Foods and Raw Materials. 2023;11(1)

ISSN 2308-4057 (Print)
ISSN 2310-9599 (Online)

Research Article
Open Access

Available online at http://jfrm.ru/en
https://doi.org/10.21603/2308-4057-2023-1-550
https://elibrary.ru/STUWOP

Grain bran hydrolysates in the production of fruit distillates

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Received 12.04.2022; Revised 17.05.2022; Accepted 07.06.2022; Published online 11.11.2022

Abstract:
Currently, there is an urgent need for domestic fermentation activators based on low-cost secondary raw materials. We aimed to study the effect of microbial enzyme preparations with different action on the hydrolysis of proteins and phytin of grain bran to obtain fermentation activators that could become an alternative to imported ones.
We studied wheat and rye brans; microbial enzyme preparations with cytolytic, proteolytic, and phytase action; multi-enzyme compositions; and grain bran hydrolysates. Firstly, we determined the kinetic characteristics of enzyme preparations. Secondly, we evaluated their effectiveness in the hydrolysis of the brans. Thirdly, we developed multi-enzyme compositions. Finally, we determined the concentration of soluble forms of phosphorus and free amino acids in the hydrolysates.
We determined optimal temperature and pH values for the enzyme preparations. The multi-enzyme compositions contributed to a high accumulation of reducing substances, water-soluble protein, and phosphorus. The concentration of free amino acids in the hydrolysates obtained under the action of the bran's own enzymes was about 20% higher in the wheat samples, compared to the rye samples. However, when using multi-enzyme compositions in addition to the bran's own enzymes, the concentration of free amino acids was 1.5 times higher in the rye hydrolysates, compared to the wheat hydrolysates.
The use of multi-enzyme compositions under optimal conditions can double the content of phosphorus and free amino acids available for yeast, compared to the control. Our results can be used for further research into using grain bran hydrolysates as an alternative source of nitrogen and phosphorus nutrition for yeast at the fermentation stage of fruit distillate production

Keywords: Grain bran, microbial enzyme preparations, multi-enzyme compositions, hydrolysates, free amino acids, soluble forms of phosphorus

Funding: The study was funded by the Ministry of Science and Higher Education of the Russian Federation (Minobrnauka) as part of the state assignments completed by the All-Russian Scientific Research Institute of Grain and Products of its Processing (VNIIZ) (Topic FGUS-2022-0006) and the All-Russian Scientific Research Institute of Brewing, Beverage and Wine Industry (VNIIPBiVP) (Topic FNEN-2019-00024).

Please cite this article in press as: Krikunova LN, Meleshkina EP, Vitol IS, Dubinina EV, Obodeeva ON. Grain bran hydrolysates in the production of fruit distillates. Foods and Raw Materials. 2023;11(1):35–42. https://doi.org/10.21603/2308-4057-2023-1-550

INTRODUCTION

Enzyme preparations of microbial origin with different action are widely used in various branches of the food industry [1–7]. Enzymatic modification of the main biopolymers of raw materials used in fermentation and winemaking facilitates the conversion of material components into a soluble state. This is due to the release of some substances from the bound state, as well as the hydrolysis of the main high-molecular polymers to soluble forms. Enzymatic hydrolysis intensifies certain stages of production, increases the yield of the end product, and improves its quality. Grain bran is a by-product of flour milling used as a substrate for enzymes [8–11].

In Russia, grain (wheat and rye) bran averages 21.3% of flour production. It is classified as a low-cost renewable raw material [7, 12, 13]. Hydrolysates

Table 1 Enzyme preparations based on standard substrates

Enzyme preparation	Producer	Standard activity	Optimal pH	Optimal temperature, °C
Cellulolytic enzyme preparations				
Agroksil Premium	Agroferment, Russia	3000 units of carboxymethyl cellulase activity 4000 units of xylase activity 2500 units of *β*-xylase activity	5.0	50
Phytase				
Agrofit	Agroferment, Russia	5000 units of phytase activity	5.0	40
Proteolytic enzyme preparations				
Penicillopepsin + Orizin (1:1)	Agroferment, Russia	serine proteinase, 6500 units of proteolytic activity	10.5	40
		acid proteinase, 300 units of proteolytic activity	4.7	30
Neutrase 0.8 L	Novozymes, Denmark	800 units of proteolytic activity	5.5–7.5	45–55

Table 2 Grain bran samples

Type of bran	Content, %			
	Moisture	Total protein (N×6.25)	Ash	Phosphorus
Wheat	13.78	13.38	4.86	0.48
Rye	10.44	13.82	5.17	0.55

obtained through the enzymatic modification of grain bran are used to produce distillates for fruit vodkas.

Fermentation is the key stage in the preparation of raw materials for distillation. Ethanol and secondary fermentation products result from complex biochemical processes under the action of the enzymatic system of *Saccharomyces* yeast.

Fruit materials used in the production of fruit vodkas have a reduced content of nitrogenous components and organic phosphorus [14–18]. Moreover, when processing highly acidic fruit materials, pulp or juice is traditionally diluted with softened water, which further reduces the concentration of components necessary for the normal functioning of the yeast population.

The lack of nitrogen and phosphorus in the fermented medium slows down the reproduction of yeast, increasing the process time and the risk of foreign microflora contamination [19]. Finally, the unbalanced biochemical composition changes yeast metabolism, leading to the accumulation of undesirable volatile substances such as acetaldehyde, acetic acid, propionic acid, and others [20–22].

The fermentation of fruit materials can be controlled by using foreign-produced fermentation activators based on sedimentary yeast autolysates [23–25]. Russia does not produce such preparations on a commercial scale.

Alternatively, grain bran could be used as a low-cost material with a high content of nitrogen and phosphorus compounds. Currently, grain bran is widely used as a source of dietary fiber in the production of bread and functional products. Previous studies have shown that grain bran can be an additional source of nitrogen nutrition for yeast in distillate production [27]. In our former study [12], we found that bran, primarily rye bran, has a high content of insoluble phosphorus compounds (phytin) which can be converted into a soluble form through enzymatic hydrolysis. The enzymatic hydrolysis of phytin during the fermentation of rye must is described by Polyakov *et al.* [28].

We aimed to determine process conditions for obtaining hydrolysates for fruit distillates from wheat and rye bran through the directed enzymatic destruction of proteins and phytin.

STUDY OBJECTS AND METHODS

Wheat and rye bran samples were obtained from two grain-processing enterprises in the Moscow region, Zernoprodukt (Noginsk) and Istra-khleboprodukt (Istra).

We used enzyme preparations of domestic and foreign production (Table 1).

The effectiveness of cellulolytic, proteolytic, and phytase enzyme preparations was determined by how well they accumulated reducing substances, soluble protein, and PO_4^{3-} ions, respectively. PO_4^{3-} ions were quantified by the colorimetric method using a calibration curve [29].

We created two multi-enzyme compositions (MEC) with cellulolytic, proteolytic, and phytase action, namely MEC 1 (Agroksil Premium + Agrofit + Penicillopepsin + Orizin) and MEC 2 (Agroksil Premium + Agrofit + Neutrase 0.8 L).

Wheat and rye bran samples are characterized in Table 2.

The enzyme preparations were introduced with an activity of 0.5 to 1.5 units/g of bran. The substrate's concentration varied from 20 to 120 mg/mL. Optimal temperature and pH were determined by studying enzyme activity at 30–70°C and 3.0–8.0, respectively.

The incubation mixture was composed of ground bran and water (1:10), citrate or phosphate-citrate buffer 0.1 M (20% of volume) with an appropriate pH value, and an enzyme preparation with an activity of

0.5 to 1.5 units/g of bran. Hydrolysis was carried out for 30 min, which corresponded to the zero order of the enzymatic reaction.

We used four control samples hydrolyzed under the action of bran's own enzymes, namely:

– wheat bran:water (1:10) hydrolyzed at pH 4.5, for 4 h at 40°C (Control 1);
– rye bran:water (1:10) hydrolyzed at pH 4.5, for 4 h at 40°C (Control 2);
– wheat bran:water (1:10) hydrolyzed at pH 5.5, for 4 h at 50°C (Control 3); and
– rye bran:water (1:10) hydrolyzed at pH 5.5, for 4 h at 50°C (Control 4).

Our experimental samples were prepared as follows:

– wheat bran + MEC 1 hydrolyzed at 40°C, pH 4.5 (Experiment 1);
– rye bran + MEC 1 hydrolyzed at 40°C, pH 4.5 (Experiment 2);
– wheat bran + MEC 2 hydrolyzed at 50°C, pH 5.5 (Experiment 3); and
– rye bran + MEC 2 hydrolyzed at 50°C, pH 5.5 (Experiment 4).

The wheat and rye brans were modified with the multi-enzyme compositions. The incubation mixture consisted of 10 g of ground bran, 100 mL of distilled water (20% of volume), and a buffer. The enzyme and substrate mixtures were preincubated at 40 or 50°C for 10 min. Enzymatic modification was carried out in two stages, 2 h each. First, we introduced the Agroksil Premium + Agrofit enzyme preparation and then, the Penicillopepsin + Orizin or Neutrase 0.8 L enzyme preparation in optimal amounts. The enzymes were inactivated by rapidly heating the incubation mixture to 85°C for 5 min. The supernatant was separated by centrifugation at 6000 rpm to use the resulting hydrolysate for further studies.

The contents of total protein, soluble protein, and reducing sugars were determined by the Kjeldahl method (N×6.25), the Lowry method, and the Bertrand method, respectively. Ash was measured by burning flour to determine the mass of the residue (State Standard 27494-2016). Moisture was determined according to State Standard 9404-88.

The content of phosphorus in the bran samples was determined in accordance with State Standard 30615-99. For this, we dry-mineralized the sample, dissolved ash, carried out a color reaction with a molybdenum-vanadium reagent, and measured the intensity of the yellow color at 440 nm in 10-mm cuvettes on an SF-2000 spectrophotometer (LOMO, Russia). The concentration of phosphorus in the liquid phase (water extracts from bran and experimental hydrolysates) was determined in accordance with State Standard R 51430-99. For this, we carried out a reaction of phosphate with molybdate in an acidic medium, resulting in a molybdate-phosphorus complex. Then, the complex was selectively reduced to molybdenum blue in the presence of ascorbic acid. Finally, we measured the optical density of the colored solution, which was directly proportional to the phosphorus content in the sample, at 720 nm in 10-mm cuvettes on the SF-2000 spectrophotometer.

Amino acids were separated by high-performance liquid chromatography on an Agilent Technologies 1200 Series instrument (Agilent, USA) with a Luna 5u C18(2) 150×4.6 mm 5 µ chromatographic column (Phenomenex, USA) with a pre-column in accordance with State Standard 34230-2017. The eluent (acetonitrile/acetate buffer solution) flow rate was 1.0 cm^3/min. The gradients for 0–28, 29–40, 41–50, 51–55, 56–60, and 61–63 min were 10/90, 28/72, 25/75, 50/50, 90/10, and 10/90%, respectively.

The results were processed by the methods of mathematical statistics in Microsoft Excel (Excel 19.0, 2018, Microsoft, USA) [29]. In particular, mean values, standard deviations, and confidence intervals were determined from three to five measurements for each sample.

RESULTS AND DISCUSSION

Firstly, we studied the main kinetic characteristics of the enzyme preparations used to modify wheat and rye bran. Figures 1 and 2 show the effect of temperature and pH on the activity of various enzyme preparations used with wheat bran.

As we can see, in the hydrolysis of non-starch polysaccharides of wheat and rye brans with Agroxil Premium (Figs. 1 and 2), the optimal pH was 4.5–5.0 (with a slight shift to the acidic region) and the optimal temperature was 40°C (with 80% of maximum activity retained at 50°C). In the hydrolysis of proteins with Penicillopepsin + Orizin, the optimal pH was 5.0 (with no activity from the alkaline serine proteinase included in the enzyme preparation), while the optimal temperature was 40°C. In the hydrolysis of proteins with Neutrase 0.8 L, the optimal pH ranges were 5.0–5.5 and 5.5–6.0, whereas the optimal temperatures were 45–50 and 50–55°C for wheat and rye brans, respectively. In the hydrolysis of phytin (inositol hexa phosphoric acid) with the Agrofit enzyme preparation, the optimal pH was 5.0 and the optimal temperature was 40°C for both brans.

The optimal amounts of the enzyme preparations at saturating concentrations of the substrate were 1.2, 1.0, 0.7, and 0.5 units of activity/g of bran for Agroksil Premium, Penicillopepsin + Orizin, Neutrase, and Agrofit, respectively.

Secondly, we studied the effectiveness of the enzyme preparations in their action on wheat and rye brans. It was determined by the accumulation of reducing substances, soluble protein, and PO_4^{3-} ions (Fig. 3). As we can see, all the enzyme preparations hydrolyzed the substrates quite actively. However, the Neutrase 0.8 L preparation was significantly more effective than the Penicillopepsin + Orizin complex under the given conditions of the enzymatic reaction. In addition, the activity of phytase was 30% higher when used on rye bran compared to wheat bran, which is

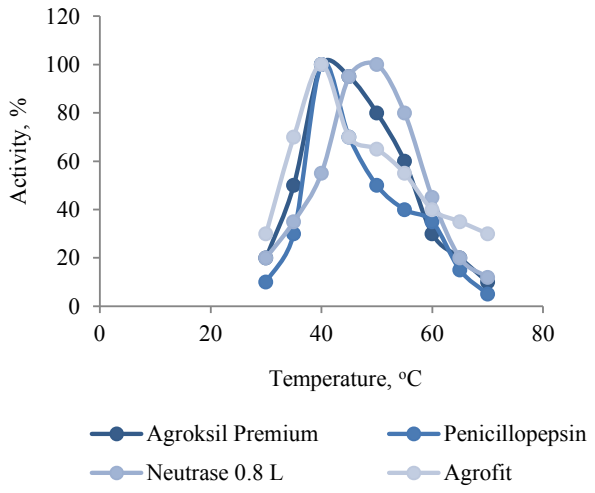

Figure 1 Effect of temperature on the activity of enzyme preparations modifying wheat bran

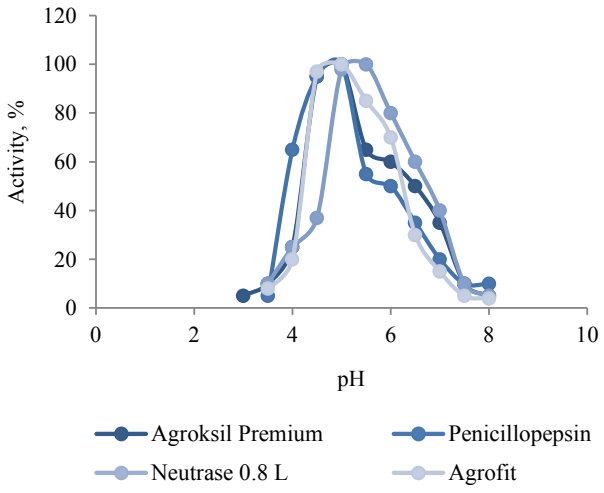

Figure 2 Effect of pH on the activity of enzyme preparations modifying wheat bran

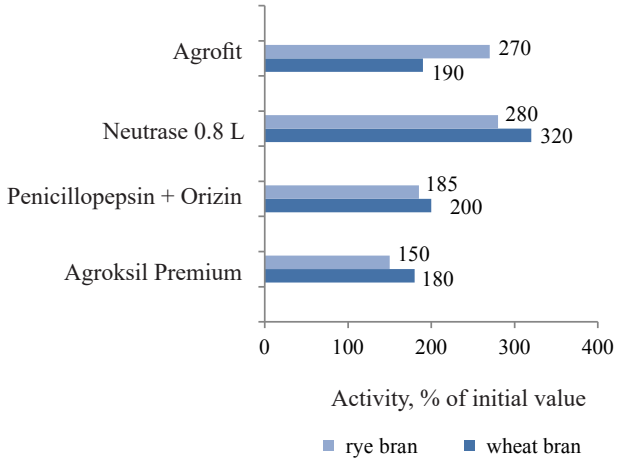

Figure 3 Effectiveness of enzyme preparations in their action on wheat and rye bran

obviously associated with the high activity of rye's own phytase [30, 31].

Thirdly, based on the kinetics of enzymatic reactions and the effectiveness of the enzyme preparations in their action on wheat and rye brans, we designed two multi-enzyme compositions (MEC) with cellulolytic and proteolytic action, as well as phytase, namely MEC 1 (Agroksil Premium + Agrofit + Penicillopepsin + Orizin) and MEC 2 (Agroksil Premium + Agrofit + Neutrase 0.8 L).

When using microbial enzyme preparations with different specificity of action, we should take into account endogenous enzyme systems, mainly acidic proteinase and grain phytase concentrated in the peripheral parts of the grain [5, 7, 30]. We used four control samples with wheat and rye brans differing in hydrolysis pH and temperature. The choice of pH was determined by the optimal values for the substrate's

Table 3 Effectiveness of the multi-enzyme compositions in their action on wheat and rye brans

Sample	Reducing substances, %	Protein (Lowry method), mg/mL	PO_4^{3-}, g/L or mg/mL
Wheat bran			
Control 1 (pH 4.5, 40°C)	0.18	0.070	0.017
Experiment 1 (with MEC 1)	0.34	0.146	0.035
Control 3 (pH 5.5, 50°C)	0.20	0.078	0.017
Experiment 3 (with MEC 2)	0.32	0.158	0.039
Rye bran			
Control 2 (pH 4.5, 40°C)	0.22	0.094	0.020
Experiment 2 (with MEC 1)	0.33	0.180	0.055
Control 4 (pH 5.5, 50°C)	0.22	0.110	0.020
Experiment 4 (with MEC 2)	0.30	0.218	0.060

MEC – multi-enzyme composition

own acidic proteinases and phytases, as well as those of the studied enzyme preparations. The results of the experiments are presented in Table 3.

The results (Table 3) indicate that MEC 1 and 2 contributed to an active accumulation of reducing substances, water-soluble protein, and PO_4^{3-} ions when used with both wheat and rye brans. Their content increased by an average of 1.3–2.3 times, with higher values for wheat bran compared to rye bran. In particular, wheat bran exposed to MEC 1 and MEC 2 had increases (compared to the controls) in reducing substances, water-soluble protein, and PO_4^{3-}

Table 4 Phosphorus concentrations in experimental and control samples of wheat and rye bran hydrolysates

Sample	Dissolved phosphorus concentration, mg%	Dissolved phosphorus, % of initial phosphorus in bran
Wheat bran		
Initial sample	26.9	5.6
Control 1 (pH 4.5, 40°C)	79.1	16.6
Experiment 1 (with MEC 1)	151.2	31.5
Control 3 (pH 5.5, 50°C)	88.5	18.4
Experiment 3 (with MEC 2)	177.6	34.7
Rye bran		
Initial sample	33.4	6.1
Control 2 (pH 4.5, 40°C)	94.9	17.4
Experiment 2 (with MEC 1)	165.4	30.3
Control 4 (pH 5.5, 50°C)	110.3	20.2
Experiment 4 (with MEC 2)	199.7	36.6

MEC – multi-enzyme composition

ions of 88 and 60%, 87 and 102%, as well as 105 and 129%, respectively. For rye bran, these indicators were somewhat lower: the increases in reducing substances, water-soluble protein, and PO_4^{3-} ions amounted to 50 and 36%, 91 and 98%, as well as 175 and 200%, when used with MEC 1 and MEC 2, respectively.

However, the absolute concentration of phosphorus in all the rye bran hydrolysates was higher than in the wheat bran hydrolysates, and the efficiency of phytase in the multi-enzyme compositions was higher at pH 5.5 (Table 4). Thus, we found that using Agrofit within a multi-enzyme composition more than doubled the amount of soluble phosphorus available for yeast, compared to the control.

Finally, we determined the composition and concentration of free amino acids in the wheat and rye bran hydrolysates obtained using two multi-enzyme compositions.

Based on their effect on yeast growth and reproduction, amino acids can be divided into easily-assimilated (aspartic acid, arginine, valine, histidine, isoleucine, and tryptophan) and poorly-assimilated (leucine, methionine, tyrosine, threonine, serine, and lysine). It has been proven that the yeast cell can assimilate most amino acids (except for proline), di- and tripeptides, as well as ammonia nitrogen. Noteworthily, the enzymatic activity of yeast cells can significantly increase in natural nutrient media with a cultivar of amino acids, such as aqueous bran extracts. In this case, yeast can directly assimilate amino acids during reproduction [32, 33]. The content of assimilable nitrogen in the medium determines the rates of yeast growth, sugar utilization, and fermentation, as well as biomass yield. The assimilation of amino acids as a result of enzymatic deamination produces various volatile components which contribute to the aroma and taste of alcoholic beverages based on distillates.

Regardless of raw materials (starch-, inulin-, sugar-containing or fruit), distillates contain predominantly higher alcohols, especially propyl, isobutyl, and isoamyl. It is known that these volatile compounds can be synthesized from the corresponding amino acids, namely threonine, valine, and leucine, respectively. Their content in bran is 8–10% of total free amino acids [27]. We found aspartic and glutamic acids, as well as asparagine to dominate in the control and experimental samples of wheat and rye bran hydrolysates (Tables 5 and 6).

We found that the concentration of free amino acids in the control wheat bran hydrolysates (obtained under the action of bran's own enzymes) was on average 20% higher than in the rye hydrolysates. However, the total concentration of amino acids in the rye hydrolysates was more than 1.3 times higher than in the wheat hydrolysates.

As can be seen in Tables 5 and 6, the multi-enzyme compositions (MEC 1 and MEC 2) increased the total concentration of free amino acids in the experimental hydrolysates by an average of 1.5–2.0 times compared to the controls. Noteworthily, the type of bran or multi-enzyme composition had almost no effect on the increase in free amino acids in the hydrolysates compared to their initial content in the raw material. This increase ranged from 192 to 205%.

We also found that the multi-enzyme compositions increased the content of the most valuable amino acids for nitrogen nutrition of yeast in the experimental bran hydrolysates [34]. Particularly, the concentrations of aspartic acid in the wheat and rye bran hydrolysates increased 1.8 and 2.5 times, respectively. The content of valine in the experimental hydrolysates increased 4–5 times compared to the initial sample.

Our analysis of the experimental samples versus the controls showed changes not only in the total concentration of free amino acids, but also in their ratios. Importantly, the total content of threonine, valine, and leucine – essential amino acids for the production of distillates – almost doubled (15–20%) compared to the initial bran samples (8–10%). Such findings have never been reported before.

CONCLUSION

We managed to scientifically substantiate the use of enzyme preparations with cellulolytic, proteolytic, and phytase action to produce wheat and rye bran hydrolysates with a high content of free amino acids and soluble phosphorus compounds as an alternative to fermentation activators based on sedimentary yeast autolysates.

We studied the kinetic characteristics of the enzyme preparations included in the multi-enzyme compositions. Also, we determined the optimal conditions for enzymatic reactions with wheat and rye brans used as a substrate, namely the initial rate of enzymatic reaction,

Table 5 Free amino acids in wheat bran hydrolysates

Amino acid	Concentration, mg/L				
	Initial sample	Control 1 (pH 4.5, 40°C)	Experiment 1 (with MEC 1)	Control 3 (pH 5.5, 50°C)	Experiment 3 (with MEC 3)
Aspartic acid	63.0	64.5	115.5	69.5	115.5
Glutamic acid	46.0	49.5	90.0	76.5	97.5
Asparagine	209.0	219.0	322.5	217.5	321.0
Histidine	21.0	34.5	49.5	36.0	48.0
Serene	12.0	18.0	28.5	21.0	24.0
Glutamine	9.0	25.5	37.5	24.0	39.0
Arginine	25.5	31.5	36.0	36.0	34.5
Glycine	66.0	105.5	162.0	97.5	157.5
Threonine	61.5	88.5	145.5	87.0	156.0
Alanine	4.5	24.0	39.0	15.0	39.0
Tyrosine	6.0	16.5	54.0	12.0	36.0
Valine	22.5	55.5	94.5	52.5	91.5
Methionine	4.5	30.0	42.0	21.0	40.5
Tryptophan	182.5	192.0	282.0	204.0	280.5
Isoleucine	15.0	37.5	60.0	34.5	58.5
Phenylalanine	13.5	63.0	100.5	43.5	97.5
Leucine	15.0	90.0	141.0	64.5	136.5
Lysine	24.0	81.0	126.0	70.5	121.5
Total	800.5	1226.0	1926.0	1182.5	1894.5
% vs. initial sample	–	130.0	205.0	125.0	202.0
% of protein nitrogen in material	5.9	8.8	13.7	8.4	13.5

Table 6 Free amino acids in rye bran hydrolysates

Amino acid	Concentration, mg/L				
	Initial sample	Control 2 (pH 4.5, 40°C)	Experiment 2 (with MEC 2)	Control 4 (pH 5.5, 50°C)	Experiment 4 (with MEC 4)
Aspartic acid	106.5	127.5	261.0	117.0	268.5
Glutamic acid	70.5	76.5	141.0	169.5	183.5
Asparagine	467.5	474.0	825.0	544.5	852.0
Histidine	18.0	42.0	72.0	40.5	73.5
Serene	13.5	22.5	34.5	24.0	33.0
Glutamine	37.5	57.0	93.0	48.0	96.0
Arginine	19.5	36.0	45.0	43.5	45.0
Glycine	58.5	114.0	175.5	88.5	174.0
Threonine	52.5	93.0	177.0	102.0	189.0
Alanine	22.5	40.5	78.0	33.0	75.0
Tyrosine	4.5	73.5	18.0	37.5	13.5
Valine	30.0	76.5	157.5	61.5	157.5
Methionine	4.5	36.0	60.0	22.5	60.0
Tryptophan	52.5	58.5	85.5	57.0	97.5
Isoleucine	13.5	48.0	91.5	37.5	99.0
Phenylalanine	15.0	75.0	138.0	46.5	148.5
Leucine	12.0	112.5	198.0	66.0	207.0
Lysine	21.0	85.5	141.0	64.5	139.5
Total	1019.5	1648.5	2791.5	1603.5	2912.0
% vs. initial sample	–	114.0	192.0	111.0	201.0
% of protein nitrogen in material	7.7	11.9	19.4	13.3	20.9

temperature, pH, enzyme concentration, and saturating substrate concentration.

We developed two multi-enzyme compositions that contained enzyme preparations with cytolytic, proteolytic, and phytase action and studied their effectiveness. According to our results, the multienzyme compositions contributed to an active accumulation of reducing substances, water-soluble protein, and soluble

phosphorus available for yeast. Their action on wheat and rye brans more than doubled the concentration of water-soluble phosphorus.

Our study showed that the concentration of free amino acids in the hydrolysates obtained under the action of bran's own enzymes was 20% higher in the wheat samples, although the absolute value of this indicator was higher in the rye samples.

The multi-enzyme compositions increased the total concentration of free amino acids in the experimental hydrolysates by an average of 1.5–2.0 times, including the most valuable amino acids for nitrogen nutrition of yeast – aspartic acid (2.5 times) and valine (4–5 times), compared to the hydrolysates obtained under the action of bran's own enzymes.

Our results can be used for further research into grain bran hydrolysates as an alternative source of nitrogen and phosphorus nutrition for yeast at the fermentation stage of fruit distillate production.

CONTRIBUTION

The authors were equally involved in writing the manuscript and are equally responsible for plagiarism.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGEMENTS

The authors thank Prof. A. P. Sinitsyn, Head of the Laboratory of the Fundamental Biotechnology Research Center, Russian Academy of Sciences, for providing the samples of enzyme preparations.

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