 2016; Aluwe et al., 2020).

Immunocastration or vaccination against anti-gonadotropin releasing hormone (GnRH) is performed by giving two injections with a gonadotropin releasing factor (GnRF) analogue-protein conjugate with at least 4 weeks apart, with the second vaccination at least 4 weeks before slaughter (Heyrman et al., 2019), and is sometimes used as an alternative to eliminate boar taint without the need for surgical castration. However, immunocastration is carried out later in life during the fattening stage and farmers are given a longer time span to apply it, and consumers could be sceptical about food safety and prices (Kress et al., 2019). Also, the available vaccine (Improvac®, Zoetis), is catalogued by the EU in the therapeutic area of sex hormones and modulators of the genital system (EMA, 2020), which limits its use in organic production systems. Outside Europe, immunocastration has been used on a large scale during the last two decades in Australia and New Zealand, and most recently in Brazil. In Europe, it is applied to 5 up to 10% of the male piglets in a number of countries, but its development is being slow, based on assumed rejection of the practice by the consumers (Bonneau & Weiler, 2019; Aluwe et al., 2020).

Consumer attitudes and beliefs are mainly determined by price, taste, health and convenience (Font-i-Furnols et al., 2019). Attitude can be described as a learned

pre-disposition to respond in a consistently favourable or unfavourable manner with respect to a given object, and is typically viewed as a latent or underlying variable that is assumed to guide or influence behaviour (Fishbein & Ajzen, 1975), and beliefs are the cognitive knowledge related to information that an individual has about something that can be linked to some attribute, action or event. Consumer knowledge regarding piglet castration is generally low but a higher number of consumers are aware of organic production (Font-i-Furnols et al., 2019). Different methodologies are used via face-to-face or online surveys to evaluate consumer acceptability: alternatives for surgical castration evaluation of the acceptability or assessing the willingness to pay for meat and meat products are used to measure the perception of piglet castration (Aluwe et al., 2020). Some studies using focus groups or online surveys regarding consumer attitudes towards surgical castration of piglets have been reported from specific countries or regions such as Norway (Fredriksen et al., 2011; Sodring et al., 2020), France, Germany, Netherlands and Belgium (Vanhonacker & Verbeke, 2011), Italy (Di Pasquale et al., 2019), Brazil (Hötzel et al., 2020), Eastern Europe (Tomasevic et al., 2020a; Tomasevic et al., 2020b), and also a more recent study performed in 16 European countries, including Portugal (Aluwe et al., 2020).

This research aims to investigate Portuguese consumers awareness, beliefs, preferences and experience in issues like boar taint, castration and pork meat quality, observing possible demographic differences. It is also intended to identify clusters of consumers with similar attitudes, crossing them with demographic data in order to verify the existence of behavioural and consumption patterns in Portugal, following the methodologies of previous studies by Vanhonacker & Verbeke (2011) and Tomasevic (2020a).

MATERIALS AND METHODS

Research sampling and questionnaire

A consumer's survey was carried out online in 2020, between March 10th and April 10th, using the CAWI (Computer-Assisted Web Interviewing) data collection method. Data was collected using a web questionnaire, produced by Cognito Forms (https://www.cognitoforms.com, Cognito LLC©). The questionnaire was reviewed using a limited group of respondents in order to identify errors and also to verify if all questions were understandable and well interpreted by the respondents. The recruitment of the consumers was done randomly, publicizing a link via social networks and also further dissemination through institutional email addresses. A total of 158 respondents completed the survey, which took approximately 10 minutes and was only available in Portuguese. The questionnaire could only be submitted and saved into the database when all questions were filled by the respondents.

Prior information regarding piglet castration or boar taint was not provided to respondents, and the only information available at the first page of the questionnaire was related to its scope: 'The present questionnaire is intended to gather information on the opinion and perception of Portuguese consumers in relation to pork, about surgical castration, immunocastration and boar taint (entire male pig scent).' Participants' general demographic information and educational level about and frequency of consumption of pork was collected (Table 1). Also, 6 questions related to consumers' knowledge about castration and boar taint were made (Table 2).

Questions regarding consumer awareness, beliefs and preferences about castration and pig meat were based on previous studies conducted by Tomasevic et al. (2020a) and Vanhonacker & Verbeke (2011), which consisted in questions with multi-choice answers and sentences where respondents had to rank their opinion using the available scales (Tables 3 and 4). These scales varied from 'Very easy' to 'Very difficult' (5-point scale), 'Bad', 'Neither good nor bad', 'Good' (5-point scale) and 'Totally disagree' to 'Totally agree' (7-point scale).

Table 1. Demographic profile and percentage of pork meat consumption of the respondents

	Overall (n = 158)
Gender	
Male	39.9
Female	60.1
Age	
21–39	50.0
40–55	34.2
> 55	15.8
Place of growing up	
Rural	55.7
Urban	44.3
Household members	
1–2	35.4
3–4	58.2
5 or more	6.3
Residence area	
North	64.6
Centre	16.5
South/Islands	19.0
Education level	
Elementary/Higher	22.8
University	77.2
Frequency of pork consumption	
More than once a week	46.8
Once a week	29.1
Fortnightly	10.1
Rarely/Never	13.9

Statistical analysis

Data were analysed using Statistica 7.0 software (Stat Soft Inc., USA). Hierarchical clustering with Ward's method and squared Euclidean distance was measured using attribute importance scores on beliefs and preferences about castration and meat quality as segmentation variables (in a total of 21 variables), followed by a K-Means cluster analysis. The number of initial clusters was set based in the hierarchical procedure.

Descriptive statistics of the data was determined, and the differences between clusters were studied by one-way analysis of variance (ANOVA) and separated by Tukey's honest significant differences test ($p < 0.05$).

RESULTS AND DISCUSSION

Demographic profile and consumption of pork

The demographic profile of the 158 Portuguese residents that completed the questionnaire (Table 1) showed that the majority of respondents were female (60.1%), 50% were less than 40 years old, 55.7% grew up in a rural area, and almost two-thirds lived in the North of Portugal. The most prevalent household members number were 3 to 4, and 77.2% had a university diploma. Regarding meat consumption, 46.8% stated eating pork more than once a week, while 13.9% rarely or never eats this meat.

Consumer experience and awareness

As stated in materials and methods, the population sample was randomly chosen, depending on the consumers' willingness to fulfil the questionnaire, their access to the internet and the dissemination of the link that started the questionnaire. Since the topics

covered are not normally in the general public domain, except for veterinary professionals, farmers, researchers or people with knowledge acquired by being born in a rural area, consumers were expected to exhibit limited knowledge of or no knowledge about boar taint and castration issues.

In order to assess the respondents' previous experience and knowledge related to boar taint and castration of male pigs, six questions were formulated (Table 2). Results showed that nearly half (46.2%) of the respondents did not know what boar taint was or even never heard about it, and 63.9% never had contact with tainted meat. When questioned about castration, consumers have revealed more knowledge about surgical castration than chemical castration (57.6% and 35.4% respectively), maybe because of the traditional practice of neutering piglets.

Table 2. Consumers' boar taint experience and awareness of castration methods

Question	%
Boar taint in pig's meat	
Never heard of	32.9
I heard about this, but I do not know what it is	13.3
I know what this is	53.8
Have you ever had contact with boar tainted pig's meat?	
I do not know	41.1
No (but I can identify the smell)	22.8
Yes	36.1
Surgical castration of pigs	
Never heard of	22.8
I heard about this, but I do not know what it is	19.6
I know what this is	57.6
Chemical castration of pigs	
Never heard of	33.5
I heard about this, but I do not know what it is	31.0
I know what this is	35.4
Surgical castration is an effective method to eliminate unpleasant smell in pig's meat	
I disagree	7.0
I do not know	53.8
I agree	39.2
Chemical castration is an effective method to eliminate unpleasant smell in pig's meat	
I disagree	7.0
I do not know	74.7
I agree	18.4

In concern to the effectiveness of the methods to eliminate boar taint, it appears that the lack of knowledge of both methods prevails in the responses of consumers. This answer is being more frequent in the chemical castration, where 74.7% stated their unknowledge. Also, 7% of respondents do not agree that both methods can eliminate boar taint, which can be just a primary emotive reaction against 'castration', or on the other hand it may also be due to a deep knowledge of the topic, since castrates and females can have high skatoles levels due to bad rearing conditions (Hansen et al., 1994).

In a similar study in four European countries, 88.7% of consumers expressed that they had never heard about boar taint or did not know what it was, and only 2% stated to know about immunocastration (Vanhonacker & Verbeke, 2011). Data retrieved from

a European exploratory survey showed that 71% of Portuguese consumers are aware of piglet castration, 33% have experienced a bad smell or taste in pig's meat and also only 3% of the respondents have negative feelings towards vaccination (Aluwe et al., 2020).

Segmentation analysis

Segmentation studies frequently utilize a single behavioural criterion to differentiate buyers by employing cross-sectional data (Assael & Roscoe, 1976). When consumer studies are performed it is relevant to detect segments of consumers to have a clear idea of their beliefs and attitudes: market segmentation is one area of behavioural research in which an understanding of attitudinal structure is applied directly to the development of marketing strategy. The combination of consumers' beliefs about product attributes with its demographic characteristics can result in a segmentation into defined groups (Bearden & Durand, 1977).

In order to divide the consumers into groups by its beliefs and attitudes and to characterize those groups by demographics, clustering procedure was followed as described in the methods section. Three clusters were set as the most adequate. Clusters were identified as A, B and C, containing 67, 41 and 50 consumers respectively. Variable scores for each cluster can be found in Tables 3 and 4.

Table 3. Consumers' attitudes and beliefs overall and cluster scores[1]

Question	Overall	Cluster		
		A	B	C
	(n = 158)	(n = 67)	(n = 41)	(n = 50)
In my opinion, the process of surgical castration of pigs is…*	2.97	2.91 [a]	2.51 [a]	3.44 [b]
In my opinion, the process of chemical castration of pigs is…*	2.75	2.87 [a]	2.34 [b]	2.94 [a]
I think chemical castration of pigs is something…**	2.45	2.70 [a]	3.00 [a]	1.66 [b]
Surgical castration causes pain to the animal***	4.97	4.45 [a]	4.29 [a]	6.24 [b]
Castration is unnecessary***	4.08	4.07 [a]	2.46 [b]	5.42 [c]
Castration is a wild act***	4.08	3.51 [a]	2.66 [b]	6.02 [c]
I would prefer alternatives to be found instead of male pigs' castration***	5.13	4.34 [a]	4.63 [a]	6.60 [b]

[1]Cluster items in the same row with different superscripts are significantly different ($p < 0.05$); *1: Very easy; 2: Easy; 3: Neither easy of difficult; 4: Difficult; 5: Very difficult; **1: Bad; 2: Bad but necessary; 3: Neither good nor bad; 4: Good but unnecessary; 5: Good; ***1: Totally disagree; 4: Neither … nor … 7: Totally agree.

Attitudes and beliefs about castration

The general opinion of Portuguese consumers about the ease or difficulty of chemical or surgical castration had a score equivalent to 'neither good nor bad'. As they were required to answer all questions in order to complete the survey, consumers who probably had no knowledge about the topic probably chose to answer in a way that did not compromise the objective of the question. The opinion of the consumers about the difficulty to perform surgical castration might be related to the knowledge they have about it or by the information they have received about it during their life (Tomasevic et al., 2020a).

Respondents' overall scores classified the chemical castration as something bad but necessary, slightly agreed that surgical castration can cause pain to the animal and they had the 'neither agree nor disagree' classification regarding the necessity of castration

and the cruelty of such act. However, consumers slightly agreed that alternatives to castration should be found.

In line with these results, in a study assessing attitudes and beliefs of European consumers towards piglet castration and meat from castrated pigs, respondents agreed that surgical castration produces pain to the animal (score of 4.9), also did not have a clear opinion about the necessity of castration (score of 3.7) and were not sure if surgical castration is savage, with a score of 4.0 (Tomasevic et al., 2020a). Contrary to these results, Fredriksen et al. (2011) found that Norwegians considered castration a necessary procedure.

Beliefs and preferences about meat quality and buying behaviours overall and cluster scores are shown in Table 4. The opinion about most of the statements is neutral, that is, the average score corresponds to the category of 'neither agree nor disagree'. These results could be interpreted as divided opinions or lack of knowledge about this subject. However, consumers slightly disagree that meat from castrated pigs is leaner, which could be sign of some degree of knowledge about the differences in meat quality.

Table 4. Beliefs and preferences about meat quality and buying behaviours overall and cluster scores[1,2]

Question	Overall (n = 158)	Cluster A (n = 67)	B (n = 41)	C (n = 50)
Traditional smoked products have the same quality if made with entire male's meat	3.68	3.70	3.37	3.90
Charcuterie products have the same quality if made with entire male's meat	3.71	3.78	3.39	3.88
Young male pig's meat (5 months) tastes better than from an adult (8 months)	3.82	4.25 [a]	4.00 [a]	3.08 [b]
Entire male's pig meat is healthier	3.82	3.48 [a]	3.68 [ab]	4.38 [b]
I do not like the idea of buying young pig's meat (5 months)	4.15	3.63 [a]	3.34 [a]	5.50 [b]
Chemical castration alters the quality of the meat	4.16	3.73 [a]	4.12 [ab]	4.76 [b]
Meat from castrated pigs has better quality	3.96	3.42 [a]	5.73 [b]	3.22 [a]
Meat from castrated pigs is leaner	3.06	3.43 [a]	3.51 [a]	2.20 [b]
I prefer to eat meat from castrated pigs	4.08	3.13 [a]	6.37 [b]	3.48 [a]
The meat from castrated pigs is more expensive	3.54	3.61	3.54	3.44
I am willing to pay more for meat from castrated pigs	3.33	3.36 [a]	4.88 [b]	2.02 [c]
Would you buy entire male's meat?	4.21	3.84 [a]	4.07 [ab]	4.82 [b]
I would buy charcuterie products made from entire male's meat	3.99	4.06	3.54	4.28
Pork purchased from butchers in Portugal has a pleasant smell / taste	4.28	4.25	4.70	3.98

[1] Cluster items in the same row with different superscripts are significantly different ($p < 0.05$);
[2] Scores 1: Totally disagree; 4: Neither … nor … 7: Totally agree.

Regarding buying behaviours, consumers also slightly disagreed that meat from castrates is more expensive and they are not willing to pay more for meat from castrated pigs. The results of the research of Aluwe et al. (2020) showed that Portuguese consumers consider that the most important attributes at purchase are good taste of meat (85%) and animal welfare (52%). However, Sodring et al. (2020) found that animal

welfare is clearly important to consumers but at the point of purchase, and often becomes secondary to other criteria.

When cluster scores about consumer attitudes and beliefs are analysed (Table 3), it can be noticed some behaviour patterns among them: cluster C individuals totally agree on the search for alternatives to castration and that surgical castration causes pain to the animal, also agree that castration is a wild act and unnecessary and they consider that chemical castration is something bad and surgical castration process is difficult.

In Table 4 the cluster analysis showed some differences and trends between groups of individuals, particularly in cluster C. This group disagrees that young male meat tastes better than meat from adult males, they do not like the idea of buying young pig's meat and also agree with that chemical castration changes que quality of meat. Moreover, cluster C individuals disagree that meat from castrated pigs is leaner and are willing to buy entire male's meat. Cluster B opinions are very different: they agree that castrated pork has better quality and they also prefer to eat meat from castrated pigs. Cluster A has mixed opinions comparatively to clusters B and C.

Clusters' attitudes, beliefs and preferences scores divide individuals into groups that could be categorized into ethical and chemicals-free orientations or more conservative, being flavour-oriented and choosing meat quality over animal welfare. Following this, cluster C consumers are mainly against castration and have welfare concerns, preferring a natural approach. Cluster B consumers are characterized for being more conservative, with a marked preference for castrated pigs' meat and assuming the necessity of castration, denying that it is a wild act. In cluster A, consumer's opinions tend to be moderate: they are sensitive to animal welfare but also aware of changes in the meat quality.

Table 5 shows the demographic characteristics of the clusters. Clusters B and C, which reveal the greatest differences of opinion between them, have some peculiarities in their demographic characteristics, which may explain some of their beliefs and preferences. Cluster B, the one with more conservative opinions, is mainly constituted by females (70.7%), are

Table 5. Clusters' demographics

	Cluster		
	A (n = 67)	B (n = 41)	C (n = 50)
Gender			
Male	43.3	29.3	44.0
Female	56.7	70.7	56.0
Age			
21–39	61.2	51.2	34.0
40–55	26.9	39.0	40.0
> 55	11.9	9.8	26.0
Place of growing up			
Rural	49.3	68.3	54.0
Urban	50.7	31.7	46.0
Household members			
1–2	34.3	41.5	32.0
3–4	56.7	56.1	62.0
5 or more	9.0	2.4	6.0
Residence area			
North	74.6	65.9	50.0
Centre	9.0	17.1	26.0
South/Islands	16.4	17.1	24.0
Education level			
Elementary/Higher	16.4	9.8	42.0
University	83.6	90.2	58.0
Frequency of pork consumption			
More than once a week	38.8	48.8	56.0
Once a week	31.3	31.7	24.0
Fortnightly	14.9	12.2	2.0
Rarely/Never	14.9	7.3	18.0

highly educated individuals (90.8%) and 68.3% grew up in a rural area. In cluster C, more welfare-oriented, 66% of the consumers have more than 40 years old and this is the group with the lowest percentage of individuals with a university degree.

CONCLUSIONS

This study revealed that Portuguese consumers have different awareness's, beliefs and preferences towards piglet castration methods and also about meat quality. Overall scores showed only a few defined opinions about the addressed issues, due mainly to the lack of information and knowledge of respondents. Castration of male piglets and boar taint are subjects that are normally not on the daily agenda of generalist information, and since it is a matter with some specificity, it can be assumed that a large part of the population does not have knowledge about the topics covered. However, cluster classification showed marked differences among groups of consumers, especially regarding meat quality and welfare issues, allowing classifying the behaviour and preferences of groups as moderate, conservative or oriented towards animal welfare and preferring a natural approach.

As a suggestion for future work, the network of contacts and dissemination of the questionnaire should be extended, as well as the duration of the study to increase the robustness of research findings.

ACKNOWLEDGEMENTS. This work is a result of the project TECH - Technology, Environment, Creativity and Health, Norte-01-0145-FEDER-000043, supported by Norte Portugal Regional Operational Program (NORTE 2020), under the PORTUGAL 2020 Partnership Agreement, through the European Regional Development Fund (ERDF). To UIDB/05937/2020 and UIDP/05937/2020 - Centre for Research and Development in Agrifood Systems and Sustainability (CISAS) - funded by national funds, through FCT - Fundação para a Ciência e a Tecnologia.

REFERENCES

Aldal, I., Andresen, O., Egeli, A.K., Haugen, J.E., Grodum, A., Fjetland, O. & Eikaas, J.L.H. 2005. Levels of androstenone and skatole and the occurrence of boar taint in fat from young boars. *Livestock Production Science* **95**(1–2), 121–129. doi: 10.1016/j.livprodsci.2004.12.010

Aluwe, M., Heyrman, E., Almeida, J.M., Babol, J., Battacone, G., Citek, J., Furnols, M.F.I., Getya, A., Karolyi, D., Kostyra, E., Kress, K., Kusec, G., Morlein, D., Semenova, A., Skrlep, M., Stoyanchev, T., Tomasevic, I., Tudoreanu, L., Van Son, M., Zakowska-Biemans, S., Zamaratskaia, G., Van den Broeke, A. & Egea, M. 2020. Exploratory Survey on European Consumer and Stakeholder Attitudes towards Alternatives for Surgical Castration of Piglets. *Animals*, **10**(10), Article 1758. doi: 10.3390/ani10101758

Assael, H. & Roscoe, A.M. 1976. Approaches to Market-Segmentation Analysis. *Journal of Marketing* **40**(4), 67–76. doi: 10.2307/1251070

Bearden, W.O. & Durand, R.M. 1977. Consumers' beliefs, demographics and development of market-segmentation strategy. *Psychological Reports* **40**(3), 867–874. doi: 10.2466/pr0.1977.40.3.867

Bonneau, M. & Weiler, U. 2019. Pros and Cons of Alternatives to Piglet Castration: Welfare, Boar Taint, and Other Meat Quality Traits. *Animals* **9**(11) Article 884. doi: 10.3390/ani9110884

Bonneau, M., Čandek-Potokar, M., Škrlep, M., Font, I.F.M., Aluwé, M. & Fontanesi, L. 2018. Potential sensitivity of pork production situations aiming at high-quality products to the use of entire male pigs as an alternative to surgical castrates. *Animal* **12**(6), 1287–1295. doi: 10.1017/s1751731117003044

Claus, R., Weiler, U. & Herzog, A. 1994. Physiological Aspects of Androstenone and Skatole Formation in the Boar - A review with experimental data. *Meat Science* **38**, 289–305.

De Briyne, N., Berg, C., Blaha, T. & Temple, D. 2016. Pig castration: will the EU manage to ban pig castration by 2018? *Porcine Health Management* **2**, Article 29. doi: 10.1186/s40813-016-0046-x

Di Pasquale, J., Nannoni, E., Sardi, L., Rubini, G., Salvatore, R., Bartoli, L., Adinolfi, F. & Martelli, G. 2019. Towards the Abandonment of Surgical Castration in Pigs: How is Immunocastration Perceived by Italian Consumers? *Animals* **9**(5), Article 198. doi: 10.3390/ani9050198

Doran, E., Whittington, F.W., Wood, J.D. & McGivan, J.D. 2002. Cytochrome P450IIE1 (CYP2E1) is induced by skatole and this induction is blocked by androstenone in isolated pig hepatocytes. *Chemico-Biological Interactions* **140**(1), 81–92. doi: 10.1016/s0009-2797(02)00015-7

Duarte, D.A.S., Schroyen, M., Mota, R.R., Vanderick, S. & Gengler, N. 2021. Recent genetic advances on boar taint reduction as an alternative to castration: a review. *Journal of Applied Genetics* **62**, 137–150. doi: 10.1007/s13353-020-00598-w

EC (2010). European Declaration on Alternatives to the Surgical Castration of Pigs. European Commission. https://ec.europa.eu/food/sites/food/files/animals/docs/aw_prac_farm_pigs_castalt_declaration_en.pdf (accessed 30 January 2021).

EFSA (2004). Welfare aspects of the castration of piglets. Opinion of the Scientific Panel on Animal Health and Welfare on a request from the Commission related to welfare aspects of the castration of piglets (Question No. EFSA-Q-2003-091) *The EFSA Journal* **91**, 1–18. doi: 10.2903/j.efsa.2004.91

EMA (2020). EU Veterinary Medicinal Product Database. European Medicines Agency. http://vet.eudrapharm.eu/vet/ (accessed 29 January 2021).

Fishbein, M. & Ajzen, I. 1975. *Belief, Attitude, Intention and Behavior: An Introduction to Theory and Research.* Reading, MA. Addison-Wesley Publishing Company.

Font-i-Furnols, M., Skrlep, M. & Aluwe, M. 2019. Attitudes and beliefs of consumers towards pig welfare and pork quality. *IOP Conference Series-Earth and Environmental Science* **333**, 012002. doi: 10.1088/1755-1315/333/1/012002

Fraser, D., Weary, D.M., Pajor, E.A. & Milligan, B.N. 1997. A scientific conception of animal welfare that reflects ethical concerns. *Animal Welfare* **6**(3), 187–205.

Fredriksen, B., Johnsen, A.M.S. & Skuterud, E. 2011. Consumer attitudes towards castration of piglets and alternatives to surgical castration. *Research in Veterinary Science* **90**, 352–357.

Hansen, L.L., Larsen, A.E., Jensen, B.B., Hansen-Moller, J. & Barton-Gade, P. 1994. Influence of stocking rate and faeces deposition in the pen at different temperatures on skatole concentration (boar taint) in subcutaneous fat. *Animal Production* **59**, 99–110. doi: 10.1017/s0003356100007546

Heyrman, E., Kowalski, E., Millet, S., Tuyttens, F.A.M., Ampe, B., Janssens, S., Buys, N., Wauters, J., Vanhaecke, L. & Aluwe, M. 2019. Monitoring of behavior, sex hormones and boar taint compounds during the vaccination program for immunocastration in three sire lines. *Research in Veterinary Science* **124**, 293–302. doi: 10.1016/j.rvsc.2019.04.010

Hötzel, M.J., Yunes, M.C., Vandresen, B., Albernaz-Goncalves, R. & Woodroffe, R.E. 2020. On the Road to End Pig Pain: Knowledge and Attitudes of Brazilian Citizens Regarding Castration. *Animals* **10**(10), Article 1826. doi: 10.3390/ani10101826

Kress, K., Millet, S., Labussiere, E., Weiler, U. & Stefanski, V. 2019. Sustainability of Pork Production with Immunocastration in Europe. *Sustainability* **11**(12), Article 3335. doi: 10.3390/su11123335

Mathur, P.K., ten Napel, J., Bloemhof, S., Heres, L., Knol, E.F. & Mulder, H.A. 2012. A human nose scoring system for boar taint and its relationship with androstenone and skatole. *Meat Science* **91**(4), 414–422. doi: 10.1016/j.meatsci.2012.02.025

Morlein, D., Schiermann, C. Meier-Dinkel, L., Trautmann, J., Wigger, R., Buttinger, G. & Wicke, M. 2015. Effects of context and repeated exposure on food liking: The case of boar taint. *Food Research International* **67**, 390–399.

Škrlep, M., Tomasevic, I., Moerlein, D., Novakovic, S., Egea, M., Garrido, M.D., Font-i-Furnols, M. 2020. The Use of Pork from Entire Male and Immunocastrated Pigs for Meat Products-An Overview with Recommendations. *Animals* **10**(10). doi:10.3390/ani10101754

Sodring, M., Nafstad, O. & Haseth, T.T. 2020. Change in Norwegian consumer attitudes towards piglet castration: increased emphasis on animal welfare. *Acta veterinaria Scandinavica* **62**, Article 22.

Tomasevic, I., Bahelka, I., Candek-Potokar, M., Citek, J., Djekic, I., Djurkin Kusec, I., Getya, A., Guerrero, L., Iordachescu, G., Ivanova, S., Nakov, D., Solowiej, B.G., Szabo, C., Tudoreanu, L., Weiler, U. & Font-i-Furnols, M. 2020a. Attitudes and beliefs of Eastern European consumers towards piglet castration and meat from castrated pigs. *Meat Science* **160,** Article 107965. doi: 10.1016/j.meatsci.2019.107965

Tomasevic, I., Bahelka, I., Citek, J., Candek-Potokar, M., Djekic, I., Getya, A., Guerrero, L., Ivanova, S., Kusec, G., Nakov, D., Solowiej, B., Stoica, M., Szabo, C., Tudoreanu, L., Weiler, U. & Font-i-Furnols, M. 2020b. Attitudes and Beliefs of Eastern European Consumers Towards Animal Welfare. *Animals* **10**(7), Article 1220 doi: 10.3390/ani10071220

Vanhonacker, F. & Verbeke, W. 2011. Consumer response to the possible use of a vaccine method to control boar taint v. physical piglet castration with anaesthesia: a quantitative study in four European countries. *Animal* **5**(7), 1107–1118. doi:10.1017/s1751731111000139

von Borell, E., Bonneau, M., Holinger, M., Prunier, A., Stefanski, V., Zols, S. & Weiler, U. 2020. Welfare Aspects of Raising Entire Male Pigs and Immunocastrates. *Animals*, **10**(11), Article 2140. doi: 10.3390/ani10112140

Wauters, J., Vercruysse, V., Aluwe, M., Verplanken, K. & Vanhaecke, L. 2016. Boar taint compound levels in back fat versus meat products: Do they correlate? *Food Chemistry* **206**, 30–36.

Wauters, J., Verplanken, K., Vercruysse, V., Ampe, B., Aluwe, M. & Vanhaecke, L. 2017. Sensory evaluation of boar meat products by trained experts. *Food Chemistry* **237**, 516–524. doi: 10.1016/j.foodchem.2017.05.128

Weiler, U. & Bonneau, M. 2019. Why it is so difficult to end surgical castration of boars in Europe: Pros and cons of alternatives to piglet castration. *IOP Conference Series-Earth and Environmental Science* **333**, 012001. doi:10.1088/1755-1315/333/1/012001

Whittington, F.M., Nute, G.R., Hughes, S.I. McGivan, J.D., Lean, I.J., Wood, J.D. & Doran, E. 2004. Relationships between skatole and androstenone accumulation, and cytochrome P4502E1 expression in Meishan x Large White pigs. *Meat Science* **67**(4), 569–576.

Agronomy Research **19**(S3), 1285–1307, 2021
https://doi.org/10.15159/AR.21.083

Improving single cell protein yields and amino acid profile via mutagenesis: review of applicable amino acid inhibitors for mutant selection

K. Spalvins*, S. Raita, K. Valters and D. Blumberga

Riga Technical University, Institute of Energy Systems and Environment, Azenes street 12/1, LV 1048 Riga, Latvia
*Correspondence: kriss.spalvins@rtu.lv

Received: February 4th,2021; Accepted: April 24th, 2021; Published: May 18th, 2021

Abstract. Single cell protein (SCP) is a good alternative for substituting plant and animal derived dietary proteins, since SCP production is more environmentally friendly, consumes less water, requires smaller land areas and its effect on climate change is much less pronounced than it is in the case of agriculturally derived proteins. Another advantage of SCP is that it is possible to use a wide variety of biodegradable agro-industrial by-products for the cultivation of SCP producing microorganisms. However, to make single cell protein technology more widely available and improve its economic viability in such markets as animal and fish feed industries, it is necessary to improve the protein yields and amino acid profiles in microorganism strains capable of using agro-industrial by-products. One way to improve the strains used in the process is to create and select SCP-rich mutants. In this review authors propose a novel approach to create SCP-rich mutants with improved total protein content and essential amino acid profiles. In this approach amino acid inhibitors are used to create selective pressure on created mutants. It is expected that mutants with the most pronounced growth would either have higher total protein content, increased essential amino acid concentrations or both, when cultivated on selective plates containing one or multiple amino acid inhibitors. This paper reviews the most suitable groups of amino acid inhibitors that could be used for selection of new strains of SCP-producing microorganisms.

Key words: mutagenesis, microbial protein, essential amino acids, amino acid inhibitors, herbicides, low-cost substrate, agricultural residues.

INTRODUCTION

Single cell protein as sustainable feed ingredient

Proteins are a group of nutrients that both humans and animals use as a major source of amino acids. Of the twenty-one amino acids found in living creatures, nine are not synthesized in the human body and need to be consumed via diet to maintain proper functionality of the human body. Four of these nine essential amino acids - lysine, methionine, threonine, and tryptophan, are not available in sufficient quantities in plant-derived products (Wang et al., 2017), thus animal sourced foods need to be included in

diet to prevent various health problems related to protein deficiency in the long term (Wang et al., 2017).

Livestock products are the main source of complete protein worldwide (Martin, 2001). Today, livestock products (meat, milk, eggs) provide more than 33% of the total protein intake in human diets (Martin, 2001). Rapidly growing human populations and growing consumer demand are generating large demand for animal products (Chadd et al., 2002). This leads to the need to identify alternative sources of protein to ensure a sustainable supply of animal feeds.

Although livestock farming is a major source of protein, calorie wise it is a very inefficient industry. Only 3–7% of the calories consumed by farm animals are converted into live weight (Shepon et al., 2016). For this reason, 83% of the world's agricultural land is used for production of livestock feeds (Poore & Nemecek, 2018). For example, in order to produce 1 kg of meat (beef), it requires 25 kg of grain and 15,000 litres of water (Mekonnen & Hoekstra, 2010). If these areas were used to grow direct human foods, our planet could feed an additional 3.5 billion people (Cassidy et al., 2013). Increasing the use of single cell protein in livestock feeds would reduce the need for intensive farming, which has a very negative impact on local ecosystems and species diversity worldwide.

Globally, seafood is also a very important source of protein for humans. On average, fish and crustaceans account for 17% of the world's protein intake (FAO, 2014). Since 2014, most of the fish and crustaceans consumed by humans are produced in captivity (FAO, 2016). This increase in farmed fish and crustaceans is mainly driven by rapidly growing demand for fish products, the depletion of fish populations in the wild and rapidly developing aquaculture industry (Tacon & Metian, 2015). Although the aquaculture industry has surpassed the wild capture fisheries in terms of production volume (Tacon & Metian, 2015), wild capture fisheries is still the main source of feed for aquaculture industry. Wild capture has remained stagnant over the last 20 years and now is no longer able to adequately supply the aquaculture industry with fishmeal (protein source) used as feed for farmed fish (Tacon & Metian, 2015). Thus, aquaculture had to look for new sources of feeds and currently the main source of protein for aquaculture fish is soy (Tacon & Metian, 2015). Soy lacks essential amino acids recommended for use in animal feeds. As a result, the aquaculture industry, same as livestock industry, has also become dependent on agricultural inputs. In addition, plant derived feeds are unsuitable for intestinal tract of predatory fish (salmonids etc.), which is one of the causes of poor health of aquaculture fish, fish are more likely to die and large amounts of antibiotics are needed to treat the various diseases (FAO, 1980).

In general, both livestock and aquaculture industries need to find new sources of protein-rich feeds that contain all the amino acids needed for a complete diet for farmed animals. Single cell proteins produced by using agro-industrial by-products are considered a very promising alternative. The authors have reviewed applicability of most of these by-products in previous articles (Spalvins et al., 2017; Spalvins et al., 2018; Spalvins & Blumberga 2018; Spalvins & Blumberga 2019; Spalvins et al., 2019). This technology is based on the cultivation of protein-producing microorganisms (bacteria, yeasts, fungi, and microalgae) using biodegradable agricultural residues and production by-products as the main source of nutrients for microbial growth. Not only would this technology produce protein-rich feed containing all the essential amino acids, but the feed itself would be cheaper, since inexpensive by-products of other industries would be

used as production substrates (Spalvins et al., 2017; Spalvins et al., 2018; Spalvins et al., 2020). Additionally, by replacing agriculture derived feeds with SCP, the health of the fish is considerably increased because these proteins are more easily digested (FAO 1980) and in its composition SCP is much more similar to feed these species of fish can acquire in wild (plankton: microalgae, bacteria, fungi etc.) (Finco et al., 2017; Spalvins & Blumberga 2018).

Creation of improved SCP-producing strains via mutagenesis

The single-cell protein production technologies have been extensively researched for decades, and the single-cell protein market in 2017 reached a total revenue of $ 5.3 billion (P&S Intelligence 2018). Although SCP market has been steadily growing and more and more SCPs produced from by-products are being introduced to the market (Ritala et al., 2017), there is always room to improve novel or already well-known SCP-producing microorganism strains. Regarding increased SCP yields and production efficiency, various properties can be improved in microorganism strains, such as, biomass concentration/cell density, protein concentration in biomass, growth speed, utilization efficiency of the selected by-products, tolerance to harmful compounds present in selected by-products etc.

Existing strains can be replaced by novel species or different strains by isolating microorganisms from areas which have been polluted with contaminants (agro-industrial by-products) that could be used as the main feedstock for SCP production. By isolating strains from contaminated areas it is possible to find strains that have adapted to utilize specific by-products more efficiently. Examples of such approach has been widely reported (Mehta 1973; Wong & Chan, 1980; Kim & Lebeault, 1981; Ivarson & Morita, 1982; Baldensperger et al., 1985; Kornochalert et al., 2014; Yadav et al., 2016; De Gregorio et al., 2002). In this approach appropriate strains can be selected from nature by looking for various beneficial factors such as: growth speed, protein content, temperature optimum, maximum tolerated temperature, salinity, shear tolerance, growth on particular substrates, growth in selective environment etc. (Borowitzka & Moheimani, 2013).

Another approach is to introduce the desired properties in existing strains. Acquisition of better microorganism strains is very important for the development and improvement of technological solutions based on SCP production. Microorganisms can be improved using both classical mutagenesis and modern genetic engineering methods combined with advanced screening methods. Although use of modern solutions is increasing (Yan et al., 2018; Leavell et al., 2020; Tatenhove-Pel et al., 2020), classical mutagenesis and random screening methods are still considered to be simple and efficient for short-term strain development (Rowlands, 1984; Anderson, 1995; Winston, 2008; Atzmüller et al., 2019) and are still widely used (Sivaramakrishnan & Incharoensakdi, 2017; Yamada et al., 2017; Zhu et al., 2018; Atzmüller et al., 2019). Mutagenesis accelerates the mutations frequency rate up to 100 times when compared to natural mutation rate (Winston, 2008). Mutagens can be divided into physical, such as UV-light, gamma and X-rays, and chemical, such as ethyl methane sulphonate (EMS), nitrosomethyl guanidine (NTG), etc. (Rowlands, 1984; Anderson, 1995). After treatment with the mutagen, the surviving strains must be selected, and this can be done by screening as many mutants as practically possible or by using selective media in which only those mutants that have acquired the desired properties will develop. For example,

UV-mutagenesis has been widely used for creation of mutants with improved lipid production capabilities (Sivaramakrishnan & Incharoensakdi, 2017; Yamada et al., 2017; Zhu et al., 2018; Atzmüller et al., 2019). It is possible to select suitable mutants using fatty acid biosynthesis inhibitors such as cerulenin (Omura, 1976; Tapia et al., 2012; Katre et al., 2017; Atzmüller et al., 2019), and it has been shown that a mutant with a higher lipid production capacity will form larger colonies on a selective plate, thus making mutant selection quick, convenient and simple. If produced lipids are used for example as a feedstock for biodiesel production, changes in fatty acid composition due to mutation is not that critical for the biodiesel production process (Atzmüller et al., 2019). If lipids are used as feed, then changes in fatty acid composition need to be accounted for as that directly affect health and feed conversion ratios of the farm animal (Long et al., 2020). It is also the same in case with SCP when used for animal or fish feed. Although total protein concentration in microbial biomass is a significant factor, the concentration of essential amino acids is the main factor that determines the value of the obtained protein. For example, such essential amino acids as lysine, methionine, threonine and tryptophan are very important components in fish feeds, as they are available in lower amounts in conventional protein sources such as soy (Al-Marzooqi et al., 2010; Hardy et al., 2018). Thus, in creating SCP mutants, it is important not only to increase the total protein concentration in the biomass of microorganisms, but also to increase the proportion of essential amino acids. Just as in the case with using cerulenin to select mutant strains with enhanced fatty acid synthesis capabilities (Omura, 1976; Tapia et al., 2012; Katre et al., 2017; Atzmüller et al., 2019), it could be possible to use amino acid inhibitors to select for improved SCP-rich mutants. Mutants with increased protein and specific amino acid concentrations would form larger colonies on selective plates that contain one or multiple amino acid inhibitors. The largest colonies could be then picked for further screening, i.e., testing of growth rate, cell density, protein content, amino acid composition etc. Unlike fatty acid inhibitors, there are no universal inhibitors of amino acids that inhibit the synthesis of all amino acids, so it is necessary to select different amino acid inhibitors, each individually or in various combinations to select for mutants not only with increased protein concentration but also with improved essential amino acid concentrations.

To the best of the authors knowledge this is the first review to analyse the possibility of using amino acid inhibitors to select for SCP-rich mutants.

AMINO ACID INHIBITORS

Most amino acid inhibitors that are available are used in agriculture as herbicides and this is the intended application also for most of the amino acid inhibitors that are currently in development (HRAC 2002; Berlicki, 2008; Cobb & Reade, 2010; Hall et al., 2020). Therefore, majority of research conducted on using these compounds are regarding their practical and cost effectiveness in weed management (Llewellyn et al., 2016; Hall et al., 2020). Also most of the research available on these herbicides is done on their inhibitory activity on plant biosynthetic pathways, while information on their activity on single-celled organisms such as bacteria, microscopic algae, yeasts, fungi, unicellular protists, archaea etc. is limited. These aspects need to be considered when selecting an amino acid inhibitor for use in SCP-producing mutant selection, as the actual inhibitor response may differ significantly from what was expected. Although

information is lacking most of the amino acid biosynthesis pathways are very similar and many enzymes that are the main targets of inhibitors are the same in different kingdoms (Herrmann & Weaver, 1999; Joshi et al., 2006; Shearer, 2007; Binder, 2010; Hall et al., 2020), therefore most of the inhibitors should promote inhibitory response in microorganisms as well. One additional difference that might be encountered when applying these herbicides on microorganisms is that even if amino acid biosynthesis pathway is shared across kingdoms, in plants many amino acids are synthesized in plastids (Herrmann & Weaver, 1999; Hall et al., 2020), while, for example, bacteria and fungi does not have such structures and the same pathways are localized in cytosol. This fact most likely will affect the inhibitor concentration required to perform the mutant selection, but also might affect some other properties of the inhibitor's effect or promote previously unnoticed side effects such as level of cytotoxicity, level of sensitivity to inhibitory effects, extent of DNA synthesis inhibition, extent of nutrient assimilation impairment, extent of pathway intermediates accumulation, extent of metabolic disruptions etc. (Zhou et al., 2007; Cobb & Reade, 2010; Fucile et al., 2011; Orcaray et al., 2012; Hall et al., 2020). For example, enzyme from histidine biosynthesis pathway imidazole glycerol-phosphate dehydratase can be inhibited by 2-hydroxy-3-(1,2,4-triazol-1-yl) propylphosphonate (Rawson et al., 2018). This inhibitor is much more potent in yeasts than in plants, due to additional β-strand which enhances the binding of inhibiting compound to the yeast enzyme (Rawson et al., 2018), therefore inhibitor concentration should be decreased appropriately if used for selection of yeast mutants. Other inhibitors are isolated from microorganisms e.g., cornexistin is isolated from fungi *Paecilomyces variotii*. If this inhibitor is used for selection of related fungal strains, then it might be the case that these microorganisms show resistance to inhibitory effect (Takahashi et al., 1994). Therefore, considering that the use of herbicides in the selection of microorganisms may lead to unpredictable side effects or render the inhibitor completely ineffective, each compound needs to be tested individually during mutant selection.

Ten of the twenty-one amino acids found in living beings cannot be synthesized by the fish, these are: arginine, phenylalanine, valine, threonine, tryptophan, methionine, leucine, isoleucine, lysine and histidine (Andersen et al., 2016). Of these ten, four - lysine, methionine, threonine and tryptophan, are present at lower concentrations in plant derived proteins than recommended in animal and fish feeds (FAO, 1980; Al-Marzooqi et al., 2010; Finco et al., 2017; Hardy et al., 2018). Therefore, if the produced SCP is intended for use in animal or fish feed, during the mutant selection it would be desirable to use amino acid inhibitors that inhibit the biosynthetic pathways of these four amino acids. In this way, selective pressure is applied directly to those mutants that are able to synthesize the relevant four amino acids in larger quantities than respective wild types. If SCP is intended for other applications - human diet supplements, other animal and pet feeds, cosmetics, building block chemicals etc., value of the produced SCP can be increased also if the concentration of any other amino acid is increased via utilization of appropriate inhibitor during the mutant selection. All amino acid inhibitors and their properties regarding mutant selection have been summarized in Table 1.

Table 1. Properties of amino acid (AA) inhibitors and their target enzymes

Inhibitor	Target enzyme	Target present in	Inhibited AA	Pros (+), cons (–), side effects, etc.
Aromatic amino acid inhibitors				
Glyphosate[1]	5-*enolpyruvyl*-shikimate 3-phosphate synthase (EPSPS)[1]	Plants, archea, bacteria, fungi, algae[1]	Phe, Trp, Tyr	(+) Inhibits microbes[41-43] (+) Well studied effects[2,40] (+) Widely available (–) Deregulates carbon metabolism[2] (–) Shikimate accumulation[2]
7-deoxy-sedoheptulase[3] (7dSh)	3-dehydro-quinate synthase (DHQS)[4]		Phe, Trp, Tyr	Cyanobacteria metabolite[5] (+) Inhibits microbes[4] (–) Limited availability[5]
3-indoleacrylic acid[6]	tryptophan synthase (TS)[4]		Trp	Bacteria metabolite[7] (+) Inhibits microbes[7,44] (+) Widely available
Branched chain amino acid inhibitors[8]				
Sulfonylureas Imidazolinones Triazolopyrimidines Pyrimidinyl (thio)benzoates Sulfonylaminocarbonyl triazolinones	Acetolactate synthase /acetohydroxy-acid synthase (ALS/AHAS)[4]	Plants, archaea, bacteria, fungi[10]	Ile, Leu, Val	(+) Inhibits microbes[45-49] (+) Well studied effects[45-49] (+) Widely available (–) Cause intermediates accumulation[4,9] (–) DNA synthesis inhibition[4,9] (–) Disrupted assimilates transport[4,9]
Histidine inhibitors				
2-hydroxy-3-(1,2,4-triazol-1-yl) propylphosphonate[16]	Imidazole glycerol-phosphate dehydratase (IGPD)[16]	Plants, bacteria, fungi, archaea[17]	His	(+) Inhibits microbes[16] (+) More potent in yeasts than plants[16] (+) Widely availability (–) Further research required
3-(diethoxy-phophoryl)-3-(1H-1,2,3-triazol-4-one-1-yl)propan-2-ylcarboxylic esters[18]				(–) Further research required
Monopyrrole aldehydes[19]				(–) Further research required
S-1-(4-biphenyl)-4-(4-imidazolyl)-3-amino-2-butanone[20] (BPIAB)	Histidinol dehydrogenase (HDH)[20]			(–) Inhibitor activity unclear[20] (–) Further research required
Glutamine inhibitors[15]				
L-Phosphinothricin[11] Methionine sulfoximine[14] Tabtoxinine β-lactam[14] Bialaphos[4]	Glutamine synthetase (GS)[11]	Prokaryotes, eukaryotes[12,13]	Gln, Asp, Pro, Arg, Lys, Met, Thr, Ile	(+) Inhibits microbes[50] (+) Well studied effects[50-54] (+) Widely available (–) Ammonia accumulation[11]

Table 1 continued

Glutamate-derived amino acid inhibitors				
Cornexistin[21,22]	Aspartate trans-aminase (AST)[21,22]	Archaea, bacteria, eukaryotes [4,38,39]	Asp, Met, Thr, Ile, Lys	Fungal metabolite[21] (–) No activity in bacteria and fungi[23] (–) Limited availability (–) Further research required
Phaseolotoxin[24]	Ornithine carbamoyl-transferase (OCT)[24]		Arg	Bacterial metabolite[24] (+) Inhibits microbes[55] (–) Limited availability (–) Further research required
Aminomethylene-bisphosphonates[25] (AMBP)	δ1-pyrroline-5-carboxylate reductase (P5CR) and GS[25]		Pro, Gln, Asp, Arg, Lys, Met, Thr, Ile	(+) More potent in bacteria than plants[26] (+) Inhibits microbes[26] (–) Ammonia accumulation[56] (–) Limited availability
Aspartate-derived amino acid inhibitors				
2-(1-cyclohexen-3(R)-yl)-S-glycine[27] (CHG)	Threonine deaminase (TD)[27]	Plants, bacteria, fungi[28]	Ile	(+) Inhibits microbes[57,58] (–) Limited availability[27] (–) Further research required
DL-Propargylglycine[29,30] (PAG)	Cystathionine γ-synthase (Cγ-S)[29,30]	Plants, bacteria, fungi[35]	Met	(+) Inhibits microbes[59-62] (+) Widely available
Rhizobitoxine[31,32]	Cysteine-S-conjugate β-lyase (Cβ-L)[31,32]	Plants, archaea, bacteria, fungi, animals[36]	Met	Bacteria metabolite[31] (–) Limited availability[34] (–) Further research required
S-(2-aminoethyl)-L-cysteine[33] (AEC)	Aspartate kinase (AK) and dihydrodi-picolinate synthase (DHDPS)[33]	Plants, bacteria, archaea, fungi[37]	Lys	(+) Inhibits microbes[63-67] (+) Widely available
L-α-(2-amino ethoxy-vinyl) glycine[33] (AVG)	DHDPS			

[1](Herrmann & Weaver, 1999); [2](Orcaray et al., 2012); [3](Schultz & Coruzzi, 1995); [4](Hall et al., 2020); [5](Brilisauer et al., 2019); [6](Widholm, 1981); [7](Wang et al., 2013); [8](HRAC 2002); [9](Zhou et al., 2007); [10](Binder, 2010); [11](Cobb & Reade, 2010); [12](Forde & Lea, 2007); [13](Patrick et al., 2018); [14](Maughan & Cobbett, 2003); [15](Berlicki, 2008); [16](Rawson et al., 2018); [17](Shearer, 2007); [18](Jin et al., 2015); [19](Schweitzer et al., 2002); [20](Dancer et al., 1996); [21](Amagasa et al., 1994); [22](Nakajima et al., 1991); [23](Takahashi et al., 1994); [24](Mitchell & Bieleski, 1977), [25](Forlani et al., 2013); [26](Forlani et al., 2012); [27](Szamosi et al., 1994); [28](Joshi et al., 2006); [29](Ravanel et al., 1998b); [30](Ravanel et al., 1998a); [31](Okazaki et al., 2007); [32](Giovanelli et al., 1971); [33](Soares da Costa et al., 2018); [34](Okazaki et al., 2007); [35](Goyer et al., 2007); [36](Cooper et al., 2011); [37](Pearce et al., 2017); [38](Singh et al., 2019); [39](Zúñiga et al., 2002); [40](Steinrücken & Amrhein, 1980); [41](Leino et al., 2020); [42](Funke et al., 2006); [43](Morjan et al., 2002); [44](Nonomura et al., 1996); [45](Jia et al., 2000); [46](Kreisberg et al., 2013); [47](Landstein et al., 1995); [48](Lee et al., 2013); [49](Burnet & Hodgson, 1991); [50](Ahmad & Malloch, 1995); [51](Myrold & Posavatz, 2007); [52](Ahmad et al., 1995); [53](Kim & Rhee, 1987); [54](Kulkarni et al., 2006); [55](Staskawicz, 1979); [56](Giberti et al., 2017); [57](Keller-Schierlein et al., 1969); [58](Szamosi et al., 1994); [59](Jin et al., 2004); [60](Piotrowska & Paszewski, 1986); [61](Johnston et al., 1979); [62](Lockwood & Coombs, 1991); [63](Zabriskie & Jackson 2000); [64](Ekwealor & Obeta, 2006); [65](Han et al., 1991); [66](Sano, 1970); [67](Rupp et al., 1989).

1. Aromatic amino acid inhibitors

Aromatic amino acids are phenylalanine, tyrosine and tryptophan (Hall et al., 2020). Aromatic amino acids are synthesized in the shikimate pathway (Tohge et al., 2013). This pathway is found in plants, fungi, bacteria, archaea, microscopic algae and other eukaryotes and prokaryotes (Hall et al., 2020). One of the pathway enzymes - 5-enolpyruvalshikimate-3-phosphate synthase (EPSPS), is a target for the widely used and commercially available herbicide glyphosate/N-(phosphonomethyl)glycine (Steinrücken & Amrhein, 1980). Glyphosate inhibits EPSPS, resulting in the synthesis cessation of all three aromatic amino acids (Fig. 1, A) (Herrmann & Weaver, 1999). Although glyphosate is highly specific for EPSPS, it is suspected that glyphosate also causes shikimate accumulation, which in turn deregulates carbon metabolism (Orcaray et al., 2012). Almost all species of fungi, bacteria and algae are sensitive to glyphosate (Morjan et al., 2002; Funke et al., 2006; Leino et al., 2020). Some EPSPS isoforms are not sensitive to glyphosate (Fucile et al., 2011). While glyphosate-sensitive EPSPS class I is present in most bacteria (Tohge et al., 2013), glyphosate-resistant EPSPS class II has been isolated from certain bacterial species, such as *Ochrobactrum anthropi* (Tian et al., 2010) and agrobacteria (Fucile et al., 2011). In general, the specific effect of glyphosate on EPSPS allows it to be used to select for a large proportion of SCP-producing bacteria, fungi and microscopic algae, however, potential adverse side effects and possible resistance may in some cases complicate mutant selection.

Brilisauer et al., 2019 reported on the isolation of a new inhibitor, 7-deoxy-sedoheptulose, from the cyanobacterium *Synechococcus elongates* (Brilisauer et al., 2019). This inhibitor targets another shikimate pathway enzyme 3-dehydroquinate synthase (DHQS) (Fig. 1, A) (Brilisauer et al., 2019). Cyanobacteria treated with this inhibitor could be rescued by adding amino acids to the medium, suggesting that the inhibitory effect was caused by amino acid starvation (Hall et al., 2020). This observation suggests that the use of 7-deoxy-sedoheptulose may be more appropriate for mutant selection, as this inhibitor may not cause as pronounced side effects as glyphosate. The disadvantage of 7-deoxy-sedoheptulose is that it is not currently commercially available and requires hemoenzymatic synthesis and purification in laboratory (Brilisauer et al., 2019). Although 7-deoxy-sedoheptulose has been isolated from cyanobacteria, studies to date have shown that this compound is able to inhibit the growth of plant, yeast, and even other cyanobacteria species (Brilisauer et al., 2019), suggesting that resistance to this inhibitor is rare and is therefore likely to be effective in the selection of other microorganisms, although this has yet to be tested.

Another enzyme in the shikimate pathway, tryptophan synthase, is inhibited by 3-indoleacrylic acid, thus stopping tryptophan synthesis (Fig. 1, A) (Widholm, 1981; Sachpatzidis et al., 1999; Hall et al., 2020). 3-indoleacrylic acid is commercially available because it is widely used to induce gene transcription. 3-indoleacrylic acid has been isolated from multiple species of bacteria (Wikoff et al., 2009; Wang et al., 2013; Zhang & Davies 2016), indicating that the use of this compound in the selection of some bacteria strains may be limited. In general, 3-indoleacrylic acid inhibition of the growth of bacteria, cyanobacteria and fungi have been reported (Nonomura et al., 1996; Wang et al., 2013).

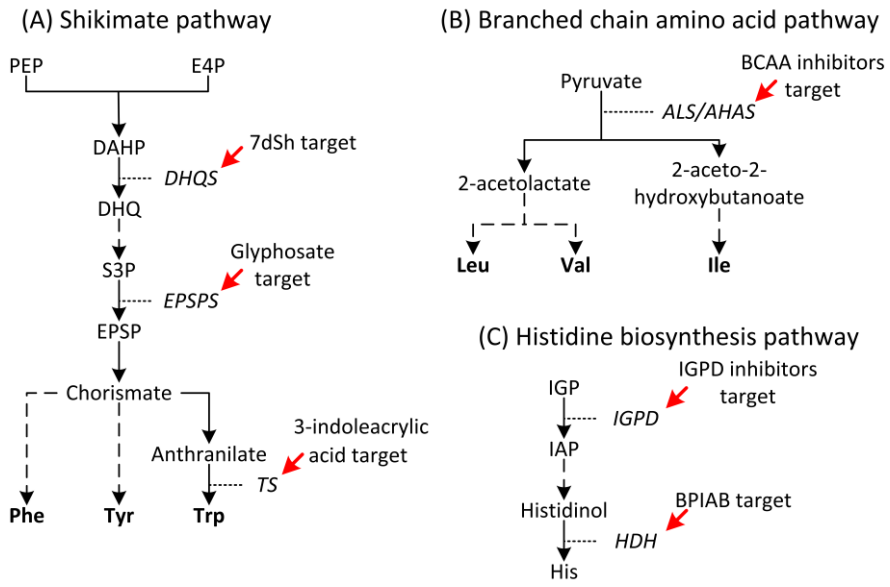

Figure 1. (A) Simplified shikimate pathway, (B) simplified branched chain amino acid biosynthesis pathway, (C) simplified histidine biosynthesis pathway. In shikimate pathway (A), through condensation of phosphoenolpyruvate (PEP) and erythrose 4-phosphate (E4P), 3-deoxy-arabino heptulosonate 7-phosphate (DAHP) is produced. DAHP is then converted to 3-dehydroquinate (DHQ) and this process is catalized by enzyme DHQ synthase (DHQS). DHQS can be inhibited by 7-deoxy-sedoheptulose (7dSh). Further down the shikimate pathway shikimate 3-phosphate (S3P) is converted to 5-enolpyruvyl-shikimate 3-phosphate (EPSP) and this reaction is catalized by EPSP synthase (EPSPS). EPSPS is target enzyme of popular herbicide - glyphosate. Inhibition of either DHQS or EPSPS causes cessation of phenylalanine (phe), tyrosine (tyr) and tryptophan (trp) biosynthesis. In tryptophan biosynthesis branch (A) intermediate anthranilate is converted to tryptophan by tryptophan synthase (TS). TS is targeted by inhibitor 3-indoleacrylic acid. In branched chain amino acid (BCAA) pathway (B) acetolactate synthase/acetohydroxyacid synthase (ALS/AHAS) catalyzes condensation of pyruvate which produces 2-acetolactate. 2-acetolactate is early precursor of leucine (leu) and valine (val) biosynthesis. ALS/AHAS also catalyzes reaction where pyruvate is used to produce 2-aceto-2-hydroxybutanoate which is intermediate for isoleucine (ile) synthesis. There are various inhibitors available which specifically target ALS/AHAS (Table 1). In histidine (his) biosynthesis pathway (C) initial step is dehydration of imidazole glycerol-phosphate (IGP) to produce imidazoleacetol phosphate (IAP) which is catalized by IGP dehydratase (IGPD). There are various IGPD inhibitors available (Table 1). Last step of histidine biosynthesis is histidinol conversion to histidine by enzyme histidinol dehydrogenase (HDH). HDH is targeted by inhibitor S-1-(4-biphenyl)-4-(4-imidazolyl)-3-amino-2-butanone (BPIAB). Images and pathway descriptions adapted from Hall et al., 2020, Tzin & Galili, 2010.

2. Branched chain amino acid inhibitors

Leucine, isoleucine and valine are synthesized via branched-chain amino acids biosynthesis (BCAA) pathway (Binder, 2010). This pathway is found in plants, bacteria, fungi, archaea, microscopic algae and other microorganisms (Singh & Shaner, 1995; Binder, 2010; Duan et al., 2019; Hall et al., 2020). Although all three amino acids are synthesized in separate pathway branches, the synthesis of all three amino acid

precursors is catalyzed by the enzyme acetolactate synthase/acetohydroxyacid synthase (ALS/AHAS), which is also a target enzyme for various inhibitors (Fig. 1, B) (Hall et al., 2020). Many of these inhibitors are commercially available, which can be categorized into five groups: sulfonylureas, imidazolinones, triazolopyrimidines, pyrimidinyl(thio)benzoates and sulfonylaminocarbonyl-triazolinones (HRAC 2002). Full list of ALS/AHAS inhibitors are provided by (HRAC 2002). In addition to amino acid depletion, all ALS/AHAS inhibitors are characterized by side effects such as branched chain amino acid biosynthesis pathway intermediate accumulation, inhibition of DNA synthesis, and impaired assimilates transport (Zhou et al., 2007). These side effects can lead to errors in mutant selection, because mutants with increased resilience to side effects might be selected rather than mutants with increased ability to synthesize more BCAAs. Using ALS/AHAS inhibitors, branched chain amino acid biosynthesis has been successfully inhibited in yeasts (Lee et al., 2013; Jia et al., 2000; Duggleby et al., 2003), bacteria (Massey et al., 1976; Allievi & Gigliotti, 2001; Kreisberg et al., 2013), microscopic algae (Landstein et al., 1995, 1993), fungi (Allievi & Gigliotti, 2001; Lee et al., 2013) and in other microorganisms (Burnet & Hodgson, 1991), which indicate that these inhibitors can be used effectively to select mutants with improved SCP production capacity, however, possible selection errors due to existing side effects must also be taken into consideration.

3. Histidine inhibitors

Histidine biosynthesis occurs in both plants and microorganisms (Rawson et al., 2018; Stepansky & Leustek, 2006). Although commercial histidine inhibitors are not available on the market, recent findings have reported on inhibitor called 2-hydroxy-3-(1,2,4-triazol-1-yl) propylphosphonate, targeting enzyme responsible for the sixth step in histidine biosynthesis named imidazole glycerol-phosphate dehydratase (IGPD) (Fig. 1, C) (Rawson et al., 2018; Hall et al., 2020). Interestingly it has been demonstrated that this inhibitor is significantly more potent in yeasts than in plants (Rawson et al., 2018). Apart from IGPD inhibition there have been no reports on other side effects caused by 2-hydroxy-3-(1,2,4-triazol-1-yl) propylphosphonate, but it needs to be considered that as this novel inhibitor is further tested some cytotoxic effects might be found. Overall, 2-hydroxy-3- (1,2,4-triazol-1-yl) propylphosphonate is currently the only available inhibitor of histidine biosynthesis that could be used for improved SCP-rich mutant selection.

There have been reports on other inhibitors targeting IGPD, such as 1-(diethoxy-phosphoryl)-3-(4-one-1H-1,2,3-triazol-1-yl)-propan-2-yl carboxylic esters (Jin et al., 2015) and monopyrrole aldehydes (Schweitzer et al., 2002), but further research is required to confirm their applicability in microorganism mutant selection.

Histidinol dehydrogenase is the last enzyme in histidine biosynthesis, which is targeted by inhibitor S-1-(4-biphenyl)-4-(4-imidazolyl)-3-amino-2-butanone (Fig. 1, C) (Dancer et al., 1996). The activity of this compound on histidinol dehydrogenase is also ambiguous and further studies are needed.

4. Glutamine inhibitors

Glutamine is the most abundant amino acid in living beings (Cruzat et al., 2018). Therefore, it is reasonable to assume that selecting mutants for their glutamine synthesis capacity might result in discovery of strains with significantly increased total protein

contents. Because glutamine is a major precursor in the glutamate-derived amino acid biosynthesis pathway, which results in the synthesis of aspartate, proline, and arginine, inhibition of glutamine synthesis results in arrest of multiple amino acids biosynthesis (Fig. 2, D, E). Further cascading effect of glutamine inhibition will also prevent biosynthesis of aspartate-derived amino acids - lysine, methionine, threonine, and isoleucine (Fig. 2, F). Thus, by using only glutamine biosynthesis inhibitors, it is possible to inhibit the synthesis of eight amino acids, of which four are essential amino acids in animals. Thus, glutamine biosynthesis inhibitors in theory seem to be the most promising inhibitors to be used in the selection of mutants with increased capacity for the production of essential amino acids and increased protein content in general.

Glutamine biosynthesis occurs in both prokaryotes and eukaryotes (Forde & Lea, 2007; Patrick et al., 2018; Hall et al., 2020). To inhibit the glutamine biosynthetic pathway, all inhibitors target enzyme glutamine synthetase (GS) (Fig. 2, D). GS differs between prokaryotes and eukaryotes, with prokaryotic GS having twelve active sites and eukaryotes ten (Unno et al., 2006; Almassy et al., 1986; Berlicki, 2008), respectively, and differences in prokaryotic and eukaryotic GS susceptibility to different inhibitors have also been reported (Kim & Rhee 1987; Ahmad & Malloch, 1995; Ahmad et al., 1995; Kulkarni et al., 2006; Myrold & Posavatz, 2007). For example, the GS inhibitor phosphinothricin in soil at a concentration of 1 mM reduced the bacterial population by 40% and the fungal population by 20% (Ahmad & Malloch, 1995). Therefore, the effects of the same GS inhibitor may differ significantly from one species of microorganism to another. A number of inhibitors are available for GS inhibition, which can be divided into four groups: methionine sulfoximine and its analogues, glufosinate isomer (phosphinothricin) and its analogues, bisphosphonates, and other GS inhibitors (Berlicki, 2008; Hall et al., 2020). As side effects for most of these inhibitors, ammonia assimilation disorders have been reported in both prokaryotes (Myrold & Posavatz, 2007;) and eukaryotes (De Block et al., 1987; Boussiba & Gibson, 1991; Ahmad et al., 1995; Maughan & Cobbett, 2003; Kulkarni et al., 2006), which is rational because all these inhibitors target the same enzyme. Ammonia assimilation inhibition (Maughan & Cobbett, 2003; Cobb & Reade, 2010) might cause errors in selection of mutants since it is likely that mutants with increased resilience to ammonia might be selected instead of those with increased glutamine synthesis capability. Therefore, vigorous testing of GS inhibitors is required for the SCP-producing microorganisms, followed by further analysis of the selected strains for their total protein content and amino acid composition. GS inhibitors have been described in detail by Berlicki, 2008.

5. Glutamate-derived amino acid inhibitors

In glutamate-derived amino acid biosynthesis aspartate, proline and arginine are synthesized from glutamate via three separate pathways resulting in respective amino acids (Fig. 2, E) (Hall et al., 2020).

Similarly as with glutamine inhibition, but to smaller extent, inhibition of aspartate biosynthesis also affects production of aspartate-derived amino acids (Hall et al., 2020). Thus, by inhibiting aspartate biosynthesis, production of five amino acids – aspartate, lysine, methionine, threonine and isoleucine is prevented, which makes aspartate biosynthesis inhibitors promising candidates for use in SCP-rich mutant selection (Fig. 2, E, F). Aspartate biosynthesis is catalysed by an enzyme aspartate transaminase which is targeted by fungal metabolite cornexistin (Fig. 2, E) (Amagasa et al., 1994;

Nakajima et al., 1991). Low or no inhibitory activity of cornexistin has been reported in bacteria and fungi (Takahashi et al., 1994). Therefore, cornexistin applicability for SCP-rich bacteria, yeast, fungi and microscopic algae mutant selection needs to be tested for each species of interest. Additionally, cornexistin is not commercially available and it need to be produced and purified in laboratory (Steinborn et al., 2020). If, cornexistin or some other aspartate inhibitor will be proven to be viable for inhibition of aspartate biosynthesis in microorganisms, then this hypothetical inhibitor would be very useful in mutant selection, because, unlike glutamine inhibitors, aspartate inhibitors have not yet demonstrated adverse side effects such as ammonia accumulation.

In arginine biosynthesis one of the enzymes ornithine carbamoyltransferase is targeted by bacterial metabolite phaseolotoxin (Fig. 2, E) (Mitchell & Bieleski, 1977). Its activity has been demonstrated in *E. coli* (Staskawicz, 1979), but lack of analysis on other microorganisms suggests that similarly as in case with cornexistin, phaseolotoxin applicability for mutant selection need to be checked on case by case basis.

In proline biosynthesis enzyme δ1-pyrroline-5-carboxylate reductase (P5CR) can be inhibited by aminomethylene-bisphosphonates (Fig. 2, E) (Forlani et al., 2013). In study done by Forlani et al., 2012, several of the evaluated bisphosphonates were more potent on bacterial P5CR than on plant P5CR. Fungi and bacteria inhibition has been confirmed in other studies as well (Kunda et al., 2012; Shaik et al., 2020). Most of these compounds are not readily available and require synthesis in laboratory (Kunda et al., 2012; Shaik et al., 2020). Two of the aminomethylene-bisphosphonates - 3,5-dichlorophenylamino-methylenebisphosphonic acid and 3,5-dibromophenyl aminomethylenebis phosphonic acid simultaneously targeted P5CR and glutamine synthetase (GS) from glutamine biosynthesis pathway (Giberti et al., 2017). Both of these aminomethylene-bisphosphonates showed higher potency on GS inhibition than on P5CR inhibition (Giberti et al., 2017) and as discussed previously (see section 4. Glutamine inhibitors), for SCP-rich mutant selection GS inhibition might be preferable. As with other GS inhibitors toxic ammonia accumulation is expected.

6. Aspartate-derived amino acid inhibitors

Aspartate-derived amino acids are methionine, threonine, isoleucine and lysine (Fig. 2, F). In comparison to other herbicides, aspartate-derived amino acid inhibitors have been scarcely studied and no commercial herbicide is currently available on the market to inhibit any of aspartate-derived amino acids (Hall et al., 2020). However, there have been reports on compounds capable of inhibiting certain pathway enzymes (Keller-Schierlein et al., 1969; Ravanel et al., 1998b, 1998a; Szamosi, Shaner & Singh 1994; Hall et al., 2020).

In isoleucine biosynthesis enzyme threonine deaminase can be inhibited by 2-(1-cyclohexen-3(R)-yl)-S-glycine (CHG) (Fig. 2, F) (Ravanel et al., 1998a; Hall et al., 2020; Szamosi et al., 1994). This herbicidal compound is not commercially available and needs to be synthesized in laboratory (Szamosi et al., 1994). Threonine deaminase inhibition by CHG, has also been observed in bacteria (Keller-Schierlein et al., 1969; Szamosi et al., 1994). To confirm the use of CHG in SCP-rich mutant selection in the future, its efficacy in inhibiting other microorganisms needs to be tested.

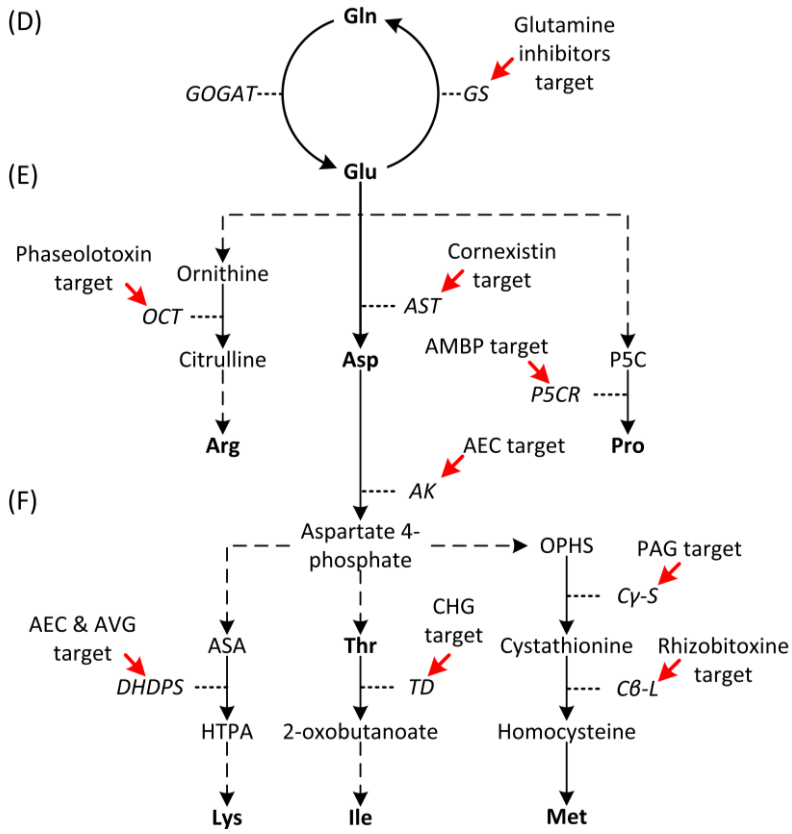

Figure 2. (D) Simplified glutamine biosynthesis pathway, (E) simplified glutamate-derived amino acid biosynthesis pathway, (F) simplified aspartate-derived amino acid biosynthesis pathway. In glutamine biosynthesis pathway (D), glutamine (gln) is converted to glutamate (glu) by glutamine oxoglutarate aminotransferase (GOGAT). Glutamate is converted back to glutamine by enzyme glutamine synthetase (GS), which is target of various GS specific inhibitors (Table 1). In arginine (arg) synthesis branch from glutamate-derived amino acid biosynthesis pathway (E) one of the intermediate steps involves ornithine convertion to citrulline by ornithine carbamoyltransferase (OCT). OCT is targeted by inhibitor phaseolotoxin. Proline (pro) is produced from δ1-pyrroline-5-carboxylate (P5C) by P5CR reductase. P5CR is target of various aminomethylene-bisphosphonates (AMBP). Some of AMBP are dual-target inhibitors which target P5CR and GS (D). Aspartate (asp) is produced from glutamate by aspertate transaminase (AST) which is targeted by fungal metabolite cornexistin. Aspartate-derived amino acid biosynthesis pathway (F) starts with convertion of aspartate to aspartate 4-phosphate by aspartate kinase (AK). AK is targeted by S-(2-aminoethyl)-L-cysteine (AEC). If AK is inhibited it causes cessation of methionine (met), threonine (thr), isoleucine (ile) and lysine (lys) synthesis. Further down the pathway one of the intermediates of lysine biosynthesis branch called aspartate semialdehyde (ASA) is converted to 4-hydroxy-tetrahydrodipicolinate (HTPA) by dihydrodipicolinate synthase (DHDPS). DHDPS can be inhibited by previously mentioned AEC or L-α-(2-amino ethoxy-vinyl) glycine (AVG). In isoleucine biosynthesis branch threonine is converted to 2-oxobutanoate by threonine deaminase (TD). TD is targeted by 2-(1-cyclohexen-3(R)-yl)-S-glycine (CHG). In methionine biosynthesis branch O-phosphohomoserine (OPHS) is converted to cystathionine by cystathionine γ-synthase (Cγ-S). Cystathionine is then converted to homocysteine by cysteine-S-conjugate β-lyase (Cβ-L). Cγ-S can be inhibited by DL-Propargylglycine (PAG) and Cβ-L can be inhibited by rhizobitoxine. Images and pathway descriptions adapted from Hall et al., 2020, Choi & Coloff, 2019.

In methionine biosynthesis enzyme cystathionine γ-synthase can be inhibited by DL-Propargylglycine (Fig. 2, F) (Ravanel et al., 1998a, 1998b). It has been demonstrated, that plants inhibited by DL-Propargylglycine can be rescued using methionine supplementation, thus indicating that the herbicidal activity comes from amino acid starvation (Ravanel et al., 1998b, 1998a). This observation is also a good indicator on the potential use of this compound in mutant selection. DL-Propargylglycine can be purchased as chemical reagent, thus no synthesis in laboratory is required (Yoshioka et al., 2014). Studies have demonstrated propargylglycine inhibitory activity in microorganisms as well (Johnston et al., 1979; Piotrowska & Paszewski, 1986; Lockwood & Coombs, 1991; Jin et al., 2004).

Another enzyme in methionine biosynthesis - cysteine-S-conjugate β-lyase, can be inhibited by bacteria metabolite rhizobitoxine and its analogues (Fig. 2, F) (Giovanelli et al., 1971; Okazaki et al., 2007), but further assessment on its use on microorganisms is required.

In lysine biosynthesis enzyme dihydrodipicolinate synthase can be inhibited by S-(2-aminoethyl)-L-cysteine and L-α-(2-aminoethoxyvinyl)glycine (Fig. 2, F) (Soares da Costa et al., 2018). Both compounds have also shown inhibitory activity in bacteria and fungi and can be used for selection of mutants with improved lysine accumulation (Sano, 1970; Rupp et al., 1989; Han et al., 1991; Zabriskie & Jackson 2000; Ekwealor & Obeta, 2006). Both inhibitors are also commercially available.

In general, the use of aspartate-derived amino acid inhibitors in mutant selection may facilitate the discovery of new mutants with improved production capacity of essential amino acids. However, all compounds in this group inhibit only single amino acid, and if the goal is to improve the overall SCP production capacity as well as the ability to synthesize multiple essential amino acids, then a better approach would be to use either aspartate or glutamine inhibitors.

CONCLUSIONS

Single cell protein is a good alternative to substitute plant-derived proteins in animal and fish feeds. SCP production technologies offer a number of environmental benefits over conventional protein sources, and these proteins can be produced from biodegradable agro-industrial by-products from other industries. SCP-producing microorganism strains are at the heart of all SCP technological solutions, so improving the properties and productivity of these strains is vital to increasing the competitiveness of SCP. In order to create microorganisms with better properties for the production of SCP, one of the possible approaches is the creation and selection of mutants. After treatment with mutagen, to select mutants with the highest protein and essential amino acid synthesis capacity, in this article we have reviewed various amino acid inhibitors that could theoretically be used in the selection of such mutants. Most amino acid inhibitors are designed for use as herbicides in weed control in agriculture, so information on the effects of these inhibitors on microorganisms is often limited. To the best of our knowledge, using amino acid inhibitors to select SCP-producing mutants with increased total protein content and improved essential amino acid profiles is a novel idea.

In total, 6 groups of amino acid inhibitors were reviewed in the article, of which glutamine inhibitors are the most promising, because it is possible to stop the synthesis of eight amino acids by using only one inhibitory compound. Many glutamine synthetase

inhibitors have been introduced into the market and their activity has been tested not only in plants, but in bacteria, fungi, yeasts and microscopic algae as well. Therefore, it should be possible to find the most suitable glutamine synthetase inhibitor for the selection of the mutant microorganisms. As all glutamine synthetase inhibitors are also causing toxic ammonia accumulation, during selection it should be taken into consideration that mutants with increased ammonia tolerance might be selected by mistake and these false positives should be removed later during further mutant strain testing.

Other promising inhibitors for SCP-rich mutant selection are glyphosate, as this popular herbicide inhibits the synthesis of three aromatic amino acids, two of which are essential amino acids in animals. In addition, the effects of glyphosate have been extensively studied in a variety of organisms, making it much easier to predict its effects on mutated microorganisms. As with glutamine inhibitors, glyphosate induced side effect should also be considered during the selection of mutant strains.

Another promising group of amino acid inhibitors are branched chain amino acid inhibitors, which target acetolactate synthase/acetohydroxyacid synthase (ALS/AHAS). These inhibitors cease synthesis of three essential amino acids. The effects of these inhibitors have been extensively studied on a wide variety of microorganisms, and ALS/AHAS inhibition can be achieved by using wide range of inhibitors from five chemically distinct groups. The side effects of these inhibitors should also be considered during mutant selection.

Inhibition of aspartate biosynthesis also results in arrest of several (five) amino acid syntheses. Unfortunately, currently only one compound has been found to inhibit aspartate biosynthesis, which has low activity in both bacteria and fungi, so its use in SCP-rich mutant selection may be severely limited.

In addition to the selection of individual inhibitors, it is possible to use combinations of multiple inhibitors in the selective medium to pick mutants with specifically improved amino acid profiles for use in specialized animal feeds or other higher value-added market segments. However, it should also be taken into consideration that the combination of inhibitors can be very time consuming until optimal concentrations are found for each applied inhibitor, and the combination of inhibitors may cause previously unobserved side effects or disproportionately amplify known effects, which would again complicate mutant selection.

In general, due to the lack of studies to date on the use of amino acid inhibitors in the selection of SCP-rich mutants, most of the hypotheses proposed here will need to be tested in a practical laboratory setting.

REFERENCES

Ahmad, I., Bissett, J. & Malloch, D. 1995. Effect of phosphinothricin on nitrogen metabolism of trichoderma species and its implications for their control of phytopathogenic fungi. *Pesticide Biochemistry and Physiology*. doi:10.1006/pest.1995.1054

Ahmad, I. & Malloch, D. 1995. Interaction of soil microflora with the bioherbicide phosphinothricin. *Agriculture, Ecosystems and Environment* **54**, 165–174. doi:10.1016/0167-8809(95)00603-P

Al-Marzooqi, W., Al-Farsi, M.A., Kadim, I.T., Mahgoub, O. & Goddard, J. 2010. The effect of feeding different levels of sardine fish silage on broiler performance, meat quality and sensory characteristics under closed and open-sided housing systems. *Asian-Australasian Journal of Animal Sciences*. doi:10.5713/ajas.2010.10119

Allievi, L. & Gigliotti, C. 2001. Response of the bacteria and fungi of two soils to the sulfonylurea herbicide cinosulfuron. *Journal of Environmental Science and Health - Part B Pesticides, Food Contaminants, and Agricultural Wastes* **36**, 161–175. doi:10.1081/PFC-100103741

Almassy, R.J., Janson, C.A., Hamlin, R., Xuong, N.H. & Eisenberg, D. 1986. Novel subunit - Subunit interactions in the structure of glutamine synthetase. *Nature*. doi:10.1038/323304a0

Amagasa, T., Paul, R.N., Heitholt, J.J. & Duke, S.O. 1994. Physiological Effects of Cornexistin on Lemna pausicostata. *Pesticide Biochemistry and Physiology*. doi:10.1006/pest.1994.1032

Andersen, S.M., Waagbø, R. & Espe, M. 2016. Functional amino acids in fish health and welfare. *Frontiers in Bioscience - Elite*. doi:10.2741/757

Anderson, P. 1995. Mutagenesis. *Methods in Cell Biology* **48**, 31–58. doi:10.1016/S0091-679X(08)61382-5

Atzmüller, D., Hawe, F., Sulzenbacher, D. & Cristobal-Sarramian, A. 2019. Wheat straw and lipids: UV-mutagenized yarrowia lipolytica for the conversion of wheat straw hydrolysate into lipids. *Agronomy Research* **17**, 2172–2179. doi:10.15159/AR.19.197

Baldensperger, J., Le Mer, J., Hannibal, L. & Quinto, P.J. 1985. Solid state fermentation of banana wastes. *Biotechnology Letters*. doi:10.1007/BF01032289

Berlicki, L. 2008. Inhibitors of Glutamine Synthetase and their Potential Application in Medicine. *Mini-Reviews in Medicinal Chemistry* **8**, 869–878. doi:10.2174/138955708785132800

Binder, S. 2010. Branched-Chain Amino Acid Metabolism in Arabidopsis thaliana. *The Arabidopsis Book*. doi:10.1199/tab.0137

Borowitzka, M.A. & Moheimani, N.R. 2013. Algae for biofuels and energy. *Algae for Biofuels and Energy*, 1–288. doi:10.1007/978-94-007-5479-9

Boussiba, S. & Gibson, J. 1991. Ammonia translocation in cyanobacteria. *FEMS Microbiology Letters* **88**, 1–14. doi:10.1016/0378-1097(91)90692-4

Brilisauer, K., Rapp, J., Rath, P., Schöllhorn, A., Bleul, L., Weiß, E., Stahl, M., Grond, S. & Forchhammer, K. 2019. Cyanobacterial antimetabolite 7-deoxy-sedoheptulose blocks the shikimate pathway to inhibit the growth of prototrophic organisms. *Nature Communications*. doi:10.1038/s41467-019-08476-8

Burnet, M. & Hodgson, B. 1991. Differential effects of the sulfonylurea herbicides chlorsulfuron and sulfometuron methyl on microorganisms. *Archives of Microbiology* **155**, 521–525. doi:10.1007/BF00245344

Cassidy, E.S., West, P.C., Gerber, J. S. & Foley, J.A. 2013. Redefining agricultural yields: From tonnes to people nourished per hectare. *Environmental Research Letters*. doi:10.1088/1748-9326/8/3/034015

Chadd, S.A., Davies, W.P. & Koivisto, J.M. 2002. Practical production of protein for food animals. In: *Protein Sources for the Animal Feed Industry. FAO Expert Consultation and Workshop, Bangkok, Thailand, 29 April-3 May 2002*.

Choi, B.H. & Coloff, J.L. 2019. The diverse functions of non-essential amino acids in cancer. *Cancers*. doi:10.3390/cancers11050675

Cobb, A.H. & Reade, J.P.H. 2010. *Herbicides and Plant Physiology: Second Edition*. *Herbicides and Plant Physiology: Second Edition*. doi:10.1002/9781444327793

Cooper, A.J.L., Krasnikov, B.F., Niatsetskaya, Z.V., Pinto, J.T., Callery, P.S., Villar, M.T., Artigues, A. & Bruschi, S.A. 2011. Cysteine S-conjugate β-lyases: Important roles in the metabolism of naturally occurring sulfur and selenium-containing compounds, xenobiotics and anticancer agents. *Amino Acids*. doi:10.1007/s00726-010-0552-0

Cruzat, V., Rogero, M.M., Keane, K.N., Curi, R. & Newsholme, P. 2018. Glutamine: Metabolism and immune function, supplementation and clinical translation. *Nutrients* **10**, 1–31. doi:10.3390/nu10111564

Dancer, J.E., Ford, M.J., Hamilton, K., Kilkelly, M., Lindell, S.D., O'Mahony, M.J. & Saville-Stones, E.A. 1996. Synthesis of potent inhibitors of histidinol dehydrogenase. *Bioorganic and Medicinal Chemistry Letters*. doi:10.1016/0960-894X(96)00384-8

De Block, M., Botterman, J., Vandewiele, M., Dockx, J., Thoen, C., Gosselé, V., Movva, N.R., Thompson, C., Van Montagu, M. & Leemans, J. 1987. Engineering herbicide resistance in plants by expression of a detoxifying enzyme. *The EMBO Journal*. doi:10.1002/j.1460-2075.1987.tb02537.x

De Gregorio, A., Mandalari, G., Arena, N., Nucita, F., Tripodo, M.M. & Lo Curto, R.B. 2002. SCP and crude pectinase production by slurry-state fermentation of lemon pulps. *Bioresource Technology* **83**, 89–94. doi:10.1016/S0960-8524(01)00209-7

Duan, L., Chen, Q. & Duan, S. 2019. Transcriptional analysis of chlorella pyrenoidosa exposed to bisphenol A. *International Journal of Environmental Research and Public Health* **16**. doi:10.3390/ijerph16081374

Duggleby, R.G., Pang, S.S., Yu, H. & Guddat, L.W. 2003. Systematic characterization of mutations in yeast acetohydroxyacid synthase: Interpretation of herbicide-resistance data. *European Journal of Biochemistry* **270**, 2895–2904. doi:10.1046/j.1432-1033.2003.03671.x

Ekwealor, I.A. & Obeta, J.A.N. 2006. Screening of UV-irradiated and S-2-aminoethyl-L-cysteine resistant mutants of Bacillus megaterium for improved lysine accumulation. *African Journal of Biotechnology* **5**, 2312–2314. doi:10.5897/AJB06.124

Finco, A.M. de O., Mamani, L.D.G., Carvalho, J.C. de, de Melo Pereira, G.V., Thomaz-Soccol, V. & Soccol, C.R. 2017. Technological trends and market perspectives for production of microbial oils rich in omega-3. *Critical Reviews in Biotechnology* **37**, 656–671. doi:10.1080/07388551.2016.1213221

Food and Agricultural Organization of United Nations (FAO). 2016. *FishStatJ: a tool for fishery statistics analysis, Release 3.0.0. Universal software for fishery statistical time series. Global capture and aquaculture production: Quantities 1950–2014; Aquaculture values 1984–2014.*

Food and Agricultural Organization of United Nations (FAO) 1980. *Fish Feed Technology. Chapter 12. Unconventional Feed Ingredients for Fish Feed by Spinelli J, National Marine Fisheries Services, Seattle, Washington*.

Food and Agricultural Organization of United Nations (FAO) 2014. *Report highlights growing role of fish in feeding the world*. Retrieved from http://www.fao.org/news/story/en/item/231522/icode/

Forde, B.G. & Lea, P.J. 2007. Glutamate in plants: Metabolism, regulation, and signalling. In: *Journal of Experimental Botany*. doi:10.1093/jxb/erm121

Forlani, G., Berlicki, Ł., Duò, M., Dzièdzioła, G., Giberti, S., Bertazzini, M. & Kafarski, P. 2013. Synthesis and evaluation of effective inhibitors of plant δ1-pyrroline-5-carboxylate reductase. *Journal of Agricultural and Food Chemistry* **61**, 6792–6798. doi:10.1021/jf401234s

Forlani, G., Petrollino, D., Fusetti, M., Romanini, L., Nocek, B., Joachimiak, A., Berlicki, Ł. & Kafarski, P. 2012. δ 1-Pyrroline-5-carboxylate reductase as a new target for therapeutics: Inhibition of the enzyme from Streptococcus pyogenes and effects in vivo. *Amino Acids*. doi:10.1007/s00726-011-0970-7

Fucile, G., Garcia, C., Carlsson, J., Sunnerhagen, M. & Christendat, D. 2011. Structural and biochemical investigation of two Arabidopsis shikimate kinases: The heat-inducible isoform is thermostable. *Protein Science*. doi:10.1002/pro.640

Funke, T., Han, H., Healy-Fried, M.L., Fischer, M. & Schönbrunn, E. 2006. Molecular basis for the herbicide resistance of Roundup Ready crops. *Proceedings of the National Academy of Sciences of the United States of America*. doi:10.1073/pnas.0603638103

Giberti, S., Bertazzini, M., Liboni, M., Berlicki, Ł., Kafarski, P. & Forlani, G. 2017. Phytotoxicity of aminobisphosphonates targeting both δ1-pyrroline-5-carboxylate reductase and glutamine synthetase. *Pest Management Science*. doi:10.1002/ps.4299

Giovanelli, J., Owens, L.D. & Mudd, S.H. 1971. Mechanism of inhibition of spinach β-cystathionase by rhizobitoxine. *BBA - Enzymology*. doi:10.1016/0005-2744(71)90016-7

Goyer, A., Collakova, E., Shachar-Hill, Y. & Hanson, A.D. 2007. Functional characterization of a methionine γ-lyase in arabidopsis and its implication in an alternative to the reverse trans-sulfuration pathway. *Plant and Cell Physiology*. doi:10.1093/pcp/pcl055

Hall, C.J., Mackie, E.R.R., Gendall, A.R., Perugini, M.A. & Soares da Costa, T.P. 2020. Review: amino acid biosynthesis as a target for herbicide development. *Pest Management Science* **76**, 3896–3904. doi:10.1002/ps.5943

Han, J.K., Oh, J.W., Lee, H.H., Chung, S., Hyun, H.H. & Lee, J.H. 1991. Molecular cloning and expression of s-(2-aminoethyl)-L-cysteine resistant aspartokinase gene of Corynebacteriumglutamicum. *Biotechnology Letters*. doi:10.1007/BF01088176

Hardy, R.W., Patro, B., Pujol-Baxley, C., Marx, C.J. & Feinberg, L. 2018. Partial replacement of soybean meal with Methylobacterium extorquens single-cell protein in feeds for rainbow trout (Oncorhynchus mykiss Walbaum). *Aquaculture Research*. doi:10.1111/are.13678

Herrmann, K.M. & Weaver, L.M. 1999. The shikimate pathway. *Annual Review of Plant Biology*. doi:10.1146/annurev.arplant.50.1.473

HRAC 2002. *Herbicide Resistance Action Committee. Classification of Herbicides According to Mode of Action. Part 7.* Retrieved from http://www.aun.edu.eg/distance/agriculture/HRGW/7. Classif her.htm

Ivarson, K.C. & Morita, H. 1982. Single-Cell Protein Production by the Acid-Tolerant Fungus Scytalidium acidophilum from Acid Hydrolysates of Waste Paper †. *Applied and Environmental Microbiology* **43**, 643–647. doi:10.1128/aem.43.3.643-647.1982

Jia, M.H., Larossa, R.A., Lee, J.M., Rafalski, A., Derose, E., Gonye, G. & Xue, Z. 2000. Global expression profiling of yeast treated with an inhibitor of amino acid biosynthesis, sulfometuron methyl. *Physiological Genomics* **2000**, 83–92. doi:10.1152/physiolgenomics.2000.3.2.83

Jin, J.K., Adams, D.O., Ko, Y., Yu, C.W. & Lin, C.H. 2004. Aviglycine and propargylglycine inhibit conidial germination and mycelial growth of Fusarium oxysporum f. sp. luffae. *Mycopathologia* **158**, 369–375. doi:10.1007/s11046-004-2225-6

Jin, Y., Zhao, H., Lu, H., Kuemmel, C.M., Zhang, J. & Wang, D. 2015. Synthesis and herbicidal activity of novel 1-(diethoxy-phosphoryl)-3-(4-one-1H-1,2,3-triazol-1-yl)-propan-2-yl carboxylic esters. *Molecules*. doi:10.3390/molecules20011088

Johnston, M., Jankowski, D., Marcotte, P., Walsh, C., Tanaka, H., Esaki, N. & Soda, K. 1979. Suicide Inactivation of Bacterial Cystathionine γ-Synthase and Methionine γ-Lyase during Processing of L-Propargylglycine†. *Biochemistry*. doi:10.1021/bi00588a033

Joshi, V., Laubengayer, K.M., Schauer, N., Fernie, A.R. & Jander, G. 2006. Two Arabidopsis threonine aldolases are nonredundant and compete with threonine deaminase for a common substrate pool. *Plant Cell*. doi:10.1105/tpc.106.044958

Katre, G., Ajmera, N., Zinjarde, S. & RaviKumar, A. 2017. Mutants of Yarrowia lipolytica NCIM 3589 grown on waste cooking oil as a biofactory for biodiesel production. *Microbial Cell Factories*. doi:10.1186/s12934-017-0790-x

Keller-Schierlein, W., Poralla, K. & Zähner, H. 1969. [Metabolic products of microorganisms. 78. Isolation, identification and mechanism of action of ketomycin ((R)-3-cyclohexeneglyoxylic acid) and of the conversion product 3-cyclohexeneglycine]. *Archiv Fur Mikrobiologie*.

Kim, J.H. & Lebeault, J.M. 1981. Protein production from whey using Penicillium cyclopium; growth parameters and cellular composition. *European Journal of Applied Microbiology and Biotechnology* **13**, 151–154. doi:10.1007/BF00703044

Kim, K.H. & Rhee, S.G. 1987. Subunit interaction elicited by partial inactivation with L-methionine sulfoximine and ATP differently affects the biosynthetic and gamma-glutamyltransferase reactions catalyzed by yeast glutamine synthetase. *The Journal of Biological Chemistry* **262**, 13050–13054. doi:10.1016/s0021-9258(18)45165-4

Kornochalert, N., Kantachote, D., Chaiprapat, S. & Techkarnjanaruk, S. 2014. Use of Rhodopseudomonas palustris P1 stimulated growth by fermented pineapple extract to treat latex rubber sheet wastewater to obtain single cell protein. *Annals of Microbiology* **64**, 1021–1032. doi:10.1007/s13213-013-0739-1

Kreisberg, J.F., Ong, N.T., Krishna, A., Joseph, T.L., Wang, J., Ong, C., Ooi, H.A., Sung, J.C., Siew, C.C., Chang, G.C., Biot, F., Cuccui, J., Wren, B.W., Chan, J., Sivalingam, S.P., Zhang, L.H., Verma, C. & Tan, P. 2013. Growth inhibition of pathogenic bacteria by sulfonylurea herbicides. *Antimicrobial Agents and Chemotherapy* **57**, 1513–1517. doi:10.1128/AAC.02327-12

Kulkarni, A., Buford, T.D., Rai, R. & Cooper, T.G. 2006. Differing responses of Gat1 and Gln3 phosphorylation and localization to rapamycin and methionine sulfoximine treatment in Saccharomyces cerevisiae. *FEMS Yeast Research* **6**, 218–229. doi:10.1111/j.1567-1364.2006.00031.x

Kunda, U.M.R., Balam, S.K., Nemallapudi, B.R., Chereddy, S.S., Nayak, S.K. & Cirandur, S.R. 2012. Facile synthesis, antioxidant and antimicrobial activity of amino methylene bisphosphonates. *Chemical and Pharmaceutical Bulletin* **60**, 104–109. doi:10.1248/cpb.60.104

Landstein, D., Arad, S. (Malis), Barak, Z. & Chipman, D.M. 1993. Relationships among the herbicide and functional sites of acetohydroxy acid synthase from Chlorella emersonii. *Planta* **191**, 1–6. doi:10.1007/BF00240889

Landstein, D., Epelbaum, S., Arad, S. (Malis), Barak, Z. & Chipman, D. 1995. Metabolic response of Chlorella emersonii to the herbicide sulfometuron methyl. *Planta* **197**, 219–224. doi:10.1007/BF00202640

Leavell, M.D., Singh, A.H. & Kaufmann-Malaga, B.B. 2020. High-throughput screening for improved microbial cell factories, perspective and promise. *Current Opinion in Biotechnology* **62**, 22–28. doi:10.1016/j.copbio.2019.07.002

Lee, Y.T., Cui, C., Chow, E.W. L., Pue, N., Lonhienne, T., Wang, J.G., Fraser, J.A. & Guddat, L.W. 2013. Sulfonylureas have antifungal activity and are potent inhibitors of Candida albicans acetohydroxyacid synthase. *Journal of Medicinal Chemistry* **56**, 210–219. doi:10.1021/jm301501k

Leino, L., Tall, T., Helander, M., Saloniemi, I., Saikkonen, K., Ruuskanen, S. & Puigbò, P. 2020. Classification of the glyphosate target enzyme (5-enolpyruvylshikimate-3-phosphate synthase) for assessing sensitivity of organisms to the herbicide. *Journal of Hazardous Materials*. doi:10.1016/j.jhazmat.2020.124556

Llewellyn, R., Ronning, D., Ouzman, J., Walker, S., Mayfield, A. & Clarke, M. 2016. *Impact of Weeds on Australian Grain Production: the cost of weeds to Australian grain growers and the adoption of weed management and tillage practices Report for GRDC*. *Grains Research and Development Corporation and the Commonwealth Scientific and Industrial Research Organisation*.

Lockwood, B.C. & Coombs, G.H. 1991. Purification and characterization of methionine y-lyase from. *Biochem.J.* **279**, 675–682.

Long, S., Liu, S., Wu, D., Mahfuz, S. & Piao, X. 2020. Effects of dietary fatty acids from different sources on growth performance, meat quality, muscle fatty acid deposition, and antioxidant capacity in broilers. *Animals*. doi:10.3390/ani10030508

Martin, M.A. 2001. The future of the world food system. *Outlook on Agriculture*. doi:10.5367/000000001101293409

Massey, L.K., Sokatch, J.R. & Conrad, R.S. 1976. Branched chain amino acid catabolism in bacteria. *Bacteriological Reviews* **40**, 42–54. doi:10.1128/mmbr.40.1.42-54.1976

Maughan, S.C. & Cobbett, C.S. 2003. Methionine sulfoximine, an alternative selection for the bar marker in plants. *Journal of Biotechnology* **102**, 125–128. doi:10.1016/S0168-1656(03)00028-2

Mehta, R.J. 1973. Studies on methanol-oxidizing bacteria - I. Isolation and growth studies. *Antonie van Leeuwenhoek* **39**, 295–302. doi:10.1007/BF02578861

Mekonnen, M.M. & Hoekstra, A. 2010. The green, blue and grey water footprint of farm animals and animal products. Volume 1: Main Report. *Unesco Value of Water Research Report Series No. 48*.

Mitchell, R.E. & Bieleski, R.L. 1977. Involvement of phaseolotoxin in halo blight of beans: transport and conversion to functional toxin. *Plant Physiology*. doi:10.1104/pp.60.5.723

Morjan, W.E., Pedigo, L.P. & Lewis, L.C. 2002. Fungicidal effects of glyphosate and glyphosate formulations on four species of entomopathogenic fungi. *Environmental Entomology*. doi:10.1603/0046-225X-31.6.1206

Myrold, D.D. & Posavatz, N.R. 2007. Potential importance of bacteria and fungi in nitrate assimilation in soil. *Soil Biology and Biochemistry* **39**, 1737–1743. doi:10.1016/j.soilbio.2007.01.033

Nakajima, M., Itoi, K., Takamatsu, Y., Sato, S., Furukawa, Y., Furuya, K., Honma, T., Kadotani, J., Kozasa, M. & Haneishi, T. 1991. Cornexistin: A New Fungal Metabolite with Herbicidal Activity. *Agricultural and Biological Chemistry*. doi:10.1271/bbb1961.55.925

Nonomura, T., Toyoda, H., Tanpo, H., Matsuda, Y., Matsuda, K., Gafur, A., Dogo, M. & Ouchi, S. 1996. Spherical Swelling of Germ Tubes and Hyphae of Fusarium oxysporum by the Treatment with 5-fluoroindole. *Japanese Journal of Phytopathology*. doi:10.3186/jjphytopath.62.414

Okazaki, S., Sugawara, M., Yuhashi, K.I. & Minamisawa, K. 2007. Rhizobitoxine-induced chlorosis occurs in coincidence with methionine deficiency in soybeans. *Annals of Botany*. doi:10.1093/aob/mcm087

Omura, S. 1976. The antibiotic cerulenin, a novel tool for biochemistry as an inhibitor of fatty acid synthesis. *Bacteriological Reviews*. doi:10.1128/mmbr.40.3.681-697.1976

Orcaray, L., Zulet, A., Zabalza, A. & Royuela, M. 2012. Impairment of carbon metabolism induced by the herbicide glyphosate. *Journal of Plant Physiology*. doi:10.1016/j.jplph.2011.08.009

P&S Intelligence 2018. *Protein Extracts from Single Cell Protein Sources Market by Source, by Application, by Geography – Global Market Size, Share, Development, Growth and Demand Forecast, 2013–2023.* Retrieved from https://www.researchandmarkets.com/reports/4655419/protein-extracts-from-single-cell-protein-sources

Patrick, G.J., Fang, L., Schaefer, J., Singh, S., Bowman, G.R. & Wencewicz, T.A. 2018. Mechanistic Basis for ATP-Dependent Inhibition of Glutamine Synthetase by Tabtoxinine-β-lactam. *Biochemistry*. doi:10.1021/acs.biochem.7b00838

Pearce, F.G., Hudson, A.O., Loomes, K. & Dobson, R.C.J. 2017. Dihydrodipicolinate synthase: Structure, dynamics, function, and evolution. *Sub-Cellular Biochemistry*. doi:10.1007/978-3-319-46503-6_10

Piotrowska, M. & Paszewski, A. 1986. Propargylglycine as a fungal inhibitor: Effect on sulphur amino acid metabolism. *Journal of General Microbiology*. doi:10.1099/00221287-132-10-2753

Poore, J. & Nemecek, T. 2018. Reducing food's environmental impacts through producers and consumers. *Science* **360**, 987–992. doi:10.1126/science.aaq0216

Ravanel, S., Gakière, B., Job, D. & Douce, R. 1998a. The specific features of methionine biosynthesis and metabolism in plants. *Proceedings of the National Academy of Sciences of the United States of America*. doi:10.1073/pnas.95.13.7805

Ravanel, S., Gakière, B., Job, D. & Douce, R. 1998b. Cystathionine γ-synthase from Arabidopsis thaliana: Purification and biochemical characterization of the recombinant enzyme overexpressed in Escherichia coli. *Biochemical Journal*. doi:10.1042/bj3310639

Rawson, S., Bisson, C., Hurdiss, D.L., Fazal, A., McPhillie, M.J., Sedelnikova, S.E., Baker, P.J., Rice, D.W. & Muench, S.P. 2018. Elucidating the structural basis for differing enzyme inhibitor potency by cryo-EM. *Proceedings of the National Academy of Sciences of the United States of America*. doi:10.1073/pnas.1708839115

Ritala, A., Häkkinen, S.T., Toivari, M. & Wiebe, M.G. 2017. Single cell protein-state-of-the-art, industrial landscape and patents 2001-2016. *Frontiers in Microbiology* **8**. doi:10.3389/fmicb.2017.02009

Rowlands, R.T. 1984. Industrial strain improvement: Mutagenesis and random screening procedures. *Enzyme and Microbial Technology* **6**, 3–10. doi:10.1016/0141-0229(84)90070-X

Rupp, L.A., DeVries II, H.E. & Mudge, K.W. 1989. Effect of aminocyclopropane carboxylic acid and aminoethoxyvinylglycine on ethylene production by ectomycorrhizal fungi. *Canadian Journal of Botany* **67**, 483–485. doi:10.1139/b89-068

Sachpatzidis, A., Dealwis, C., Lubetsky, J.B., Liang, P.H., Anderson, K.S. & Lolis, E. 1999. Crystallographic studies of phosphonate-based α-reaction transition-state analogues complexed to tryptophan synthase. *Biochemistry*. doi:10.1021/bi9907734

Sano, K. 1970. Microbial production of l-lysine III. Production by mutants resistant to s-(2-aminoethyl)-l-cysteine. *The Journal of General and Applied Microbiology*. doi:10.2323/jgam.16.5_373

Schultz, C.J. & Coruzzi, G.M. 1995. The aspartate aminotransferase gene family of Arabidopsis encodes isoenzymes localized to three distinct subcellular compartments. *The Plant Journal*. doi:10.1046/j.1365-313X.1995.07010061.x

Schweitzer, B.A., Loida, P.J., CaJacob, C.A., Chott, R.C., Collantes, E.M., Hegde, S.G., Mosier, P.D. & Profeta, S. 2002. Discovery of imidazole glycerol phosphate dehydratase inhibitors through 3-D database searching. *Bioorganic and Medicinal Chemistry Letters*. doi:10.1016/S0960-894X(02)00283-4

Shaik, M.S., Nadiveedhi, M.R., Gundluru, M., Katike, U., Obulam, V.S.R. & Cirandur, S.R. 2020. Efficient catalyst free green synthesis and in vitro antimicrobial, antioxidant and molecular docking studies of α-substituted aromatic/heteroaromatic aminomethylene bisphosphonates. *Synthetic Communications* **0**, 1–18. doi:10.1080/00397911.2020.1853778

Shearer, A. 2007. *MetaCyc Pathway: L-histidine biosynthesis*. Retrieved from https://biocyc.org/META/new-image?object=HISTSYN-PWY

Shepon, A., Eshel, G., Noor, E. & Milo, R. 2016. Energy and protein feed-to-food conversion efficiencies in the US and potential food security gains from dietary changes. *Environmental Research Letters*. doi:10.1088/1748-9326/11/10/105002

Singh, B.K. & Shaner, D.L. 1995. Biosynthesis of branched chain amino acids: From test tube to field. *Plant Cell*. **7**, 935–944. doi:10.1105/tpc.7.7.935

Singh, R.S., Singh, T. & Pandey, A. 2019. Microbial enzymes-an overview. In: *Biomass, Biofuels, Biochemicals: Advances in Enzyme Technology*. doi:10.1016/B978-0-444-64114-4.00001-7

Sivaramakrishnan, R. & Incharoensakdi, A. 2017. Enhancement of lipid production in Scenedesmus sp. by UV mutagenesis and hydrogen peroxide treatment. *Bioresource Technology* **235**, 366–370. doi:10.1016/j.biortech.2017.03.102

Soares da Costa, T.P., Abbott, B.M., Gendall, A.R., Panjikar, S. & Perugini, M.A. 2018. Molecular evolution of an oligomeric biocatalyst functioning in lysine biosynthesis. *Biophysical Reviews*. doi:10.1007/s12551-017-0350-y

Spalvins, K. & Blumberga, D. 2019. Single cell oil production from waste biomass: review of applicable agricultural by-products. *Agronomy Research* **17**(3), 833–849. doi:10.15159/AR.19.039

Spalvins, K, Ivanovs, K. & Blumberga, D. 2017. Single cell protein production from waste biomass : comparison of various agricultural by-products. *Energy Procedia* **16**, 1493–1508. doi:10.1016/j.egypro.2018.07.111

Spalvins, K. & Blumberga, D. 2018. Production of Fish Feed and Fish Oil from Waste Biomass Using Microorganisms: Overview of Methods Analyzing Resource Availability. *Environmental and Climate Technologies* **22**, 149–164. doi:10.2478/rtuect-2018-0010

Spalvins, K., Geiba, Z., Kusnere, Z. & Blumberga, D. 2020. Waste Cooking Oil as Substrate for Single Cell Protein Production by Yeast Yarrowia lipolytica. *Environmental and Climate Technologies*. doi:10.2478/rtuect-2020-0116

Spalvins, K., Vamza, I. & Blumberga, D. 2019. Single Cell Oil Production from Waste Biomass: Review of Applicable Industrial By-Products. *Environmental and Climate Technologies*. doi:10.2478/rtuect-2019-0071

Spalvins, K., Zihare, L. & Blumberga, D. 2018. Single cell protein production from waste biomass: Comparison of various industrial by-products. *Energy Procedia* **147**, 409–418. doi:10.1016/j.egypro.2018.07.111

Staskawicz, B.J. 1979. A Rapid and Sensitive Microbiological Assay for Phaseolotoxin. *Phytopathology*. doi:10.1094/phyto-69-663

Steinborn, C., Wildermuth, R.E., Barber, D.M. & Magauer, T. 2020. Total Synthesis of (+)-Cornexistin. *Angewandte Chemie - International Edition* **59**, 17282–17285. doi:10.1002/anie.202008158

Steinrücken, H.C. & Amrhein, N. 1980. The herbicide glyphosate is a potent inhibitor of 5-enolpyruvylshikimic acid-3-phosphate synthase. *Biochemical and Biophysical Research Communications*. doi:10.1016/0006-291X(80)90547-1

Stepansky, A. & Leustek, T. 2006. Histidine biosynthesis in plants. *Amino Acids*. doi:10.1007/s00726-005-0247-0

Szamosi, I.T., Shaner, D.L. & Singh, B.K. 1994. Inhibition of threonine dehydratase is herbicidal. *Plant Physiology* **106**, 1257–1260. doi:10.1104/pp.106.4.1257

Tacon, A.G.J. & Metian, M. 2015. Feed matters: Satisfying the feed demand of aquaculture. *Reviews in Fisheries Science and Aquaculture*. doi:10.1080/23308249.2014.987209

Takahashi, S., Nakajima, M., Kinoshita, T., Haruyama, H., Sugai, S., Honma, T., Sato, S. & Haneishi, T. 1994. Hydantocidin and cornexistin - New herbicidal antibiotics. *Natural and Engineered Pest Management Agents* **551**, 74–84.

Tapia, V,E., Anschau, A., Coradini, A.L., Franco, T. & Deckmann, A. 2012. Optimization of lipid production by the oleaginous yeast Lipomyces starkeyi by random mutagenesis coupled to cerulenin screening. *AMB Express*. doi:10.1186/2191-0855-2-64

Tian, Y.S., Xiong, A.S., Xu, J., Zhao, W., Gao, F., Fu, X.Y., Xu, H., Zheng, J.L., Peng, R.H. & Yao, Q.H. 2010. Isolation from Ochrobactrum anthropi of a novel class II 5-enopyruvylshikimate-3-phosphate synthase with high tolerance to glyphosate. *Applied and Environmental Microbiology* **76**, 6001–6005. doi:10.1128/AEM.00770-10

Tohge, T., Watanabe, M., Hoefgen, R. & Fernie, A.R. 2013. Shikimate and phenylalanine biosynthesis in the green lineage. *Frontiers in Plant Science* **4**, 1–14. doi:10.3389/fpls.2013.00062

Tzin, V. & Galili, G. 2010. The Biosynthetic Pathways for Shikimate and Aromatic Amino Acids in Arabidopsis thaliana. *The Arabidopsis Book* **8**. doi:10.1199/tab.0132

Unno, H., Uchida, T., Sugawara, H., Kurisu, G., Sugiyama, T., Yamaya, T., Sakakibara, H., Hase, T. & Kusunoki, M. 2006. Atomic structure of plant glutamine synthetase: A key enzyme for plant productivity. *Journal of Biological Chemistry*. doi:10.1074/jbc.M601497200

van Tatenhove-Pel, R.J., Hernandez-Valdes, J.A., Teusink, B., Kuipers, O.P., Fischlechner, M. & Bachmann, H. 2020. Microdroplet screening and selection for improved microbial production of extracellular compounds. *Current Opinion in Biotechnology* **61**, 72–81. doi:10.1016/j.copbio.2019.10.007

Wang, G., Xu, M., Wang, W. & Galili, G. 2017. Fortifying horticultural crops with essential amino acids: A review. *International Journal of Molecular Sciences*. doi:10.3390/ijms18061306

Wang, M.H., Peng, P., Liu, Y.M., Jia, R.B. & Li, L. 2013. Algicidal activity of a dibenzofuran-degrader Rhodococcus sp. *Journal of Microbiology and Biotechnology* **23**, 260–266. doi:10.4014/jmb.1208.08018

Widholm, J.M. 1981. Utilization of Indole Analogs by Carrot and Tobacco Cell Tryptophan Synthase in Vivo and in Vitro. *Plant Physiology*. doi:10.1104/pp.67.6.1101

Wikoff, W.R., Anfora, A.T., Liu, J., Schultz, P.G., Lesley, S.A., Peters, E.C. & Siuzdak, G. 2009. Metabolomics analysis reveals large effects of gut microflora on mammalian blood metabolites. *Proceedings of the National Academy of Sciences of the United States of America*. doi:10.1073/pnas.0812874106

Winston, F. 2008. EMS and UV mutagenesis in yeast. *Current Protocols in Molecular Biology*, 1–5. doi:10.1002/0471142727.mb1303bs82

Wong, P.K. & Chan, K. yu 1980. Algal single cell protein production from sewage effluent with high salinity. *Experientia*. doi:10.1007/BF01965971

Yadav, J.S.S., Yan, S., Ajila, C.M., Bezawada, J., Tyagi, R.D. & Surampalli, R.Y. 2016. Food-grade single-cell protein production, characterization and ultrafiltration recovery of residual fermented whey proteins from whey. *Food and Bioproducts Processing* **99**, 156–165. doi:10.1016/j.fbp.2016.04.012

Yamada, R., Kashihara, T. & Ogino, H. 2017. Improvement of lipid production by the oleaginous yeast Rhodosporidium toruloides through UV mutagenesis. *World Journal of Microbiology and Biotechnology* **33**(5), 99. doi:10.1007/s11274-017-2269-7

Yan, J., Gui, X., Wang, G., Xu, L., Yan, Y., Pan, D., Zha, G., Jiao, L., Han, B., Madzak, C. & Wang, Y. 2018. Engineering Yarrowia lipolytica to Simultaneously Produce Lipase and Single Cell Protein from Agro-industrial Wastes for Feed. *Scientific Reports*, **8**.

Yoshioka, N., Akamatsu, S., Mitsuhashi, T., Todo, C., Asano, M. & Ueno, Y. 2014. A simple method for the simultaneous determination of mushroom toxins by liquid chromatography-time-of-flight mass spectrometry. *Forensic Toxicology* **32**, 89–96. doi:10.1007/s11419-013-0214-5

Zabriskie, T.M. & Jackson, M.D. 2000. Lysine biosynthesis and metabolism in fungi. *Natural Product Reports* **17**, 85–97. doi:10.1039/a801345d

Zhang, L.S. & Davies, S.S. 2016. Microbial metabolism of dietary components to bioactive metabolites: Opportunities for new therapeutic interventions. *Genome Medicine*. doi:10.1186/s13073-016-0296-x

Zhou, Q., Liu, W., Zhang, Y. & Liu, K.K. 2007. Action mechanisms of acetolactate synthase-inhibiting herbicides. *Pesticide Biochemistry and Physiology*. doi:10.1016/j.pestbp.2007.04.004

Zhu, X., Kong, J., Yang, H., Huang, R., Huang, Y., Yang, D., Shen, B. & Duan, Y. 2018. Strain improvement by combined UV mutagenesis and ribosome engineering and subsequent fermentation optimization for enhanced 6′-deoxy-bleomycin Z production. *Applied Microbiology and Biotechnology* **102**, 1651–1661. doi:10.1007/s00253-017-8705-7

Zúñiga, M., Pérez, G. & González-Candelas, F. 2002. Evolution of arginine deiminase (ADI) pathway genes. *Molecular Phylogenetics and Evolution*. doi:10.1016/S1055-7903(02)00277-4

Agronomy Research **19**(S3), 1308–1317, 2021
https://doi.org/10.15159/AR.21.053

Evaluation of ozone influence on wheat grain quality during active drying

E. Straumite[1,*], A. Rucins[2], D. Viesturs[2], J. Kleperis[3] and A. Kristins[3]

[1]Latvia University of Life Sciences and Technologies, Faculty of Food Technology, Department of Food Technology, Rigas iela 22, LV-3001 Jelgava, Latvia
[2]Latvia University of Life Sciences and Technologies, Ulbroka Research Center, Instituta iela 1, LV-2130 Ulbroka, Latvia
[3]University of Latvia, Institute of Solid State Physics, Raina blv. 19-125, LV-1586 Riga, Latvia
[*]Correspondence: evita.straumite@llu.lv

Received: February 1st, 2021; Accepted: April 24th, 2021; Published: April 29th, 2021

Abstract. The aim of this study was to evaluate the effect of ozone on the quality of wheat grain during air ventilation drying process. After harvesting, the wheat grain was placed in two storage tanks. In one storage tank for grain drying was used air but in the other - ozone as the drying agent. The following quality parameters - moisture, water activity, gluten, starch and protein content, as well as the total plate count of microorganisms were determined during storage. Wheat grain quality parameters were analysed by taking samples from the top and bottom of the storage tanks. Two-year experiments showed that ozone treatment did not significantly affect ($P > 0.05$) the moisture content, water activity, gluten, starch and protein content of the analysed wheat grain, but all parameters were significantly affected ($P < 0.05$) by the sampling location - top or bottom of the storage tank. All samples taken from the top of the tanks throughout the drying process had higher water activity ($a_w < 0.800$). It should be noted that in both series of experiments it was found that there is a very large difference (up to 10%) in grain moisture between grain sampled at the top and bottom of the tanks. Favourable conditions for the development of microorganisms are increased moisture and free water available in the products and raw materials. According to the results obtained in the experiments, it can be concluded that the total plate count in the analysed wheat grain did not exceed the permissible norms (10^5 CFU g^{-1}).

Key words: grain safety, ozone treatment, wheat quality.

INTRODUCTION

According to statistics (Worldwide production of grain 2019/2020), in 2019/2020, wheat (*Triticum aestivum* L.) was grown 764.49 million tons, which is the second crop grown after corn (1,116.34 million tons). Of the total amount of wheat grown in 2019/2020, 80% was used for food. Wheat is mainly used in the production of bread, flour confectionery, pasta, as well as in daily meals (sauces, pancakes, etc.). Therefore, it is important to ensure grain and flour quality during storage and processing, because their quality is affected by many factors.

Ozone is widely used to ensure the quality of drinking water and the processing of food raw materials and products (fruit, vegetables, and cereals). The ozone treatment is considered as an alternative method to reduce the total number of microorganisms, pests and mycotoxins in food or raw materials, as well as to reduce the risk of mould, thus extending the shelf life of products (Tiwari et al., 2010; Jian et al., 2013; Rakcejeva et al., 2014; Zhu, 2018; Hutla et al., 2020). Additional studies showed that ozone treatment can reduce the content of mycotoxins in products and raw materials and it have harmful effects by toxigenic fungi (Savi et al., 2020; Nickhil et al., 2021). The efficiency of ozone treatment depends on raw material or food product used, the level of contamination, the time and duration of ozone treatment (Wang et al., 2016; Granella et al., 2018). Many studies have been done under laboratory conditions and with a small amount of grain or flour (Rakcejeva et al., 2014; Mei et al., 2016; Granella et al., 2018; Hu et al., 2020). In order to enable the assessment of the potential use of ozone for grain treatment by farmers, it is necessary to evaluate ozone treatment performance in real farm conditions. Such research has been started by Latvian scientists (Kleperis et al., 2019; Rucins et al., 2020), and research is still ongoing. There are relatively few studies on how ozone treatment affects grain quality and if ozone can be used as the agent to enhance active drying of grain.

The aim of this study was to evaluate the effect of ozone on the quality of wheat grain during air ventilation drying process.

MATERIALS AND METHODS

The experiments were performed in 2019 and 2020 on the farm Mazkalniņi, Tervete district, Latvia.

Experimental design

Wheat grains were harvested in July 2019 and in August 2020. The wheat grain storage in 2019 was performed according to the procedure described by Rucins et al. (2020) and the location of ozone sensors in the grain storage tanks was designed as described Kleperis et al. (2019). Storage tanks were equipped with fans of equal 7,000 $m^3 h^{-1}$ capacity - one blowing only air from below, the other - air / ozone mixture (200 $g h^{-1}$). The ozone generator OPV-100.03 (ООО НПО 'Жемчужина Руси', RU) was connected to the fan (Fig. 1). The air flow above the grain layer was 0.05–0.06 $m s^{-1}$. From the experience of 2019, it was found that the fan productivity for a 5.5 m high wheat grain layer was insufficient and in 2020 the experiments were performed with a 3.0–3.5 m high wheat grain layer, which required about 10.8 t of wheat grain to fill each tank.

Drying with air active ventilation without and with ozone was performed for 185 h (2019) and 136 h (2020), approximately 7 and 6 h per day, respectively.

The ozone concentration at inlet was 15–18 ppm, while ozone concentration at the 5 m level was around 0.1 ppm in 2019, indicating that the majority of supplied ozone was absorbed at the lower levels. In the 2020 experiment, the inlet concentration remained at 15–18 ppm, but due to the reduced height of the grain layer, its concentration at the top was around 2.6–4.5 ppm.

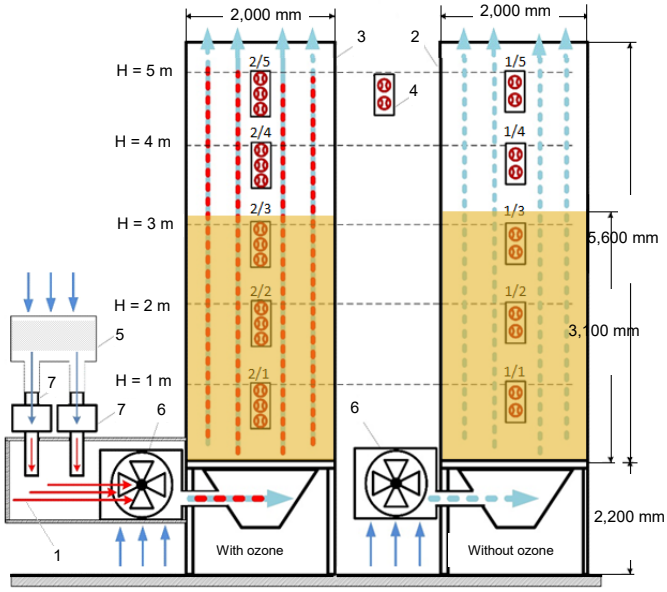

Figure 1. Experimental design of wheat grain storage tanks for year 2020.

The tank filling: to the height (coloured) to 3.2 m; 1/1–1/5; 2/1–2/5 – air humidity, temperature and ozone sensors; 1 – ozone; 2 – tank without ozone; 3 – tank with ozone; 4 – outside sensors; 5 – air dust filter; 6 – fans; 7 – the ozone generators.

Moisture content of wheat grain

Moisture content of wheat grain was determined according to AACC Method 44-15.02 Moisture - Air-Oven Methods, in the ULM 500 oven (Memmert, Germany).

Water activity of wheat grain

Water activity was determined with LabSwift-aw device (Novasina, Switzerland).

Chemical composition of wheat grain

Gluten, starch and protein content was determined using Grain Analyzer Infratec™ 1241 (FOSS, Denmark). For the analysis approximately 700 g of whole wheat grain were used.

Microbiological analysis of wheat grain

Determination of the microorganisms total plate count (TPC) was completed according to the standard ISO 4833-1:2003 Microbiology of the food chain - Horizontal method for the enumeration of microorganisms - Part 1: Colony count at 30 °C by the pour plate technique.

For the analysis (moisture content, water activity and microbiological analysis) were used approximately 100 g of ground wheat grain.

All parameters were analysed for wheat grain sampled from each storage tank (without ozone and with ozone) from the top and the bottom.

Statistical analysis

All analysis was done triplicate. In the experiments required data are given as average with standard deviation. Data were analysed Microsoft Office Excel 2016. ANOVA (one-way analysis of variance) and Tukey's test at a confidence level of 95% was used to estimate differences between means. Comparison of analysed parameters were calculated by *t-test* and significance between data was defined at $P \leq 0.05$.

RESULTS AND DISCUSSION

Grain quality after harvesting and storage may be affected by many factors (temperature, drying type, air humidity, storage conditions, raw material quality) (Granella et al., 2018). During storage moisture content, water activity, chemical composition and microbiological parameters were determined for wheat grain.

Moisture content

In Fig. 2 the experimental data on changes of the wheat grain moisture during active drying with and without ozone were collected.

In accordance with the Regulations of the Cabinet of Ministers of the Republic of Latvia No. 461 (12.08.2014.) for food quality schemes, procedures for their implementation, operation, monitoring and control, wheat must not have a moisture content higher than 14%, but the protein content not less than 12.5%. The moisture content of wheat grains, which does not exceed 14%, is optimal so that the grains can be stored for a long time and retain their quality.

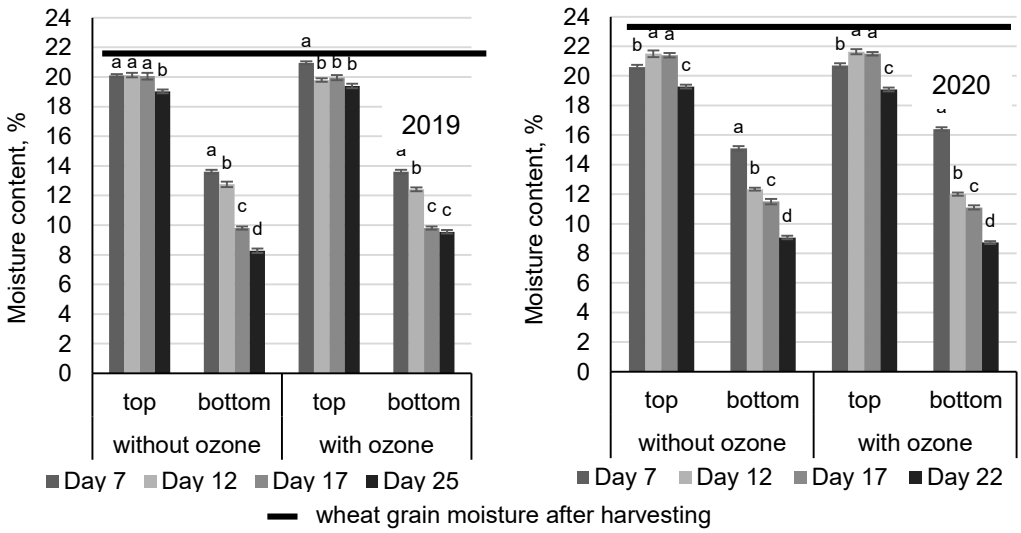

Figure 2. Changes of wheat grain moisture content during air and ozone active drying.
Different letters within sample groups indicate significant differences between samples ($P < 0.05$).

In both years of the experiment, the moisture content of wheat grain after harvesting was high - 22.97 ± 0.35% (2019) and 21.47 ± 0.22% (2020) (Fig. 2.). After seven days of active drying with and without ozone, the moisture content of the grain at the top of the storage tank was reduced by 1.87–2.77% ($P > 0.05$), but at the bottom of the storage

tank - by 7.87–8.74% ($P < 0.05$). Active drying with air and ozone shows that during the first week of storage, more moisture is released from grain at the bottom of the storage tank. In the experiment performed in 2020, after seven days of active drying, the moisture of wheat grain, which was at the bottom of the storage tanks, was already below 14.0% and they are safe for long-term storage. In turn, in 2019, the moisture content of wheat grain at the bottom of the storage tanks, which is lower than 14.0%, was reached after 12 days of active drying. It must be acknowledged that for wheat grain taken from the bottom of the tanks for analysis, the moisture content at the end of drying was relatively low: 8.28 ± 0.16–9.07 ± 0.06% (without ozone, 2020 and 2019, respectively) and 8.73 ± 0.14–9.55 ± 0.25% (with ozone, 2019 and 2020, respectively), which could affect the baking properties of cereals. All analysed samples taken from the top of the storage tanks, regardless of the type of active drying - with or without ozone - were high (above 19.0%) even on the last day of the experiment. It should be noted that in both years of the experiments it was found that on the last day of the experiment there was a very large difference (up to 10.74%) in moisture of wheat grain for those sampled from the top and bottom of the storage tanks. Therefore, regardless of the storage tank filling (5.0 or 3.5 m), it is necessary to stir the grain during active drying to even out the moisture content of the wheat grain. From the obtained results it can be concluded that active drying with ozone didn't significantly affect ($P > 0.05$) the moisture content of wheat grain. However, moisture content of wheat was affected ($P < 0.05$) by the sampling location with a quicker reduction of moisture at the bottom of the storage tank.

Water activity

During the active drying of wheat grain with and without ozone, its water activity, which characterizes the amount of free water in the products, was analysed. In the Fig. 3. the changes in wheat grain water activity during storage using active drying with and without ozone are summarized.

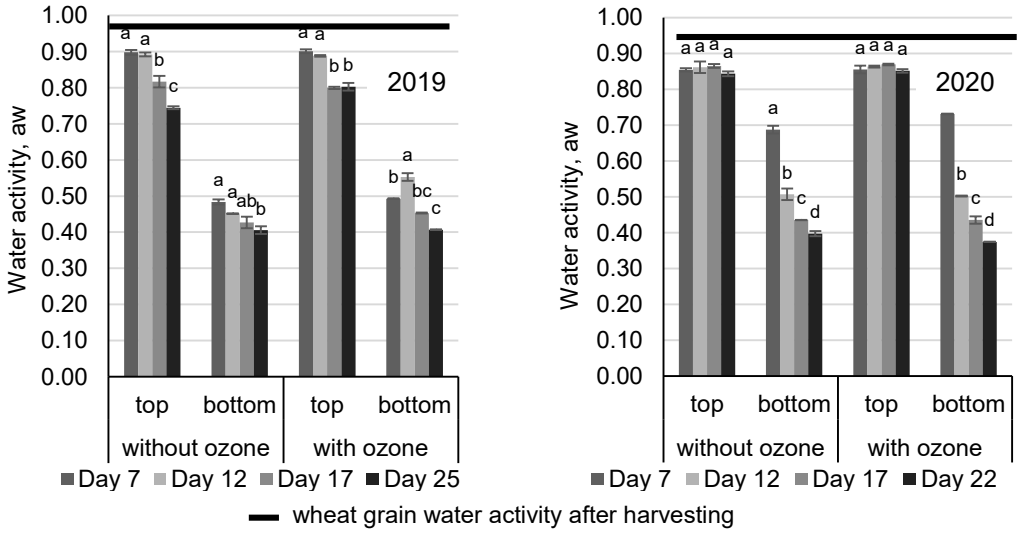

Figure 3. Changes of wheat grain water activity during active air and ozone drying.
Different letters within sample groups indicate significant differences between samples ($P < 0.05$).

The water activity of wheat grain after harvesting was very high - above 0.900, which indicates a large amount of free water available to microorganisms that can promote grain moulding process. For wheat grain harvested in 2020, the water activity was high ($a_w > 0.800$) during the whole period of active drying (22 days). Similar results were obtained in the 2019 experiments, when only after 25 days of active drying; the water activity of the samples taken from the top of the storage tank was below 0.800, which is high value. The wheat grain of 2019, after seven days of active drying, reached an $a_w < 0.500$, which is sufficient not to promote the development of microorganisms. In turn, in 2020 $a_w < 0.500$ was reached after 12 days of active drying. Analysing the results obtained in the study 2020, it can be concluded that active ozone drying didn't significantly affect ($P > 0.05$) the water activity of wheat grain, but it was significantly affected ($P < 0.05$) by the sampling location - the top or bottom of the storage tank.

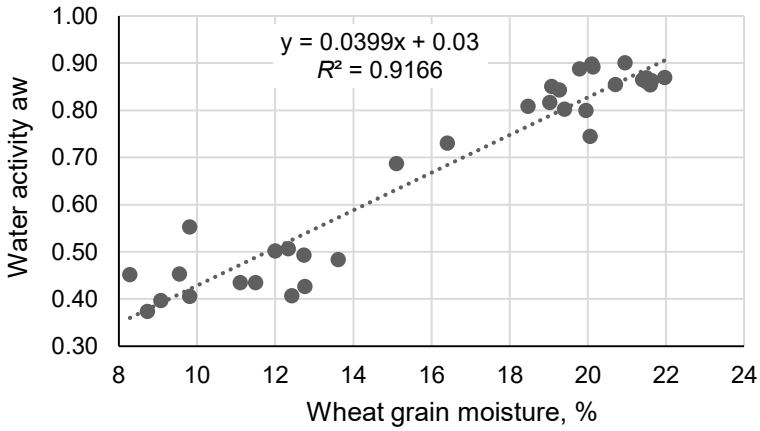

Figure 4. Correlation between wheat grain moisture and water activity during active air and ozone drying.

The results of experiments demonstrated strong linear correlation ($r = 0.957$) between wheat grain moisture and water activity (Fig. 4.) - the lower the wheat grain moisture content, the lower grain water activity.

Chemical composition

In the Table 1 wheat grain protein, gluten and starch changes during active drying are summarized.

The protein content of wheat grain ranged from 7.00 to 20.00 g 100 g^{-1}, but the optimal protein content for good baking properties is 12.00 to 14.00 g 100 g^{-1}. The analysed wheat grain of 2019 had a protein content of 15.89 ± 0.72–16.41 ± 0.17 g 100 g^{-1} (Table 1), which is relatively high. In turn, the analysed wheat grain in 2020 had 14.87 ± 0.31–13.28 ± 0.41 g 100 g^{-1}, which is optimal for obtaining good quality bread. In this research we didn't find that the protein content of wheat grain was significantly affected ($P > 0.05$) by active drying with and without ozone and the sampling place (top or bottom), but the protein content was significantly affected ($P < 0.05$) by the year.

Table 1. Wheat grain chemical composition changes during active air and ozone drying

	Protein content, g 100 g^{-1}				Gluten content, g 100 g^{-1}				Starch content, g 100 g^{-1}			
	without ozone		with ozone		without ozone		with ozone		without ozone		with ozone	
	top	bottom	top	bottom	top	bottom	top	bottom	top	bottom	top	bottom
2019												
after harvesting	16.42 ± 0.24[a]				36.46 ± 0.46[a]				65.36 ± 0.84[a]			
Day 7	16.34 ± 0.36[a]	16.42 ± 0.39[a]	16.30 ± 0.12[a]	16.27 ± 0.53[a]	36.02 ± 0.38[a]	36.71 ± 0.53[a]	35.79 ± 0.76[ab]	35.6 ± 0.65[ab]	64.87 ± 0.59[a]	63.99 ± 0.19[b]	64.15 ± 0.43[a]	64.98 ± 0.41[ab]
Day 12	16.25 ± 0.42[a]	16.30 ± 0.07[a]	16.21 ± 0.58[a]	16.01 ± 0.74[a]	36.14 ± 0.75[a]	35.09 ± 0.49[ab]	34.54 ± 0.34[b]	35.15 ± 0.41[b]	64.62 ± 0.71[a]	63.78 ± 0.65[b]	64.97 ± 0.47[a]	64.12 ± 0.23[bc]
Day 17	16.41 ± 0.17[a]	16.25 ± 0.41[a]	16.14 ± 0.23[a]	16.21 ± 0.08[a]	35.95 ± 0.65[a]	34.91 ± 0.18[b]	32.87 ± 0.34[c]	32.54 ± 0.76[c]	63.98 ± 0.45[a]	63.98 ± 0.27[b]	64.23 ± 0.46[a]	63.87 ± 0.14[c]
Day 25	16.37 ± 0.32[a]	16.22 ± 0.29[a]	16.10 ± 0.71[a]	15.89 ± 0.72[a]	35.52 ± 0.96[a]	34.81 ± 0.28[b]	32.45 ± 0.76[c]	32.41 ± 0.51[c]	63.64 ± 1.09[a]	63.21 ± 0.36[b]	64.74 ± 0.37[a]	63.76 ± 0.43[c]
2020												
after harvesting	13.67 ± 0.23[a]				28.03 ± 0.65[a]				68.73 ± 0.46[a]			
Day 7	13.57 ± 0.37[a]	14.87 ± 0.31[a]	13.77 ± 0.04[a]	13.80 ± 0.24[a]	26.30 ± 0.14[b]	31.00 ± 1.10[b]	25.470 ± 0.23[b]	28.80 ± 0.98[a]	68.90 ± 0.54[a]	65.53 ± 0.87[b]	68.33 ± 0.98[a]	67.07 ± 0.78[ab]
Day 12	13.73 ± 0.21[a]	14.00 ± 0.29[a]	13.83 ± 0.28[a]	13.63 ± 0.09[a]	26.23 ± 0.78[b]	30.07 ± 0.78[b]	25.170 ± 0.69 [b]	29.20 ± 0.48[a]	68.83 ± 0.36[a]	65.53 ± 0.45[b]	68.50 ± 0.65[a]	65.57 ± 0.34[b]
Day 17	13.60 ± 0.09[a]	13.80 ± 0.19[a]	13.80 ± 0.39[a]	13.60 ± 0.21[a]	25.70 ± 0.43[b]	29.90 ± 0.54[b]	25.700 ± 0.97[b]	28.70 ± 0.54[a]	69.00 ± 1.01[a]	65.10 ± 0.69[b]	68.90 ± 0.39[a]	65.40 ± 0.23[b]
Day 22	13.73 ± 0.15[a]	13.93 ± 0.56[a]	13.28 ± 0.41[a]	13.59 ± 0.18[a]	25.37 ± 0.21[b]	30.10 ± 0.72[b]	25.560 ± 0.51[b]	28.76 ± 0.87[a]	67.73 ± 0.69[a]	65.66 ± 0.45[b]	68.08 ± 0.65[a]	65.36 ± 0.18[b]

Values marked with the different letters in the columns per year are significantly different ($P < 0.05$).

Gluten content is an important quality indicator for the use of wheat grain in bread making. The minimum gluten content for making good quality bread is 23%. In 2019, after harvesting, the gluten content in wheat grain was 36.46 ± 0.46 g 100 g^{-1}, but in 2020 - 28.03 ± 0.65 g 100 g^{-1}, which allows to conclude that the gluten content in wheat grains was significantly affected ($P < 0.05$) by harvesting year - there were different weather conditions that could have affected the gluten content. The analysed wheat grain samples had a gluten content of more than 23% (irrespective of the year of harvest, active drying and sampling place in storage tanks), so they correspond to good quality grain that can be used for bread production. (Mei et al., 2016) found that 1.5–2.0 h of ozone treatment significantly affected ($P < 0.05$) wet gluten content. This is inconsistent with our study, but it could be related to the fact that 18.5 t and 10.6 t of wheat grain were used and active drying with ozone occurred for 185 h (2019) and 136 h (2020).

The starch content of wheat grain can be 60–70% of grain mass (Broberg et al., 2015). The analysed wheat grain of 2019 after harvest had a starch content of 65.36 ± 0.84 g 100 g^{-1} (Table 1), which did not change significantly during the active drying of wheat grain at the top of the storage tank. On the other hand, for wheat grains taken from the bottom of the storage tank, the starch content decreased by about 2.00% as a result of 25 days of active drying. In turn, in 2020, the starch content of wheat grain after harvesting was 68.73 ± 0.46 g 100 g^{-1}, which differs significantly ($P < 0.05$) from the 2019 harvest. For changes in starch content of active drying with and without ozone in 2020 were observed similar trends as in 2019.

The study showed that the protein, gluten and starch content of wheat grain was not significantly affected ($P > 0.05$) by the active drying with ozone. Our results are in accordance with the study of (Zhang et al., 2021), who reported that ozone treatment of wheat didn't affect the main chemical composition of milled flour.

Microbiological parameters

In the Fig. 5 the changes of microbiological parameter during storage using active drying with and without ozone are summarized.

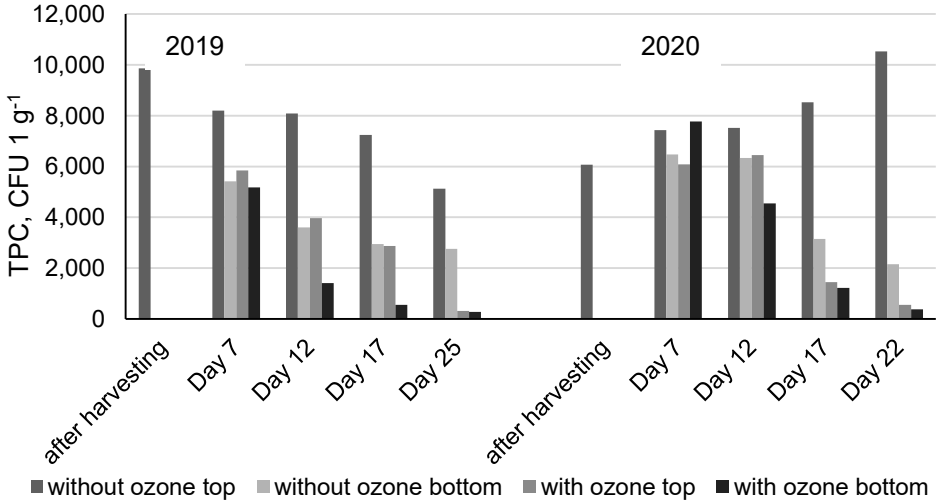

Figure 5. Total plate count (TPC) in wheat grain during active air and ozone drying.

European Commission Health & Consumer protection directorate has indicated that safe amount for consumers in the food product of microorganisms total plate count (TPC) is $< 10^5$ CFU g^{-1} (European Commission, 2012). Then, based on the results of the study, it can be concluded that the TPC in the analysed wheat grains didn't exceed the permissible norms. Active drying with ozone of wheat grains can significantly ($P < 0.05$) reduce the total amount of microorganisms (Fig. 5). What coincides with Hu et al. (2020) results, who found that in the buckwheat based composite flour total amount of microorganisms decreased significantly ($P < 0.05$). In year 2020, after 22 days of active drying, the TPC at the top of the storage tank increased significantly, which could be due to the high moisture content of the wheat grain analysed, which significantly contributed to the development of microorganisms. Jian et al. (2013) have found that ozone can reduce the count of microorganisms in grain, but this depends on the amount of ozone and the processing time with ozone.

The results obtained in this study showed that the application of ozone significantly ($P < 0.05$) reduced total microorganism count compared to the processing without ozone. However, the height of grain layer - 5.0 m in 2019 or 3.5 m in 2020 did significantly affect ($P > 0.05$) TPC. Savi et al. (2020) found similar trend in the storage of rice, and they recommend ozone treatment as antifungal agent.

CONCLUSIONS

The results obtained in the study showed that ozone treatment of wheat grain 15–18 ppm and treatment time of 185 h (2019) and 136 h (2020) did not significantly affect ($P > 0.05$) the moisture content and water activity of grain, but these parameters were significantly affected ($P < 0.05$) by the sampling location – the top or the bottom of the storage tank. Active drying with ozone significantly ($P < 0.05$) reduced the total plate count, but it was not observed that it would be significantly affected ($P > 0.05$) by the height of grain layer (2019 and 2020). The study proved that the protein, gluten and starch content of wheat grain was not significantly affected ($P > 0.05$) by the active drying with ozone.

In order to ensure a more efficient wheat grain active drying process in large quantities (more than 10 tons), it is necessary to find a solution that at least once a week grain in the storage tank is stirred.

ACKNOWLEDGEMENTS. This research is supported by Support Service project No l8-00-A01620-000003 'Technology development of grain drying with active ventilation using ozone'.

REFERENCES

AACC Method 44-15.02 'Moisture – Air-Oven Methods'. AACC Approved Methods of Analysis, 11th Edition.

Broberg, M.C., Feng, Z., Xin, Y. & Pleijel, H. 2015. Ozone effects on wheat grain quality e A summary. *Environmental Pollution* **197**, 203–213.

European Commission. 2012. *Working Document on Microbial Contaminant Limits for Microbial Pest Control Products*. September, 24.

Granella, S.J., Christ, D., Werncke, I., Bechlin, T.R. & Machado Coelho, S.R. 2018. Effect of drying and ozonation process on naturally contaminated wheat seeds. *Journal of Cereal Science* **80**, 205–211.

Hu, J., Li, X., Jing, Y., Hu, X., Ma, Z., Liu, R., Song, G. & Zhang, D. 2020. Effect of gaseous ozone treatment on the microbial and physicochemical properties of buckwheat-based composite flour and shelf-life extension of fresh noodles. *Journal of Cereal Science* **95**, 103055.

Hutla, P., Kolarikova, M., Hajek, D., Dolezal, P., Hausvater, E. & Petrackova, B. 2020. Ozone treatment of stored potato tubers. *Agronomy Research* **18**(1), 100–112.

ISO 4833-1. 2013. 'Microbiology of the food chain – Horizontal method for the enumeration of microorganisms – Part 1: Colony count at 30 °C by the pour plate technique'. International Organization for Standardization, Geneva, Switzerland.

Jian, F., Jayas, D.S. & White, N.D.G. 2013. Can ozone be a new control strategy for pests of stored grain? *Agricultural Research* **2**(1), 1–8.

Kleperis, J., Kristins, A., Veinbergs, J., Gvardina, I., Viesturs, D., Rucins, A., Straumite, E., Sloka, B. & Buveris, J. 2019. Applicataion of ozone in grain drying: Autonomous sensor system construction and pecularities. In *Proceedings of the 9th International Scientific Conference Rural Development 2019*, pp. 34–39.

Mei, J., Liu, G., Huang, X. & Ding, W. 2016. Effects of ozone treatment on medium hard wheat (Triticum aestivum L.) flour quality and performance in steamed bread making. *CYTA - Journal of Food* **14**(3), 449–456.

Nickhil, C., Mohapatra, D., Kar, A., Giri, S.K., Tripathi, M.K. & Sharma, Y. 2021. Gaseous ozone treatment of chickpea grains, part I: Effect on protein, amino acid, fatty acid, mineral content, and microstructure. *Food Chemistry* **345**, 128850.

Rakcejeva, T., Zagorska, J. & Zvezdina, E. 2014. Gassy ozone effect on quality parameters of flaxes made from biologically activated whole wheat grains. *International Journal of Nutrition and Food Engineering* **8**(4), 378–381.

Rucins, A., Viesturs, D., Kristins, A. & Bruveris, J. 2020. Investigations in intensification of grain drying by active ventilation applying ozone. In *Engineering for Rural Development* 2019, **19**, 231–237.

Savi, G.D., Gomes, T., Canever, S.B., Feltrin, A.C., Piacentini, K.C., Scussel, R., Oliveira, D., Machado-de-Avila, R.A., Cargnin, M. & Angioletto, E. 2020. Application of ozone on rice storage: A mathematical modeling of the ozone spread, effects in the decontamination of filamentous fungi and quality attributes. *Journal of Stored Products Research* **87**, 101605.

Tiwari, B.K., Brennan, C.S., Curran, T., Gallagher, E., Cullen, P.J. & O' Donnell, C.P. 2010. Application of ozone in grain processing. *Journal of Cereal Science* **51**(3), 248–255.

Wang, L., Shao, H., Luo, X., Wang, R., Li, Y., Li, Y., Luo, Y. & Chen, Z. 2016. Effect of ozone treatment on deoxynivalenol and wheat quality. *PLoS ONE* **11**(1), 1–13.

Worldwide production of grain 2019/2020, by type 2021. *https://www.statista.com/statistics/263977/world-grain-production-by-type/*. Acessed 28.01.2021.

Zhang, W., Li, L., Shu, Z., Wang, P., Zeng, X., Shen, W., Ding, W. & Shi, Y. C. 2021. Properties of flour from pearled wheat kernels as affected by ozone treatment. *Food Chemistry* **341**, 128203.

Zhu, F. 2018. Effect of ozone treatment on the quality of grain products. *Food Chemistry* **264**, 358–366.

Agronomy Research **19**(S3), 1318–1324, 2021
https://doi.org/10.15159/AR.21.063

Measurability of quality in fermentation process of rice wine by IoT in the field of industry 4.0

J. Vošahlík[1,*] and J. Hart[2]

[1]Czech University of Life Sciences Prague, Faculty of Engineering, Department of Technological Equipment of Constructions, Kamýcká 129, CZ165 00 Prague, Czech Republic
[2]Czech University of Life Sciences Prague, Faculty of Engineering, Department of Vehicles and Ground Transport, Kamýcká 129, CZ165 00 Prague, Czech Republic
[*]Correspondence: vosahlik@oikt.czu.cz

Received: February 1st, 2021; Accepted: April 7th, 2021; Published: May 4th, 2021

Abstract. The article inquiries into the issue of automation of the rice wine fermentation process in the field of industry 4.0. Fermentation is the process of converting D-glucose into ethanol along with oxidation of reduced coenzymes (fermentation). This is known as ethanol fermentation, which takes place anaerobically in the presence of yeast. The fermentation is being improved by automation (sensors, etc.). The main aim is to develop an experimental automation environment in industry 4.0 for the process of rice wine fermentation. During the rice wine fermentation process, variety of measurable attributes are created which affect the quality of the resulting product. They can be monitored with the help of automation elements (pH, temperature, humidity etc.). In case of an experimental environment development, it is therefore important to select appropriately the sensory that can record the measurable attributes. At the same time, the sensory must be at a level of reliability that guarantee their sufficient use in the mentioned experimental environment for the rice wine fermentation. The result is that, if the right environment is chosen, the quality of the fermented wine will improve.

Key words: automation, industry, 4.0, IoT, fermentation, rice wine.

INTRODUCTION

Nowadays, thanks to the development of industrial technologies, these technologies can be applied extensively in an agri-food complex. The technologies, at present, include industry 4.0, especially the Internet of Things (IoT). The Internet of things (IoT) describes the network of physical objects that are embedded with sensors, software, and other technologies for the purpose of connecting and exchanging data with other devices and systems over the internet. Industry 4.0 is the information-intensive transformation of manufacturing (and related industries) in a connected environment of big data, people, processes, services, systems and IoT-enabled industrial assets with the generation, leverage and utilization of actionable data and information as a way and means to realize smart industry and ecosystems of industrial innovation and collaboration. In the fermentation process of rice wine, technologies including standard 4.0 can be used for

data acquisition. During the fermentation process, substances, that can be monitored with the help of sensors, are produced. These substances have a significant effect on the sequent quality of the resulting product and can therefore lead to an improvement or deterioration of quality (Gilchrist, 2016).

The measured values can be interpreted via the Internet interface using IoT and the fermentation process can be virtually monitored online. Especially for collecting measured values to one place like server, PC or data storage. The measured values in the agri-food result of the complex can be used to improve the fermentation process. Thus, the possibility of deterioration of the quality of the resulting product will be minimized (Lokman et al., 2020).

Especially IoT in the fermentation process can be found as external device for measuring and collecting data from sensors. Storing data and this device can be connected to the internet for sending data to storage like external server. Measured data in fermentation process are usually about temperature, humidity, acidity etc. They are measured usually throughout the fermentation process for getting the whole information about fermentation process. Data can be throughout the fermentation process processed and presented on website or in application for better overview about fermentation process (Tomtsis et al., 2016).

Fermentation is the process in which D-glucose $C_6H_{12}O_6$ is converted into C_2H_5OH ethanol along with the oxidation of reduced coenzymes (NADH, FADH) - called fermentation. It is an alcohol fermentation (ethanol). Fermentation takes place anaerobically (under inaccessible air) and energy that is stored in ATP adenosine triphosphate molecules is being released. If the D-glucose $C_6H_{12}O_6$ is by chemical processes converted into ethanol C_2H_5OH and carbon dioxide CO_2, the alcohol fermentation takes place. This happens in the presence of yeast (Keot et al., 2020).

Any fermentation processes are greatly technologically demanding. Even the slightest mistake can completely devalue the resulting product. The fermentation can be expressed by chemical formula: $C_6H_{12}O_6 \rightarrow 2\ C_2H_5OH + 2\ CO_2$. The fermentation is also further improved by automation, which greatly helps to improve the production process, quality of the product, simplifying work and mainly the quantitative production. It also substitutes the work of man and thus minimizes the error rate of production caused by human error such as autonomic stainless-steel stirrer (Uehara, 2018).

The stirrers help in the fermentation process to better mix the substances that they gradually release during the fermentation process and to mix the dead spots. During the fermentation process, carbon dioxide was released, which is driving force to rotate the stainless-steel stirrer and mix releasing substances on its own (Cai, 2019).

Fermentation process also can be found in different fermentation environments such as milk fermentation, soya fermentation by lactic acid bacteria, vegetable and fruit fermentation and mainly in mentioned fermentation, where is goal to obtain alcohol drink (wine, whisky etc.). With combination with IoT, these processes can be improved. Mainly for getting data, from places where is not great internet connection (Slapkauskaite et al., 2019; Yang et al., 2020).

MATERIALS AND METHODS

The following experimental fermentation environment was established (Fig. 1). As the environment, in which the fermentation took place, was chosen a glass 9 liters fermentation vessel from Orion. A total of 4 vessels were used for the fermentation, two containers were without the self-stir and two containers with an autonomic stainless-steel stirrer. The resulting measured values are processed as average values. Two vessels without stirrer, represent the grey line and two vessels with stirrer represent black line in the par of results and discussion.

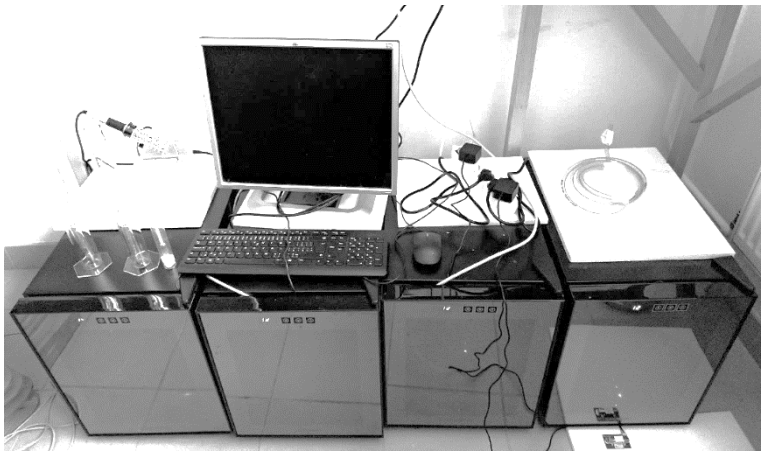

Figure 1. Experimental fermentation environment.

A fermentation solution of rice wine was implemented into this container in the following way - the solution contained 5l of water, 1kg of sugar, 1 kg of rice, 500 g of orange, 200 g of lemon, 5 pcs cloves. The water was boiled. After the overcooking, the sugar was poured in warm water for better distribution. This solution was mixed in one 30 L container to obtain the same fermentation conditions. Subsequently it was cooled to a room temperature for 24 hours. After that, the solution was poured into individual fermentation vessels together with the addition of the individual listed items.

The rice was overcooked before being put into the fermentation solution. Prior to closing the fermentation process in individual containers, Vinflora ® 20 mL yeast was poured directly into the prepared solution. Finally, the vessels were closed, the fermentation lids were fitted, and the fermentation process took place (Uehara, 2018).

Autonomic stirrer made of stainless-steel (ČSN 17240. AISI 304 in the Czech Republic) was modeled in a software SOLIDWORKS environment which is suitable for this purpose (Fig. 2). Subsequently, it was

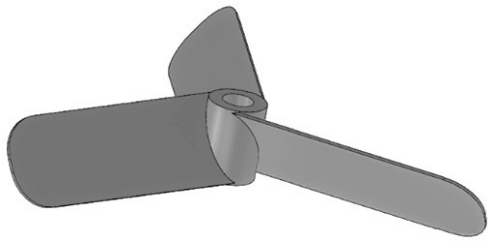

Figure 2. Part of autonomic food stainless-steel stirrer.

cut, machined, welded, fitted, and applied in two fermentation vessels. Autonomic stirrer is made of three blades due to weight reduction friction compared to the four-blade variant. Every blade is 80 mm height, 37 mm width and 8 mm thick. The ends are rounded by R12,7 and the beginnings are welded to the stainless-steel rod ø 14 mm and in the middle there is ø 8 mm bore for bar.

Before the implementation of the fermentation process, sensory technology was selected and applied on the fermentation vessels. Raspberry Pi 4 model B 4GB RAM (4 units) serves as the main calculation unit, which is responsible for the communication and data collection throughout the proposed system from sensory in the process of rice wine fermentation. For the correct gathering of information from individual sensors, it was necessary to properly install the connectors, solder the required electrical components and last but not least to program the sensors in the Python and C++ programming language. The whole system is working on the operating system of the latest Raspbian (Linux) update (Fig. 3).

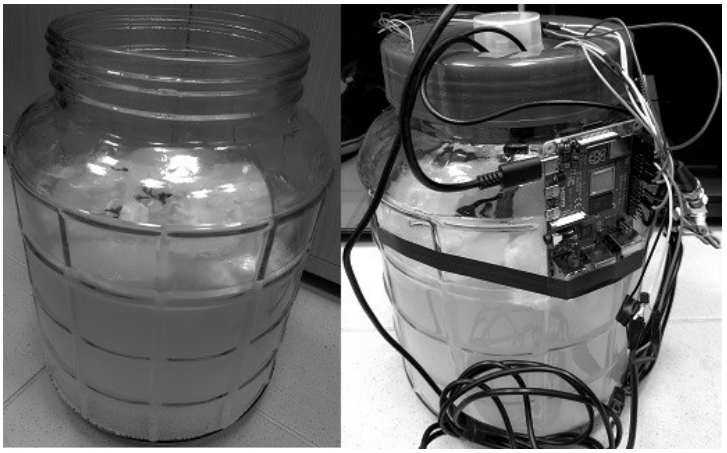

Figure 3. Vessels (from the left: Vessel with fermentation solution, vessels with sensors).

The first component that primarily measures humidity is called the BMP-280. It uses the I2C interface on Raspberry Pi 4 model B 4GB RAM programmed in Python. The measurement of the temperature both inside and outside the container in the cooling box was carried out with the help of the DS18S20 sensor. DS18S20, which was placed from inside the container was immersed in the solution. This sensor must have been in a waterproof version. Sensing of this sensor is realized in the C++ program. The third sensor that was placed in the solution was the pH probe E-201C-Blue, which was properly calibrated and programmed in C++. Data collection by the compute unit took place every 60 seconds and the data was stored in internal storage and after that externally processed (Fig. 3).

In such manned containers with sensors and prepared process for fermentation, they were placed into the cooling boxes HYUNDAI VIN12A HYUVIN12A. At the end of the fermentation process, the solution was cooled to 12 °C. During the fermentation, the sugar content of the solution was also measured using a sugar meter and the percentage of alcohol contained in the fermented solution with an alcohol meter.

RESULTS AND DISCUSSION

The first best measurable attribute is the temperature inside the vessel, which fermentation generates spontaneously by conversion of glucose especially into alcohol. The room temperature at the beginning of fermentation was 21 °C (Fig. 4). The fermentation vessels that do not contain an automatic stainless-steel stirrer have a higher temperature during the fermentation process of rice wine literally throughout the whole process. After the 8th day of fermentation, i.e., after the end of the fermentation, the solution was in cooling boxes cooled to 12 °C and this temperature was constantly maintained. The highest temperature achieved during the fermentation was 30.9 °C in the container without the stirrer and 29.7 °C in the container without the stirrer. Thus, the tendency of the fermentation temperature is rising more slightly in vessels with a self-stirring stainless-steel stirrer than in vessels without self-stirring stirrer.

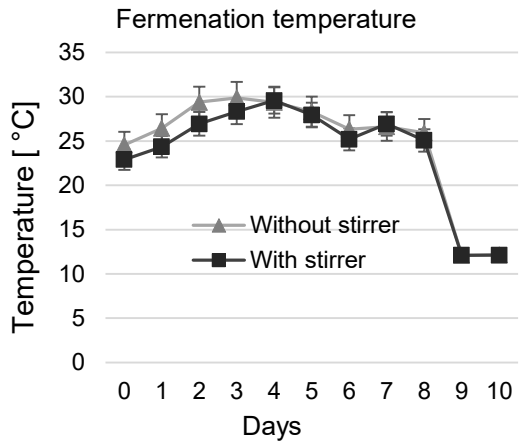

Figure 4. The course of fermentation temperature.

Another interesting and well-measurable attribute is the percentage humidity in individual containers. After closing the vessels and before starting the fermentation process, the moisture value of the vessel was on average 85% (Fig. 5).

Consequently, the percentage humidity initially during fermentation was higher in fermentation vessels without a spontaneous stirrer made of stainless food steel due to the faster onset of the yeast fermentation process. Both trends were increasing. However, the fermentation with stainless steel stirrer was slower but there was the highest humidity of 98.4% in the vessel with stainless steel stirrer vs. 97.3% in vessel without stirrer.

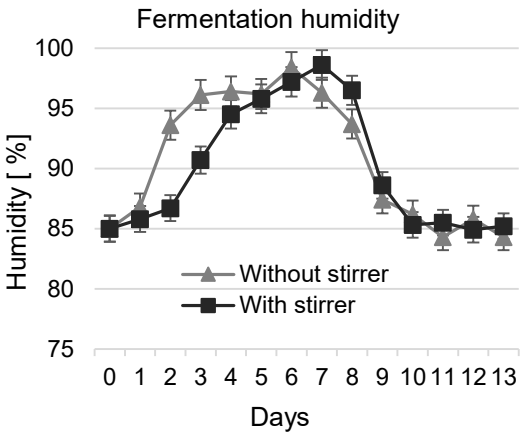

Figure 5. The course of fermentation humidity.

The third measurable aspect was acidity measurement (pH). At the beginning, the pH of the water itself was 7.4 ph. After the overcooking, adding sugar and further ingredients, it was 5.35 ph. (Fig. 6). During the process of fermentation of rice wine pH significantly decreases due to the consumption of continuous nutrients of yeast and the production of alcohol. The resulting pH was lower in containers with a self-stirrer than in containers without a self-stirrer.x

In the fourth case, the percentage of sugar content of fermented rice wine, was measured. The basic solution contained 23.5% sugar content. The decrease of percentage of sugar content was monitored in the fermentation process that was conducted in a container without a stainless-steel stirrer. In the container with the stainless-steel stirrer the consumption of sugar was slightly slower. Between the third and sixth day, the highest consumption of the sugar was measured (Fig. 7). That was the peak of exuberant fermentation. After the ninth day, the percentage of sugar content in the solution was 0% meaning there was no longer a fermentation process going on.

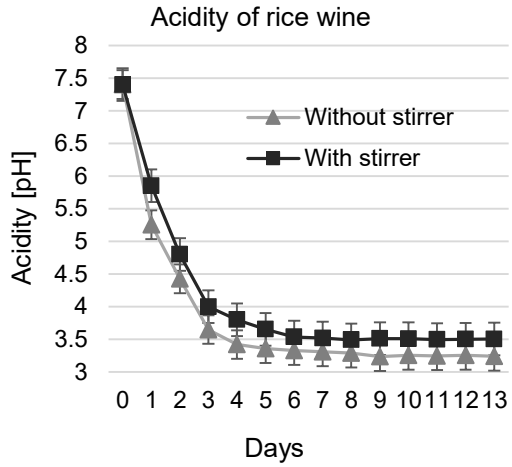

Figure 6. The course of fermentation acidity.

The fifth aspect measured was the percentage of alcohol in the fermented rice wine solution. The conversion of sugar by yeast into alcohol is proportional and therefore there is an increasing tendency. After consuming of all the sugar in the solution, the percentage of alcohol in the solution was 13,5 % in a container without a stirrer and 12,8 % in a container with a stirrer (Fig. 8). The alcohol level of regular rice wine varies in different 12-25% alcohol, so this rice wine affects the lower limit range.

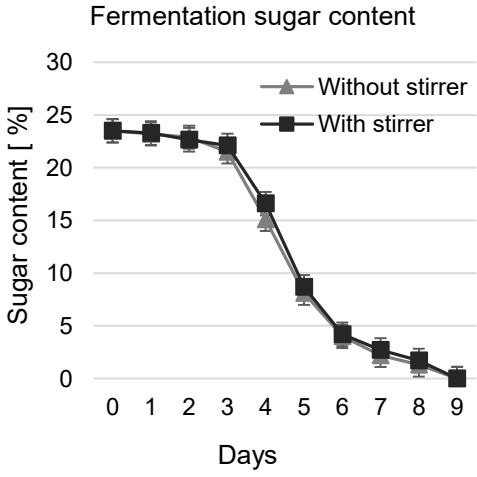

Figure 7. The course of fermentation sugar content.

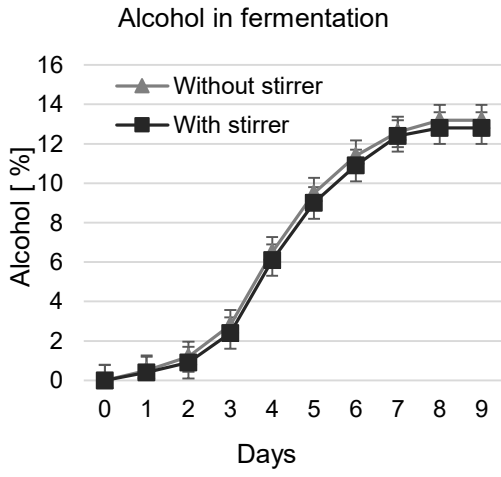

Figure 8. The course of alcohol content.

CONCLUSIONS

The use of a stainless-steel self-stir affects the fermentation process. The temperature is affected. The temperature increases slower during the fermentation in containers with a stirrer than in containers without a stirrer, where the temperature increase is faster. The percentage humidity in the container has the same tendencies as the temperature, i.e., without the stirrer it has a faster increase than with the stirrer.

The self-stir most significantly affects the pH since the substances are better released from additives to solution in containers with a stirrer than in containers without a self-free stainless-steel stirrer. This also slightly influences the conversion of sugars into alcohol. Here, in containers with stainless steel stirrer, the transformation tends to be more gradual than in containers without a stirrer.

For the future, subsequent improvements in the shape of stainless-steel self-stirs scoops and replacement of the material with e.g., plastic suitable for the food industry with the help of a 3D printer will probably make it possible to achieve greater efficiency in the release of substances from additives into fermented rice wine.

ACKNOWLEDGEMENTS. It is a project supported by the CULS IGA TF 'The University Internal Grant Agency' (2020:31150/1312/3105 and 31170/1312/3116).

REFERENCES

Cai, H., Zhang, Q., Shen, L., Luo, J., Zhu, R., Mao, J., Zhao, M. & Cai, C. 2019. Phenolic profile and antioxidant activity of Chinese rice wine fermented with different rice materials and starters. *LWT* **111**, 226–234, doi:10.1016/j.lwt.2019.05.003

Gilchrist, Alasdair. 2016. *Industry 4.0: the industrial internet of things*. 2016. New York, NY, Apress. ISBN 9781484220467

Keot, J., Bora, S., Das Kangabam, R. & Barooah, M. 2020. Assessment of microbial quality and health risks associated with traditional rice wine starter Xaj-pitha of Assam, India: a step towards defined and controlled fermentation. *3 Biotech* **10**(2). doi:10.1007/s13205-020-2059-z

Lokman, T., Islam, M. & Apple, M. 2020. Design & Implementation Of IoT Based Industrial Automation Syste. In: *2020 **11**th International Conference on Computing, Communication and Networking Technologies (ICCCNT)*, pp. 1–6. doi:10.1109/ICCCNT49239.2020.9225388

Slapkauskaite, J., Kabasinskiene, A. & Sekmokiene, D., 2019. Application of fermented soya as a bacterial starter for production of fermented milk. *Czech Journal of Food Sciences* **37**(6), 403–408. doi:10.17221/194/2018-CJFS

Tomtsis, D., Kontogiannis, S., Kokkonis, G. & Zinas, N. 2016. IoT Architecture for MonitoringWine Fermentation Process of Debina Variety Semi-Sparkling Wine. In: *Proceedings of the SouthEast European Design Automation, Computer Engineering, Computer Networks and Social Media Conference on - SEEDA-CECNSM '**16***. doi: 10.1145/2984393.2984398. New York, New York, USA: ACM Press, pp. 42–47

Uehara, Y., Ohtake, S. & Fukura, T. 2018. A Mash Temperature Monitoring System for Sake Brewing. In: *2018 IEEE International Conference on Consumer Electronics-Taiwan (ICCE-TW),* pp. 1–2. doi:10.1109/ICCE-China.2018.8448774

Yang, Y., Hu, W. & Xia, Y. 2020. Flavor Formation in Chinese Rice Wine (Huangjiu): Impacts of the Flavor-Active Microorganisms, Raw Materials, and Fermentation Technology. *Frontiers in Microbiology* [online]. 2020, **11** [cit. 2021-01-31]. ISSN 1664-302X. doi:10.3389/fmicb.2020.580247