PRODUCTION OF FRUIT WINES USING NOVEL ENZYME PREPARATIONS

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Abstract

Aim: This work describes the activities of new-generation enzymatic preparations in fruit-berry substrates engineered for use in the fruit-wine industry. The enzymes were produced after genetic modification and selection of fungi *Penicillium verruculosum*, which produce efficient cellulase and pectinase enzymatic complexes.

Methods and results: This paper covers the main characteristics of novel multi-enzyme complexes and the results of in-lab fruit-wine production with addition of enzymatic preparations, which could be used on an industrial scale. The juice yield and the content of suspended materials in the enzymatically treated samples were compared. Experiments included the sensory analysis of produced juices and fruit wines.

Conclusion: Results show a significant increase in juice yield from the fruit pulp processed with the enzymatic preparations, without any negative effect on the quality and organoleptic attributes of the final product.

Significance and impact of the study: The obtained data clearly show that the use of the new-generation enzymatic preparations in the fruit-wine industry is effective.

Key words: enzyme preparations, enzymatic treatment, *Penicillium verruculosum*, fruit wines, multi-enzyme complex, fruit-wine industry

Résumé

Objectif: Ce travail décrit les activités de la nouvelle génération de préparations enzymatiques sur substrats de fruits/baies développée pour l'application dans l'industrie du vin de fruits. Ces enzymes ont été produites après la modification génétique et la sélection de champignons *Penicillium verruculosum*, source d'efficaces complexes enzymatiques de cellulase et de pectinase.

Méthodes et résultats: Cet article présente les principales caractéristiques des nouveaux complexes multi-enzymatiques et les résultats, après application de préparations enzymatiques, de la fabrication de vins de fruits en laboratoire, production qui pourrait se décliner à une échelle industrielle. Les rendements en jus et les matières en suspension contenues dans les échantillons enzymatiques traités ont été comparés. Les paramètres physico-chimiques et les caractéristiques d'intensité de couleur de la boisson finale ont été enregistrés.

Conclusion: Les résultats montrent une augmentation significative du rendement de jus extrait de la pulpe de fruit traitée avec des préparations enzymatiques sans influence négative sur les aspects qualitatifs et les propriétés organoleptiques du produit final.

Signification et impact de l'étude: Les données obtenues montrent clairement la haute efficacité de l'utilisation de la nouvelle génération de préparations enzymatiques dans l'industrie du vin de fruits.

Mots clés: préparations d'enzymes, traitement enzymatique, *Penicillium verruculosum*, vins de fruits, complexe de multi-enzymes, industrie du vin de fruits

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INTRODUCTION

This paper addresses an important technological problem of the fruit-wine industry (Codex Alimentarius : wine made from fruit other than grapes) : how to increase juice yield from raw material without compromising the quality of the final product. Fruit-wine consumption is significantly lower compared to traditional grape wines ; however, in countries such as Great Britain, Poland or Russia, this type of beverage is well known and appreciated (Noller and Wilson, 2009 ; Kiselev *et al.*, 2013). A vast range of raw materials can be used for fruit-wine production : apple, pear, pineapple, guava, kiwi (Soufleros *et al.*, 2001), Chinese lychee, orange, cherry, cranberry, mango, passion fruit, papaya, peach, etc. Selection of the raw material is mainly determined by traditional recipes existing in the country of origin.

The fruit-wine technology is characterized by the specificity of the raw materials, which vary in their chemical content and requirements for different processing conditions. The production of such types of wine is often confronted with numerous problems such as low juice yield, difficulties with pressing, slow juice clarification, clouding and color changes in the final product (Volchok *et al.*, 2013).

Currently, preprocessing of fruits and berries with various enzymes prior to pressing and filtration is considered to be the most effective solution to these technological problems (Jayani *et al.*, 2005 ; Liew Abdullah *et al.*, 2007), ensuring better fiber maceration and juice clarification, prevention of colloidal hazes, and achievement of balanced and diverse flavors (Ageeva and Markosov, 2013). Selection of enzymes is based on their activities required for a particular fruit or berry.

The Enzyme Biotechnology Laboratory of the Bach Institute for Biochemistry, Russ. Acad. Sci. (INBI RAS) is developing new superior enzymes and enzymatic complexes with several activities at ratios allowing the efficient processing of various raw materials. This article describes the processing of several fruit substrates, containing cellulose and hemicellulose, with the new multi-enzymatic complexes BI_3-227.7 and BI_3-227.4, followed by the lab-scale production of fruit wines. Both enzyme preparations were derived from recombinant strains of *Penicillium verruculosum*. These complexes were selected in relation to earlier experiments processing fresh viburnum and strawberry juice yield from the pulp and larger content of reducing sugarspreliminary processing experiments. The main criteria were yield increasing of juice and larger content of reducing sugars (Volchok *et al.*, 2013). The results of the organoleptic analysis and the comparisons of enzyme-processed juices and wines against the non-processed samples are presented to acknowledge the efficiency of the proposed method and multi-enzymatic complexes.

MATERIALS AND METHODS

1. Substrates

Ash berry, plum and black currant provided by the Russian State Agrarian University named after K.A. Timiryazev were used as raw fruit and berry material. Table 1 shows the dates of harvest and sampling.

2. Enzyme preparations

Multi-enzyme preparations were obtained by cotransformation of the auxotrophic host strain *P. verruculosum* 537. Expression plasmid encoding *P. canescens* pectin lyase (PELA) and *Aspergillus niger* β-glucosidase (BG), and transforming plasmid pSTA 10 were used in transformation experiments. Details of the developed process for the recombinant strains and enzyme preparations are described in Bushina *et al.* (2012). Preparations are in the form of a light brown powder (easily soluble in water) obtained by lyophilization of culture filtrates (micro-filtrated and concentrated by ultrafiltration method) after fermentation of recombinant *P. verruculosum* strains, and they show stable, high enzymatic activity in the range of 25-50ºC for temperature and 4.0-5.0 for pH.

Enzyme complexes contained pectin lyase A, cellobiohydrolase, endo-1,4-glucanase and β-glycosidase. Earlier activity of enzymes was tested on apple, citrus and beet pectin (Morozova *et al.*, 2010 ; Bushina *et al.*, 2012). Their main enzymatic activities are presented in Table 2.

Table 1 - Harvest and sampling dates of raw fruit and berry material

Materials	Harvest dates	Sampling dates
Ash berry	25/09/14	27/09/14
Yellow plum	20/08/14	23/08/14
Black currant	30/07/14	02/08/14

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Table 2 - Characteristics of multi-enzymatic preparations

Показатель	Enzyme preparation	
	BI_3-227.4	BI_3-227.7
Protein content, mg/g of preparation	854±39,77	503±33,6
%RSD	1,873	2,684
Cellulase (CMCase) activity, U/g of preparation	3346±388,84	3194±310,6
%RSD	4,673	3,911
Cellulase (Avicelase) activity, U/g of preparation	170±19,92	174±14,9
%RSD	4,736	3,448
β-glycosidase activity, U/g of preparation	3999±388,11	395±25,49
%RSD	3,93	2,58
Pectinlyase activity, U/g of preparation	2694±388,38	1164±113,2
%RSD	5,86	3,925
Xylanase activity, U/g of preparation	3490±288,28	5310±334,9
%RSD	3,321	2,522

* - Sum of cellulase activities is equated to 1.

Enzymatic activities towards polysaccharide substrates were determined from the initial rates of formation of reducing sugars by the Somogyi–Nelson method (Nelson, 1944; Somogyi, 1952). Activities against p-NP-derived substrates were determined at pH 5.0 and 40ºC by measuring p-nitrophenol release, as described elsewhere (Gusakov *et al.*, 2005). All activities are expressed as international units per mg protein (U/mg) (one unit corresponds to the hydrolysis of 1 μmol of glycoside bonds from the substrate per minute). Methods of determination of enzymatic activities are described in detail in Bushina *et al.* (2012).

3. Scheme for producing wines

Fruit-wine materials were produced in the lab using the methodology presented in Table 3.

4. Analytical methods

During the experiments, juice yield, viscosity and suspension content in fermented samples were compared.

Characteristics of fruit juices

Suspension content of fruit must was evaluated gravimetrically by centrifugation. 10-cm^3 samples were put in pre-measured sedimentation tubes and centrifuged for 10 min at 3,000 rpm. Supernatant was removed, leaving the tubes upside down for 1 min. Sediment content was calculated by the equation:

$$C= (m_2-m_1)*100/V, \text{ where}$$

m_2 – mass of sedimentation tube with sediment, g;
m_1 – mass of empty sedimentation tube, g; and
V – sample volume, cm^3.

For determination of relative viscosity, samples were centrifuged for 10 min at 8,000 rpm. Then 5 cm^3 of liquid was incubated in an Ostwald viscometer for 5 min at 20ºC (Ashapkin *et al.*, 2005). Relative viscosity was calculated by the equation:

$$\eta=T_i/T_0, \text{ where}$$

T_i – flow time of selected sample, sec; and
T_0 – flow time of water, sec.

5. Sensory analysis of fruit juices and wines

Ten people were recruited for participating in the sensory analysis of produced juices and fruit wines. The range of descriptors and reference terms allowing the complete organoleptic description of the juices and wines was selected previously (Baxter *et al.*, 2005). 10-scale evaluation maps were developed using the following key terms: “weak”, “little” or “absent” for the left anchors and “strong” or “much” for the right anchorskey words. During the week (5 days), panel members participated in training sessions to ensure an homogeneous interpretation of the terms and correct filling of the score cards (Laboissiere *et al.*, 2007).

3 terms (2 for wine) were selected for color and turbidity, 5 for aroma, and 6 for flavor and aftertaste. Samples were served in 100-mL transparent plastic glasses coded with three-digit codes. Water and

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Table 3 - In lab fruit-wine production schemes

Material	Technological scheme without enzymatic treatment of fruit pulp	Technological scheme with enzymatic treatment of fruit pulp
Ash berry dry wine	1. Washing and sorting of raw material	
	2. Crushing pulp, introduction of pulp water to obtain titrated acid content 5 g/1000 mL	
	3. Sulfitation of fruit pulp ($K_2S_2O_5$, «Megahim», RU) up to 80 mg/1000 mL salt	
	4. Maceration of fruit pulp carried out in during 24 h at 50 ºC	4. Maceration of fruit pulp carried out in presence of enzyme preparation #3-227.7 (0,03% from pulp mass) during 24 h at 25 ºC
	5. Pressing pulp (laboratory mechanical press capacity 1500 mL)	
	6. Introduction sugar up to 22 g/100 mL (sugar syrup 80%), sulfitation of fruit juice up to 120 mg/1000 mL	
	7. Sedimentation (24 h, 4 ºC), separation	
	8. Must fermentation process up to 1.5 g/100 mL sugar at 20 ºC. Adding commercial yeast «France universal» (France) 500 g/625 L, introduction in fruit must 0,6 g/1000 mL $(NH_4)H_2PO_4$ (Sigma, USA)	
	9. Fermentation in during 15 days, 20 ºC	
	10. Sedimentation (24 h, 4 ºC), separation	
	11. Filtering wine materials (if necessary), storage in glass bottles for 4 ºC	
Yellow-plum, black currant sweet wine	1. Washing and sorting of raw material	
	2. Crushing pulp, introduction of pulp to obtain titr titretaed acid content 6,8 g/1000 mL	
	3. Sulfitation of fruit pulp ($K_2S_2O_5$, «Megahim», RF) up to 80 mg/1000 mL	
	4. Maceration of fruit pulp carried out in during 24 h at 50 ºC	4. Maceration of fruit pulp carried out in presence of enzyme preparation #3-227.4 (0,03% from pulp mass) during 24 h at 25 ºC
	5. Pressing pulp (laboratory mechanical press capacity 1500 mL)	
	6. Introduction sugar (sugar syrup 80%) up to 22 g/100 mL concentration, sulfitation of fruit must up to 120 mg/1000 mL concentration	
	7. Sedimentation (24 h, 4 , 4 ºC), separation	
	8. Must fermentation process up to 1,5 g/100 mL sugar at 20 ºC. Adding commercial yeast «France universal» (France) 500 g/625 L, introduction in fruit must 0,6 g/1000 mL $(NH_4)H_2PO_4$ (Sigma, USA)	
	9. Introduction sugar (sugar syrup 80%) up to 15 g/100 mL sugar concentration, mash fermentation carried out in during 30 days, 20 ºC	
	10. Sedimentation (24 h, 4 ºC), separation	
	11. Filtering wine materials (if necessary), storage in glass bottles at 4 ºC	

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unsalted biscuits were provided for clearing the palate. Spider web plots were made for graphical representation of the tasting session results (Duarte *et al.*, 2010).

6. Data analyses

Physical characteristics of the juices were measured in triplicate for each parameter. Identified Rrelative standard deviation (RSD %) and confidence interval were identified.

Student *t*-test capability on Microsoft Excel 2003 was used to determine the significance of the differences in between attributes (Doerffel, 1990). For data processing, a statistical significance level of P = 0.05 was used.

RESULTS AND DISCUSSION

The yield of free-run juice in the course of in-lab production of fruit wine is presented in Figure 1 (data obtained were recalculated for 1 ton of pulp – raw weight). It is important to note that due to high acidity the substrate pulp used for the experiment was diluted with water. The must yield from untreated pulp was used as a control.

It is clearly seen from the figure data that the use of enzymatic preparations significantly increases the yield of high quality free-run juice.

Table 4 shows the results of relative viscosity for the must produced from different raw materials and its sediment content.

The advantages of the enzymatic treatment of raw plant materials compared to non-processed samples are lower viscosity (lower biopolymer content - cellulose, hemicellulose, pectin - due to enzymatic destruction) and lower concentration of sediment in the fermented must.

Thus, enzyme preparation BI_3-227.4 was chosen for treatment of black currant and plum due to its β-glucosidase and pectin lyase activities leading to the rapid rarefaction bioconversion of pectin substrates. Another enzymatic preparation, BI_3-227.7, was chosen for the ash berry treatment due to its cellulase and hemicellulose activities. These results were expected as the preparation formula, either BI_3-227.4 or BI_3-227.7, correlates to the component composition of the cell wall of these plants.

Table 5 shows the results of the organoleptic analysis of the juices obtained with enzymes and the juices obtained by pressing after maceration. Participants in the sensory analysis especially noted more attractive color characteristics in the case of the enzyme-processed plum juice compared to the reference sample.

Figure 2 shows significant differences in aroma and appearance characteristics between the enzyme-processed samples and control.

Juices that have undergone enzymatic treatment are characterized by lower amount of suspended particles and lower turbidity, which facilitates the subsequent clarification and filtration processes. Figure 3 depicts the differences in taste attributes of

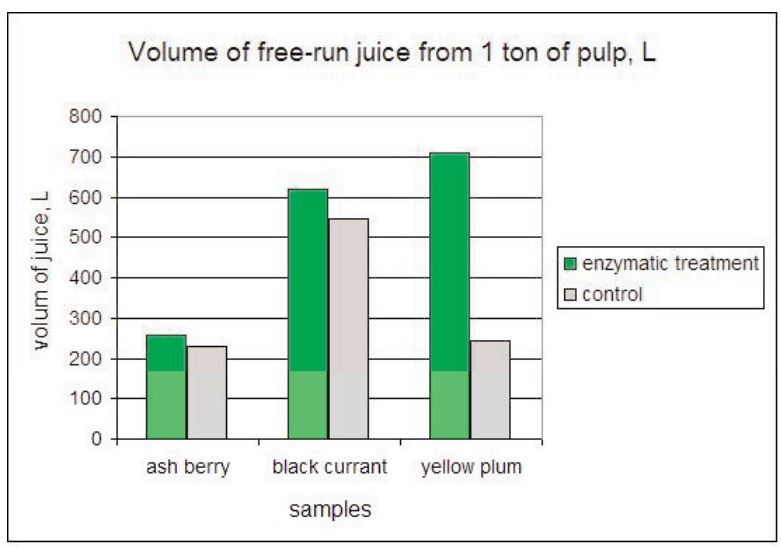


Figure 1 - Volume of free-run juice from 1 ton of pulp, L

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Table 4 - Physical attributes of fruit must

Tested samples, fresh must		Relative viscosity	%RSD	Mass fraction of suspended, g/100 mL	%RSD
Ash berry	Enzyme preparation BI_3-227.7	1,715±0,013	0,321	0,726±0,037	0,704
	control	1,855±0,015	0,329	0,935±0,012	2,529
Black currant	Enzyme preparation BI_3-227.4	2,079±0,01	0,194	1,143±0,075	5,094
	control	2,240±0,01	0,18	1,356±0,028	0,735
Yellow-plum	Enzyme preparation BI_3-227.4	1,641±0,01	0,254	1,318±0,017	0,44
	control	1,816±0,013	0,303	1,436±0,037	1,85

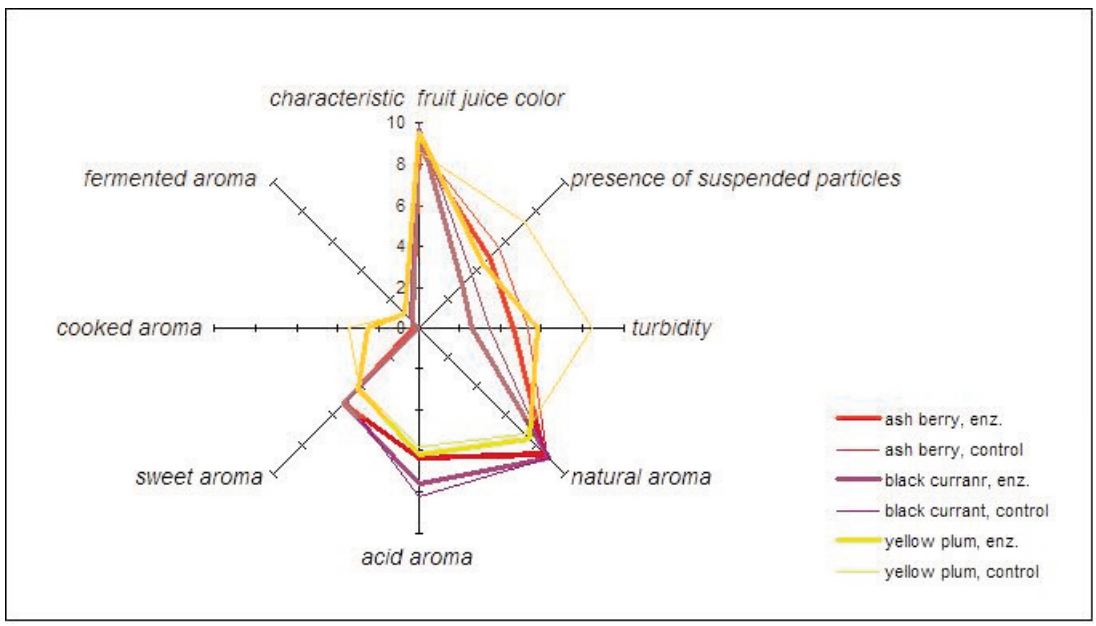


Figure 2 - Appearance and aroma attributes for simples of juices

the juices. From this spider web plot illustrating the flavor attributes of the compared juices, we can draw the conclusion that the enzyme used had a minor influence on flavor consistency.

Juices produced by traditional maceration are characterized by a stronger aroma. It happens as maceration done without multi-enzyme complexes takes significantly more time compared to enzymatic treatment. At the same time, the richness of the juice produced with enzymatic complexes can be corrected by adjusting the fermentation time.

The organoleptic study of the wines produced from analyzed juice samples included the determination of fruity and floral notes in aroma and main flavor parameters (Table 6).

Provided data show that the use of multi-enzyme complexes for fruit-wine production has a positive effect on appearance and aroma characteristics (especially noticeable in the case of plum juice) without affecting other organoleptic attributes.

Figure 4 depicts the results of the sensory analysis of produced fruit wines. In the case of yellow plum, significant positive difference can be observed. The sample produced with the developed enzyme complex is characterized by noticeably lower cooked odor and better clarity compared to the reference.

Comparing flavor parameters of enzyme-processed wine and control showed no significant differences (Figure 5).

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Table 5 - Fruit juices sensory attributes mean values

Attributes	Definitions	References	The results of the evaluation samples of juices (average)					
			Ash berry (enz.prep.)	Ash berry (control)	Black currant (enz.prep.)	Black currant (control)	Yellow plum (enz.prep.)	Yellow plum (control)
Appearance								
Characteristic juice color	Color characteristic of juice (for ash berry – intense pink, for black currant – dark purple, for plum – light yellow)	Little: 0-1 Much: 8-10	8,84	8,93	9,7	9,85	9,5[3]*	8,6[3]*
Presence of suspended particles	Presence of particles from fruit pulp	Absent: 0-1 Much: 9-10	4,83*	5,58*	2,95**	3,65**	4,35[3]*	7,35[3]*
Turbidity	Non-limpid aspect related to the difficulty of light passing through juice	Absent: 0-2 Much: 9-10	4,6*	5,3*	2,55**	3,44**	5,75[3]*	8,45[3]*
Aroma								
Natural	Characteristic aroma from natural juice	Weak: 0-1 Strong: 8-10	8,69	8,84	9	8,95	7,55[3]*	7,3[3]*
Acid	Aroma related to the presence of characteristic organic acids from fruit	Weak: 0-3 Strong: 8-10	6,35	6,4	7,55**	8,2**	6,15	5,8
Sweet	Aroma due to the presence of sucrose and other sugars from fruit	Weak: 0-3 Strong: 8-10	5,25	5,25	5,12	4,95	4,2	4,05
Cooked	Characteristic aroma from fruit submitted to thermal processing	Absent: 0-2 Strong: 9-10	0,3	0,3	0,1	0,1	2,5[3]*	3,45[3]*
Fermented	Characteristic aroma from fruit showing signs of early deterioration	Absent: 0 Strong: 10	0,5	0,5	0,5	0,6	1	1
Flavor								
Natural	Characteristic flavor from natural juice	Absent: 0-1 Strong: 8-10	9	9,05	8,5	8,7	7,8	7,6
Acid	Flavor stimulated by the presence of characteristic organic acids from fruit	Weak: 0-3 Strong: 8-10	8,22	8,35	8,7	8,95	7,1[3]*	7,65[3]*
Sweet	Flavor stimulated by the presence of sucrose and other sugars from fruit	Weak: 0-3 Strong: 8-10	6,61	6,31	5,65	5,3	5,75	5,4
Cooked	Characteristic flavor from fruit submitted to thermal processing	Absent: 0-2 Strong: 9-10	0,1	0,2	0,4	0,4	2,8	3,8
Fermented	Characteristic flavor from fruit showing signs of early deterioration	Absent: 0 Strong: 10	0,3	0,5	0,1	0,5	1	1
Astringency	Harsh sensation perceived in mouth and tongue characteristic of fruit	Weak: 0-4 Strong: 7-10	7,32	7,72	5,3	5,5	3,65	3,25
Consistency								
Consistency	Perception in mouth of juice dilution or concentration	Weak: 0-3 Strong: 8-10	7,75	8,15	9,28	9,3	7,7	7,4

*paired parameters ash berry juices (** for black currant, 3* for yellow plum) with t-test above the significance level P

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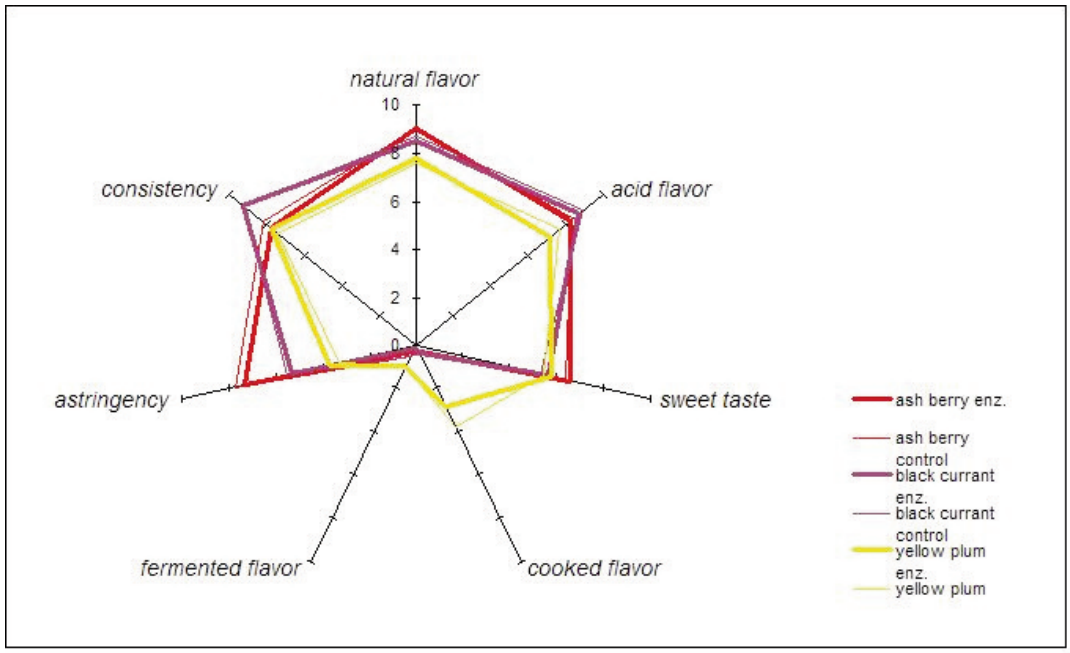


Figure 3 - Flavor and consistency attributes for simples of juices

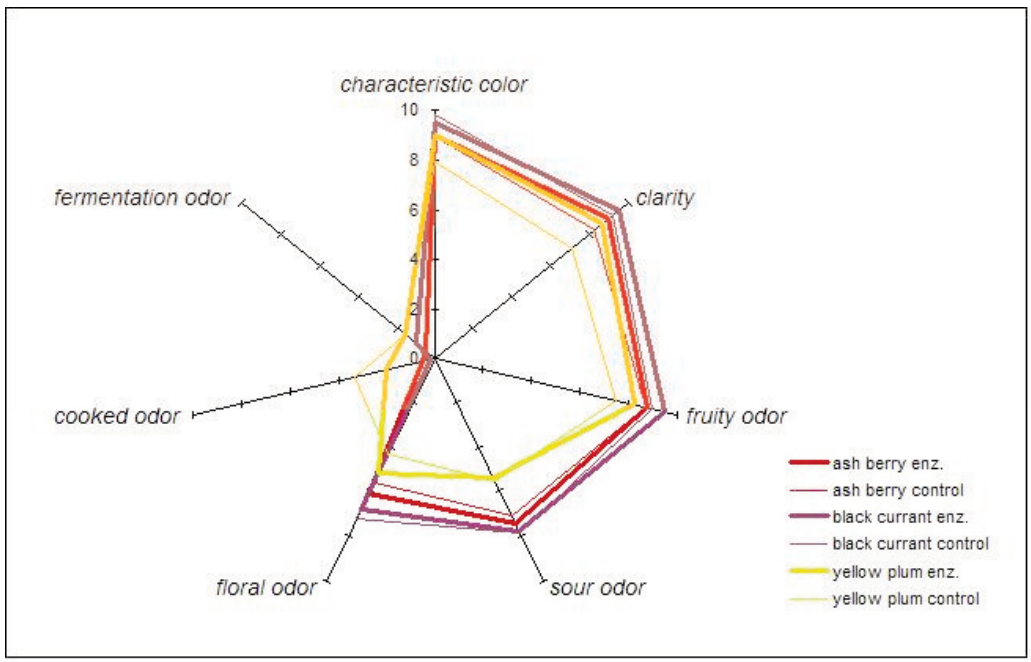


Figure 4 - Appearance and aroma attributes for simples of fruit-wines

In earlier research held in the Enzyme Biotechnology Laboratory INBI RAS, the organoleptic parameters of the red wine processed with the help of monitored enzymatic complexes were compared to the wine produced according to traditional technology. Cabernet Sauvignon and the local grape variety Tsimlyansky Black (provided by the « SARKEL » department of the « Tsimlyansky wines » company), a local grape variety, served as raw material for the wine. Results obtained during the tasting session are described in Volchok *et al.* (2014). These data show that the developed multi-enzymes applied to the grape-wine industry allow to achieve well balanced wines with rich fruity aromas.

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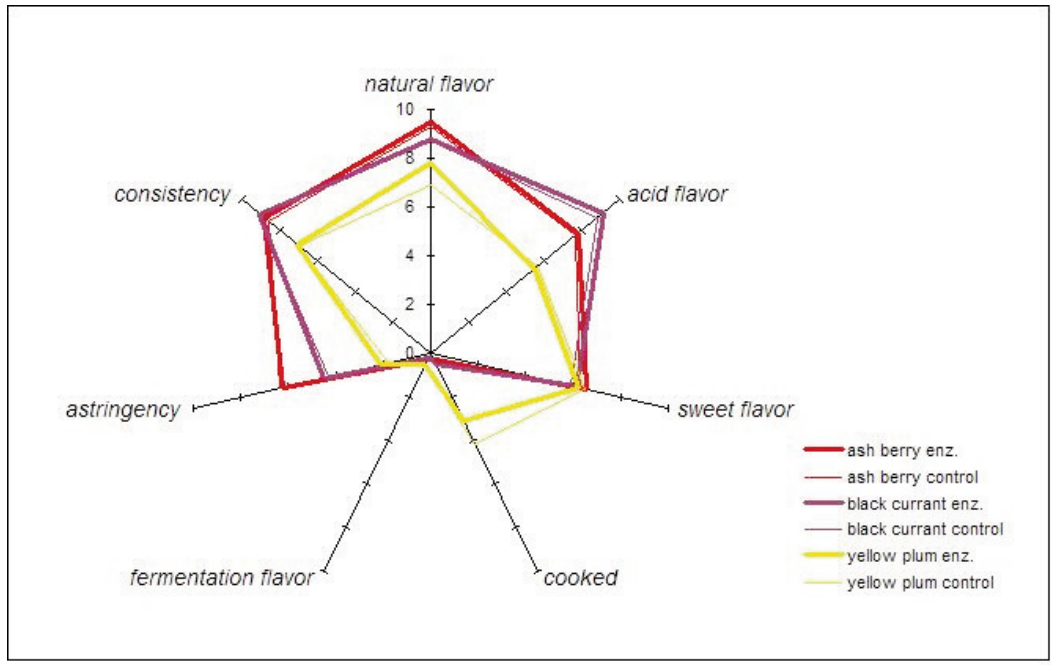


Figure 5 - Flavor and consistency attributes for simples of fruit-wines

CONCLUSIONS

Samples of fruit wines were produced in-lab, including a maceration stage with the use of new enzymatic preparations consisting of the target activities of cellulase, β-glucosidase and pectin lyase. Different ratios of the target activities allowed to apply specific enzymatic agents to a particular type of raw material. The juice yield was higher compared to the reference sample. And produced fruit-wines material was were not inferior to the reference sample in quality attributes, showing lower viscosity, lower sediment content and higher color intensity. Lower sediment content was observed in the must treated by enzymatic preparations. Organoleptic analyses of juices and fruit wines showed a positive effect of multi-enzyme complexes on the sensory characteristics of products.

Obtained data clearly show the high efficiency of the new-generation enzymatic preparations in the fruit-wine industry.

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Table 6 - Fruit wines sensory attributes mean values

Attributes	Definitions	References	The results of the evaluation samples of fruit-wines (average)					
			Ash berry (enz.prep.)	Ash berry (control)	Black currant (enz.prep.)	Black currant (control)	Yellow plum (enz.prep.)	Yellow plum (control)
Appearance								
Characteristic color	Color characteristic of wine (for ash berry – intense pink, for black currant – dark purple, for plum – light yellow)	Little: 0-1 Much: 8-10	9	8,9	9,5	9,8	9^{3}*	$7,9^{3}$*
clarity	The degree of transparency	Absent: 0-2 Much: 9-10	9*	8,1*	9,5	9,2	$8,7^{3}$*	$7,1^{3}$*
Aroma								
Fruity	Characteristic aroma from fruit	Weak: 0-1 Strong: 8-10	8,8	8,71	9,5	9	8,23	7,45
Sour	Aroma related to the presence of characteristic organic acids from fruit	Weak: 0-3 Strong: 8-10	7,4	7	7,7	7,83	5,4	5,5
Floral	Fresh floral notes in the aroma	Weak: 0-3 Strong: 8-10	6,1	5,6	6,8	7,2	$5,1^{3}$*	$4,3^{3}$*
Cooked	Characteristic aroma from fruit submitted to thermal processing	Absent: 0-2 Strong: 9-10	0,5	0,5	0,2	0,2	2^{3}*	$3,3^{3}$*
Fermentation	The aroma of the presence of yeast	Absent: 0-1 Strong: 10	0,5	0,5	1	1	1,5	1,5
Flavor								
Natural	Characteristic flavor from natural fruit	Absent: 0-1 Strong: 8-10	9,5	9,3	8,75	8,7	$7,8^{3}$*	$6,9^{3}$*
Acid	Flavor stimulated by the presence of characteristic organic acids from fruit	Weak: 0-3 Strong: 8-10	7,8	7,7	9,1	8,8	5,5	5,6
sweet	Flavor stimulated by the presence of sucrose and other sugars from fruit	Weak: 0-3 Strong: 8-10	6,6	6,25	6,1	5,9	6,2	6,5
cooked	Characteristic flavor from fruit submitted to thermal processing	Absent: 0-2 Strong: 9-10	0,3	0,2	0,5	0,5	$3,1^{3}$*	$4,2^{3}$*
fermentation	The aroma of the presence of yeast	Absent: 0 Strong: 10	0,3	0,3	0,2	0,2	0,5	0,5
astringency	Harsh sensation perceived in mouth and tongue characteristic of fruit	Weak: 0-4 Strong: 7-10	6,2	6,4	4,5	4,3	2,1	1,8
Consistency								
consistency	Perception in mouth of fruit wine dilution or concentration	Weak: 0-3 Strong: 8-10	8,8	8,6	9	9,1	7,1	6,96

*paired parameters ash berry wines (** for black currant, 3* for yellow plum) with t-test above the significance level P

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