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ENZYMATIC MODIFICATION OF WHEAT BRAN

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Analysis of recent research and publications

By-products of grain processing are a source of biologically active substances and biocorrectors of human nutrition. Their rational use is a task for modern innovative biotechnology.

Functional properties of cereals are associated with various minerals, vitamins, and numerous bioactive compounds present in the grain. Among them, special attention has recently been paid to so-called phytochemicals – non-food plant chemicals having a protective and preventive effect on health [1-3].

Physiological functions of phytochemicals are different. They can be antioxidants (carotenoids, flavonoids, polyphenols, allyl sulphides, etc.), phytoestrogens (isoflavones, lignans), enzyme effectors (indole, protease inhibitors, terpenes), antibacterial substances (allicin). Besides, they can

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Abstract. The article presents conceptual approaches to solving technological and technical problems when developing functional food products. General approaches have been suggested to change currently used technologies in order to make complex processing of raw materials more efficient and increase the output of high-quality foods and food ingredients with antioxidant properties. Cereal crops are the richest source of functional ingredients and the main component in the human diet. It has been proved that products of their processing contain a lot of useful substances. For the first time, polyphenols have been obtained from grain raw materials by biotechnological methods. It has been shown that a practical way to purify and cleave a polysaccharide matrix is pretreatment of raw materials with amylolytic and proteolytic enzymes. Based on the laws of enzymatic hydrolysis of polysaccharides, the wheat bran were treated with a multi-enzyme preparation *Viscozyme L* with hemicellulase, cellulase, pectinesterase, and feruloyl esterase activities. This caused a high effect of destruction of certain covalent bonds among polymers of the cell walls of the bran, and resulted in high extraction of polyphenols from raw materials, for example, of ferulic acid from 40.99 to 2507.9 µg/g. The effect of plant polyphenols on cultivation of probiotic microorganisms has been characterised. The prebiotic properties of polyphenols obtained from wheat bran and those of a polyphenol concentrate from grape pomace ENOANT have been compared. It has been shown that enzymatic hydrolysis of wheat bran allows increasing the content of free polyphenols. It has been established that a polyphenol extract from wheat bran can be used for its intended purpose as an effective antioxidant that has no negative effect on the body's main physiological systems.

Keywords: enzymatic hydrolysis of wheat bran, hydrolases, phenolic substances.

prevent DNA of cancer cells from replicating (saponins, capsaicin, etc.) and pathogens from adhering to the walls of human cells (proanthocyanidins) [4].

Phenolic compounds are the most common secondary metabolites of plants. Due to their low toxicity, they have a positive effect on physiological processes in the human body and increase its resistance. According to their biological activity, distribution, and prospective use, phenols fall into three groups: phenolic acids, flavonoids, and polyphenols. Today, the most promising direction of applied studies is studying the antioxidant, antibacterial, cytotoxic, and neurotoxic properties of phenolic compounds. This will help obtain environmentally safe drugs of natural origin for the pharmaceutical, food, and agricultural industries. New sources of phenolic compounds of plant origin (for example, cereal crops) are being searched for, as they can be an effective alternative to their synthetic analogues.

Cereals, as sources of phytochemicals and dietary fibres, can be widely used in food systems being added as antioxidants and prebiotic substances. Among phytomaterials contained in cereals, polyphenols are of the greatest interest due to their various positive effects on human health. Fortification of food products with phenolic extracts can be an effective technological approach to improve the physiological effects.

Research materials and methods

Food polyphenols are not absorbed in the small intestine by 90–95%. Thus, they reach the large intestine, although it should be emphasised that their absorption and metabolism largely depend on the chemical structure of polyphenols (PP). Most flavanoids are poorly absorbed in the small intestine and are well metabolised in the large intestine. It is shown by the authors [4,5] that isoflavones are among the best polyphenols with high absorption capacity; catechins, flavanones, and glycosides of flavanols are of medium absorption capacity; and proanthocyanidins, flavan-3-ols, galates, and anthocyanins are poorly absorbed in the intestine.

Cereals contain such phytochemicals as phenol type antioxidants, saponins, sterols, and phytoestrogens. Some health-improving effects of cereals are due to the structural features of their dietary fibre complexes with phenolic compounds, lignin, and other bioactive molecules. Ferulic acid is the predominant phenolic compound in cereal bran. Other polyphenols are phenolic acids, namely: p-Coumaric, sinigrin, and vanillic [5].

Phenolic antioxidants are especially widely represented in cereals by phenolic acids. They are present in cereals in the free form, or are bound through etheric bonds with fatty acids, sugars, and polysaccharides of cell walls [6]. Given the fact that most of the useful substances of grain are found in bran, using it to manufacture functional ingredients is a good alternative to synthesised substances, and this tendency is becoming more and more progressive. So, secondary products obtained after grain is processed by biotechnological methods need more research. It can help use them rationally in food products as correctors of human intestinal microbiota.

The normal microbiota of the human gastrointestinal tract (GIT), or its microbial landscape, is a result of long evolution.

The main active components of probiotic preparations are live probiotic microorganisms that have antagonist properties to a wide range of pathogenic and opportunistic pathogenic bacteria. Their main purpose is to normalise the host's microbiota.

The use of antibiotics and pharmaceutical chemicals, the influence of radio waves and chemotherapy, the impact of xenobiotics from food lead to development of diseases of the gastrointestinal

tract and other organs and systems of the body. Biological bacterial preparations have proved to be helpful in preventing and treating GIT dysbiosis. However, in recent years, a decrease has been observed in their effectiveness, and results of treatment with these preparations have been unstable, especially in sensitised organisms, because the heterogeneous microbial mass of the preparation and sometimes additional components in its composition create a significant antigen load on the body. There are also other problems of using biopreparations and functional products: they are not resistant to oxygen, heating, and GIT protective barriers.

More and more studies testify to the importance of intestinal microbiota for human health, both mental and physical. Intestine bacteria not only help the maximum absorption of nutrients and energy, but are also important for the health of the entire body [7]. In particular, microbial intoxication and imbalance in the intestinal microbiota composition result from intestinal disorders (like chronic inflammatory bowel disease), other immune disorders, the use of antibiotics, etc. [8,9]. Although genetic and environmental factors are the key ones to determine the composition of the intestinal microbiota, it has long been established that diet affects the activity of microbes, the total number of bacteria, their species composition, and their viability in the intestine. In fact, inter-individual variance in intestinal microbiota may, to some extent, reflect differences in the diet, although changes in the diet may also cause individual responses of different people's intestinal microbiota [10].

Phenolic compounds, or polyphenols, are secondary metabolites in the plant kingdom. Natural polyphenols can be classified into two main groups: flavonoids and non-flavonoids. Among flavonoids, various groups can be distinguished according to their C-heterocycle structure: flavonols, flavones, flavan-3-ols, isoflavones, flavanones, dihydroflavonols, anthocyanidins, and chalcones (Fig. 1).

It is known that there are three forms of phenolic compounds in cereals: insoluble (70–80%), conjugated soluble (15–30%), and free phenols (5–8%). Ferulic acid in wheat bran is covalently bound with arabinoxylans. Using organic solvents to extract polyphenols (PP) is not an effective method to obtain them, since there is a low yield of PP and a negative effect of toxic reagents.

Non-flavonoid phenols include phenolic acids that hydrolyse tannins and stilbenes. PP are also an integral part of human diet. A lot of them are contained in commonly consumed fruit, vegetables, and other plant-based products such as cocoa, tea, or wine. A number of epidemiological studies have shown that dieting with an excessive consumption of fruit and vegetables is directly associated with the risk of various chronic diseases, such as coronary heart disease, specific cancers, and neurodegeneration [11,12].

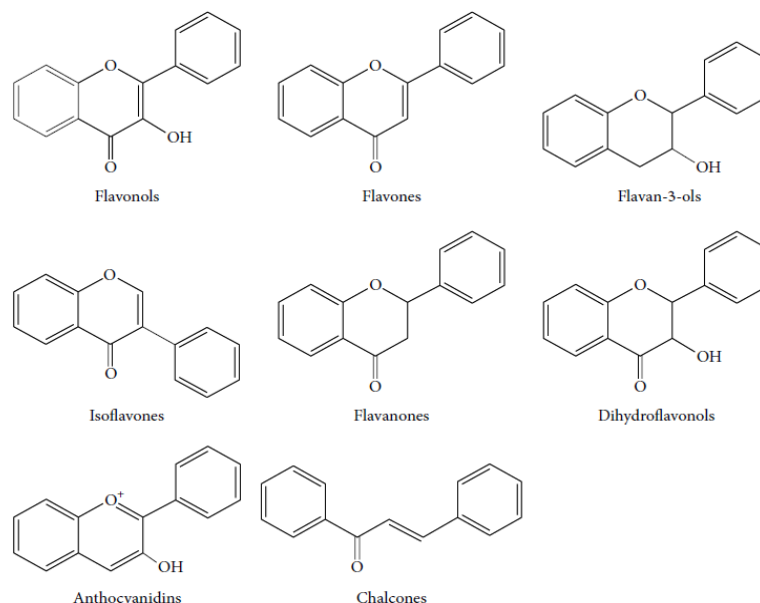


Fig. 1. Phenolic compounds present in food products

A number of pharmacological effects of various phenolic compounds, especially flavonoids, have been analysed *in vitro* and *in vivo* [13,14]. However, the effect of these compounds on human health depends mainly on their bioavailability. That is why, to understand their behaviour *in vivo*, it is important to find out how they are absorbed, converted in the course of metabolism, and excreted from the body.

Modulation of gastrointestinal microbiota by polyphenols has attracted particular interest in recent years. Various authors have done their research, their approaches ranging from the simplest experiments to test the effect of polyphenols on the growth of isolated intestinal bacteria to complex approximations involving a fundamental study of microbiota excreted with faecal masses. There have also been experiments on fermentation that consisted in compositional analysis of faecal samples of animals and humans.

Thus, some authors pay attention to the effect of food components (including polyphenols) on the gastrointestinal microbiomes, while others focus on the effect of food polyphenols on microbial modulation and their potential consequences for human health [15-19]. Selma et al. published the first review, where they considered some issues of microbial degradation of polyphenols and modulation of gastrointestinal microbiota involving PP and their metabolites [20]. The effect of PP on intestinal bacteria from grapes and teas has also been studied [21,22]. The development of biology and microbial techniques has significantly increased our knowledge of intestinal microbiota and its modulation due to food components, including polyphenols. The opportunities offered by the new metabolomic approaches in studying the effect of polyphenols on intestinal microbiota have also been considered [23].

The **purpose** of this work was to study *in vitro* the PP content in enzymatically modified wheat bran and their effect on probiotic microorganisms.

The main **objectives** of the study were:

- obtaining polyphenols (PP) from grain raw materials (wheat bran) by treating them with enzyme preparations;
- determining their quantity and quality characteristics;
- studying the effect of polyphenols on the cultivation of probiotic microorganisms.

Research materials and methods

The materials used in this study were: wheat bran with the fraction size 1.09 to 1.64 mm (from the company Sofia LLC), grape pomace of the Cabernet-Sauvignon variety harvested in 2018, the multi-enzyme preparation *Viscozim-L* (with the following activities: β -glucanasic – 100 U/g, xylanasic – 50 U/g, cellulasic – 70 U/g, pectinesterasic – 40 U/g), polyphenol concentrate from grape pomace *Enoant*, probiotic microorganisms *Lactobacillus plantarum* LB4 *Bifidobacterium bifidum* BB24 from the museum of the Department of Biochemistry, Microbiology and Nutrition Physiology, ONAFT.

A complex of traditional and modern biochemical, physicochemical, and microbiological methods of research was applied: proteins were determined by the Kjeldahl method [24], the mass fraction of lipids by the Soxhlet extraction method [25], starch by the Ewers polarimetric method [26], dietary fibres by the enzymatic method of determining soluble and insoluble dietary fibres [27].

The diagram of the research is presented in Fig. 2

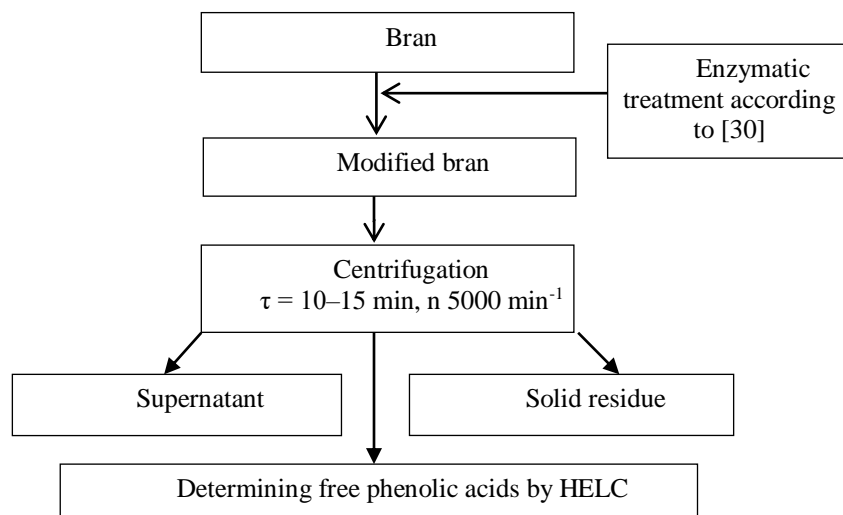


Fig. 2. Analysis chart of the PP compounds of the modified bran

Enzymatic hydrolysis was carried out at a temperature of 45°C for 5 hours at pH 6.7. After the enzymolysis and thermal inactivation of the enzyme at 45°C for 10 min, the solid residue was separated from the supernatant by centrifugation at 5000 min⁻¹, τ=10 min, with the hydromodulus G1:10 and the concentration of the enzyme preparation 0.015% of the dry weight.

Extraction of polyphenols from grape pomace was carried out with 96% ethyl alcohol during 72 hours in a dark place.

The quantitative and qualitative composition of polyphenols was determined by high-performance liquid chromatography (HPLC) using the system Prominence LC-20 Shimadzu (Japan). The system consisted of the following functional modules: degasser DGU-20A3, pumping module LC-20AD, cooling autosampler SIL-20AC, photometric detector SPD-20AV, column thermostat CTO-20A, reversed phase column Agilent Technologies Microsorb-MV-150 (silica gel with the grafted group C18 (-CH₂)₁₇CH₃), 150 mm long, 4.6 mm in diameter, the sorbent grain size 5 μm).

Preparation of samples for HELC: phenolic substances from dietary fibre and pomace were extracted by means of 80% ethyl alcohol, with the hydromodule 1:15, and kept for 72 h in a dark place. The supernatant obtained after enzymolysis was investigated by the HELC method for the content of phenolic substances without any other manipulation.

The mobile phase (eluent) composition: methanol, and 0.9% solution of phosphate acid in deionised water (Sigma-Aldrich reagents, Germany).

The substances in the extract were identified by comparing the retention time and spectral characteristics of the substances analysed with similar characteristics of standards according to the PP identification procedure shown in Fig. 2 [28]. The wavelengths of the chromatography were 225, 255, 286, and 350 nm [28–31].

The following reference substances were used to identify the substances under study, or determine what polyphenol groups they belong to: chlorogenic and caffeic acids (phenolic acids), catechin (catechins), the flavonols myricetin, quercetin, and rutin, the flavanones naringenin, naringin, hesperidin, and hesperetin, the flavones luteolin and apigenin, the anthocyanin cyanidin (anthocyanins) (Sigma-Aldrich, Germany). The identification characteristics of these standards were obtained under the chromatography conditions described above. The calibration curves “peak area – reference substance content” were linear, with an accuracy of at least r²=0.994. Free phenolic acids were determined in the way described in the works [32,33].

Before the microbiological study, the polyphenol samples from wheat bran were sterilised at t=120°C, τ=20 min. For cultivation, the *L. plantarum* LB4 *Bifidum* BB24 strains of probiotic microorganisms were used. They were cultivated on an MRS culture medium with polyphenols added in different concentrations. The cultivation was carried out by the thermostatic method at t=30°C, τ=24 h. After the cultivation, the number of viable cells was determined by the most probable number method (MPN) [34].

The medico-biological tests used three groups of eight male white rats with an average weight of 330–400 g. The first group was intact. The second group included the animals who had been given the antibiotic Lincomycin to cause abiosis: the antibiotic had been administered in 70 mg/kg doses with the drinking water for 7 days. The third group had also undergone antibiotic therapy, but with a wheat bran polyphenol concentrate added to the diet, with the fixed concentration 2 g/kg, in therapeutic doses, in combination with products containing live cells of probiotic microorganisms (lg 10.12 CFU/cm³). The experimental studies complied with the national General Ethical Principles for Experiments on Animals.

Results of the research and their discussion

The enzymatic method of receiving PP has significant advantages over chemical methods. The main mechanism of enzymatic hydrolysis is conversion of insoluble components in a solution. Enzymatic hydrolysis by multi-enzyme compositions, which include endo and exo-xylanases, endo and exo-glucanases, and β -glucosidases with ferulesterases, is performed under heterogeneous conditions. Hydrolysis of glycosidasic bonds of polysaccharides leads to changes in the matrix structure of grain raw materials (bran), and reduces the mechanical strength. Since the kinetics of the hydrolase enzyme action lies in the diffusion zone, which is explained by the substrate's low availability, the raw materials used for the experiments were of a different particle size (0.59 up to 1.64 mm).

The research results have shown that xylooligosaccharides are formed, with a simultaneous release of polyphenolic compounds, due to transformation of wheat bran matrix (pre-treated by the amylolytic enzymes α and γ -amylases) in the course of limited enzymatic hydrolysis by β -xylanases of the enzyme preparation Viscozyme L, as described in the section *Materials and methods*. The total PP yield is 2% of the bran, which results in redistribution of chemicals in the dry residue after modification, as shown in Table 1.

HELC has allowed determining which representatives of polyphenols were present in the wheat bran after their pre-extraction with ethanol. And after enzymatic treatment, polyphenols were determined in the supernatant. The results are presented in Table 2.

As it can be seen from Table 2, monomers of polyphenolic substances in bran are mainly represented by ferulic acid. After the enzymatic treatment, its content increased from 40.99 $\mu\text{g/g}$ to 2507.9 $\mu\text{g/g}$. The amounts of vanillic, syringic, and salicylic acids increased significantly, too.

These results can be explained by the following: during the enzymatic treatment, enzymes with xylanase, cellulose, and pectinesterase activity catalysed hydrolytic cleavage of xylooligosaccharides (XOS), arabinoxxylooligosaccharides (ACOS), hemicellulose, and, partially, cellulose, with the release of bound polyphenols.

Due to the action of enzymes with xylanase activity, enzymolysates accumulate 33.4% of XOS and ACOS, as shown in Table 1.

As noted in the review of literature, polyphenolic compounds, along with antioxidant activity, show prebiotic properties. To study these properties in wheat bran polyphenols, we analysed their effect on probiotic microorganisms *in vitro* in comparison with classical grape pomace polyphenols obtained by alcohol extraction of pomace. The polyphenol composition of grape pomace is shown in Table 3.

Table 1 – Chemical composition of bran ($n=3$, $p \leq 0.95$)

Parameters	Original bran	Modified bran	
		Solid residue	Supernatant
Protein substances, %	13.7	13.3	0.4
Lipids, %	3.5	3.02	0.48
Starch, %	22.0	–	22.0
Dietary fibre, %	56.3	45	–
Cellulose	16.3	16.3	–
Hemicellulose	35	4.6	31.4
Lignin	5	5	–
Phytic acid, %	4.1	1.75	2.35
Polyphenols, $\mu\text{g/g}$	1155	–	1762
Phenolic acids, $\mu\text{g/g}$	1050	–	1216
Flavonols, $\mu\text{g/g}$	327	54	273

Table 2 – Monomeric composition of wheat bran polyphenols

Group of monomeric components of polyphenols	Content, $\mu\text{g/g}$	
	before fermentation	after fermentation
Gallic acid	32.77	32.77
Protocatechuic acid	3.03	3.03
<i>p</i> -hydroxybenzoic acid	11.17	24.3
Vanillic acid	11.82	30.22
Syringic acid	18.24	52.24
<i>p</i> -Coumaric acid	24.66	136.96
Ferulic acid	40.99	2507.9
Isoferulic acid	26.61	308.81
Salicylic acid	48.2	578.25
Total monomers	217.49	3674.4

Table 3 – Polyphenol composition of grape pomace

Polyphenols	Content, $\mu\text{g/g}$	Certain substances	Content, $\mu\text{g/g}$
Phenolic acids	13.70		
Catechins	1449.16	catechin	509.48
Catechin-like substances	444.48		
Flavonols	51.57	Rutin	21.11
		Quercetin	25.35
Flavanones	7.73	Naringin	2.78
Stilbenes	21.44	Resveratrol	0
Not identified	36.72		
Total	2024.8		

As it can be seen from Table 3, polyphenols of grape pomace are mainly represented by catechins and catechin-like substances.

After determining the amounts and types of polyphenols contained in wheat bran and grape pomace, the next step was to determine the effect of polyphenols

from various plant sources on the growth of probiotic microorganisms.

The results of cultivation are shown in Fig. 4. As seen in Fig. 4, the number of probiotic microorganisms decreases with an increase in the number of polyphenols. This can be explained by the powerful antioxidant properties of polyphenols, which, in turn, leads to a decrease in the amount of free oxygen in the medium. Lactic acid microorganisms are aerobic, so the less oxygen is available, the lower the degree of accumulation of microorganisms. It can also be noted that in the samples with polyphenols (at the concentrations 5 and 10 mg/ml, respectively), there are more microorganisms than in the control sample. So it can be concluded that polyphenols of wheat bran and grape pomace have prebiotic properties. In the samples with pomace polyphenols, this parameter is lower than in the bran samples, which can be due to the peculiarities of the chemical composition of pomace. Grape pomace is known to contain quite a lot of tannins, which, along with polyphenols, are released during extraction, as shown in Fig. 5. It is also a well-known fact that tannins inhibit microbial growth, so their presence neutralises, to some extent, the prebiotic effect of polyphenols.

The study of the effect of wheat bran polyphenols compared with grape pomace has shown that wheat bran polyphenols have better prebiotic activity, because their composition includes polyphenols of different classes, and besides, because pomace polyphenols contain tannins that inhibit the growth of probiotic microorganisms.

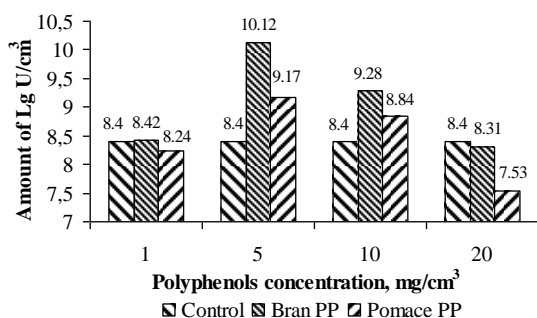


Fig. 4. Accumulation of probiotic microbiota *L. plantarum* according to the polyphenol concentration

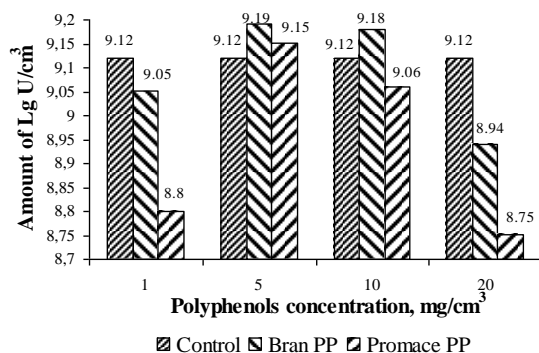


Fig. 5. Accumulation of probiotic microbiota *B. bifidum* according to the polyphenol concentration

Approbation of results

After the experiments that allowed establishing the prebiotic properties of polyphenols, it was necessary to determine how safe these substances are for living creatures. It was done at the Ukrainian Research Institute of Transport Medicine.

During the experiment involving the use of a wheat bran polyphenol concentrate, the test animals manifested no significant changes in their morphometric and physiological parameters.

Using the polyphenol concentrate normalised the morphological composition of intestinal microbiota in all test groups.

The wheat bran polyphenols introduced in the experimental animals' diet in the form of a powdered dietary supplement caused no functional changes that could have been attributed to the action of this dietary supplement.

Thus, the food concentrate of wheat bran polyphenols can be used as an effective means to normalise the intestinal microbiota composition.

Conclusion

Polyphenols present in wheat bran have been identified. High-performance liquid chromatography has shown that polyphenolic substances in cereals are mainly ferulic and salicylic acids, their contents being 2507.9 µg/g and 578 µg/g, respectively. Grape polyphenols are represented mainly by catechins and catechin-like substances, and their quantities are 1449.16 µg/g and 444.48 µg/g, respectively.

It has been found that most polyphenols are associated with the bran matrix. The use of Viscozyme L resulted in high degradation of certain covalent bonds among the cell wall polymers of the bran, and, consequently, in high extraction of polyphenols from the raw material. It has been established that this method of obtaining the target components under the optimum conditions (at 45°C, for 5 hours, with pH 6.7 and HM 1:10) increases the yield of polyphenols from 217.49 to 3674.4 µg/g.

The comparative characteristic of the polyphenol extracts obtained from wheat bran and grape pomace has shown that the extracts differed in their polyphenol composition but insignificantly.

The prebiotic properties of polyphenols have been confirmed. As shown in Fig. 4 and 5, with a certain concentration of polyphenols, the number of probiotic microorganisms increases compared to the control, which proves the prebiotic effect of polyphenols.

It has been shown that a polyphenol extract can be used as a dietary supplement with antioxidant and prebiotic properties.

Enzymatically modified wheat bran has been found to be a potential source of bioactive substances and antioxidants and can be used as functional food ingredients.

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ЕНЗИМАТИЧНА МОДИФІКАЦІЯ ПШЕНИЧНИХ ВИСІВОК

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Анотація. У статті наведено концептуальні підходи вирішення технологічних і технічних проблем при створенні функціональних продуктів харчування. Запропоновано загальні підходи щодо зміни існуючих технологій з метою підвищення ефективності комплексної переробки сировини і збільшення випуску високоякісних продуктів та інгредієнтів харчування з антиоксидантними властивостями. Злакові культури є найбагатшим джерелом функціональних інгредієнтів і основною складовою частиною в раціоні харчування людини. Доведено, що велика частина корисних речовин знаходиться у продуктах його переробки. Вперше отримано поліфеноли із зернової сировини біотехнологічним шляхом. Обґрунтовано доцільність попередньої обробки сировини амілолітичними та протеолітичними ферментами для очищення та розщеплення полісахаридного матриксу. Згідно закономірності ферментативного гідролізу полісахаридів, використано обробку пшеничних висівок мультиферментним препаратом Viscozyme L з геміцелюлазною, целюлазною, пектинестеразною та ферулоестеразною активностями, що зумовило високий ефект деструкції певних ковалентних зв'язків між полімерами клітинних стінок висівок і, як наслідок, високе вилучення поліфенолів із сировини, наприклад ферулової кислоти з 40,99 до 2507,9 мкг/г. Охарактеризовано позитивний вплив рослинних поліфенолів на культивування пробіотичних мікроорганізмів. Надано порівняльну характеристику пребіотичних властивостей поліфенолів отриманих з пшеничних висівок, та виноградних вичавок. Показана можливість за допомогою ферментолізу пшеничних висівок збільшувати частку вільних поліфенолів. Встановлено, що екстракт поліфенолів з пшеничних висівок можна використовувати за призначенням як ефективний антиоксидант, який не володіє негативним впливом на стан основних фізіологічних систем організму.

Ключові слова: ферментоліз, пшеничні висівки, гідролази, фенольні речовини

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