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REVIEWS

EXPERIMENTAL ARTICLES

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ANALYTICAL REVIEW OF BIOTECHNOLOGICAL PROBLEM OF UKRAINIAN HARD CHEESES

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An analytical review of the biotechnological process of production of various hard and semi-hard cheeses in the EU and Ukraine, as well as domestic recipes of fermented cheeses for production at craft cheese factories and at home was conducted. An analysis of the conditions of the key stages of production, including fermentation, coagulation and ripening was carried out. The composition and type of lactic acid bacteria in sourdough for fermented cheeses, as well as enzymes for fermentolysis and coagulation of milk casein was studied. Because of a complex study, 73 types of hard and semi-hard cheeses were analyzed: 35 recipes of the New England Cheesemaking Supply Company by Jim Wallace; 30 production processes of hard and semi-hard cheeses from the EU; 8 technical conditions of hard cheeses of Ukrainian producers. It was shown that the prospects for optimizing the development of new types of hard and semi-hard cheeses in Ukraine are mainly related to the regulation of the time and temperature of fermentation, cooking and ripening of cheeses, as well as the expansion of the biodiversity of the primary and secondary microbiome of starter cultures to improve the taste and aroma of the ready-to-use product.

Purpose to analyze the fermentation process and recipes for the production of hard cheeses in Ukraine with the main world samples, to compare the composition and type of lactic acid bacteria in industrial and craft starters, as well as the types of enzymes for fermentolysis and casein coagulation of milk in order to optimize production to improve the taste and aroma of ready-to-use product.

Materials and methods. Methodical analysis and abstract-logical method for summarizing the evaluation criteria of the biotechnological process of various hard and semi-hard domestic cheeses with world samples according to recommendations, requirements and standards with the development of patents, technical conditions of their production in the EU and Ukraine; DSTU 6003 (Solid cheeses); New England Cheesemaking Supply Company cheese recipes by Jim Wallace. The obtained data were processed by the methods of statistical analysis, systematization, comparison and generalization of information.

Results. The documentation was examined and the data of standards, regulations, requirements and recommendations regarding the biotechnology of hard and semi-hard cheeses were analyzed. An analysis of the market of hard cheeses and the peculiarities of the fermentation process of their production were carried out. The determination of critical points and key stages of production using industrial deposited fermentation producers and protein enzymes coagulation and biodiversity of lactic acid bacteria in sourdoughs for fermented cheeses at world productions were given. An assessment of the prospects for developing new and improving the biotechnology of Ukrainian cheese of high quality and safe hard cheeses for healthy nutrition were done.

Key words: hard cheese; semihard cheese; acidification; mesophilic and thermophilic lactic bacteria; coagulation; rennet; chymosin; renin; pepsin; cheese maturation; microbiome.

Production of fermented cheeses is represented in every country of the world, as consumers choose them for their nutrition, rich taste and aroma. The oldest and largest producers of authentic cheeses are European countries such as the Netherlands, Switzerland, Denmark, France, Italy and Spain.

The production of dairy products is one of the important sectors of global agribusiness and a point of innovative implementation of the food bioindustry [1]. According to the Union of Dairy Enterprises of Ukraine in 2019, milk production amounted to 8 million tons, and 35 Ukrainian producers were added to the list of exporters of dairy products to Saudi Arabia [2]. The results of monitoring by experts of the state and development prospects of the domestic market of milk and dairy products in 2021 in Ukraine produced 8.72 million tons of milk, including farm enterprises produced 2.75 million tons of milk. Today, there are about 200 milk fermentation enterprises in Ukraine, with the concentration of production at large milk processing enterprises. In 2020, compared to 2019, the volume of exports of dairy products to Moldova, Georgia, Azerbaijan and Kazakhstan increased by 9–11% in each country. [3].

However, there are many factors that negatively affect the innovative development of enterprises in the food industry of Ukraine, in particular, in cheese making, which reduce their competitiveness. Thus, as of January 1, 2022, the total volume of the shadow segment on the Ukrainian dairy market was estimated at 20–25%. Among the falsified goods, various types of cheese, butter, and sour cream prevailed, which were dominated by substances of vegetable origin, and the content of milk raw materials did not exceed 26% [4], and, under the influence of war uncertainty, these indicators can be even worse [5].

In the structure of dairy production in Ukraine for 2020, according to the marketing research of the Pro-Consulting Company, influencing factors, problems and development trends of one of the most important food industries of our country — the dairy market in Ukraine, including fermented milk products, were determined and studied. 4%, cheeses — 9.4%. The cheese market was characterized by multidirectional trends: the volume of fresh cheese production continued its growth in 2021 (+7.4%); the production volume of other (fermented) cheese in 2021 continued its decline (–14.2% to the level of 2020, 70 thousand tons); processed cheese production volumes in 2021 decreased slightly from their 2020 levels, returning to 2018 levels. Mainly dessert yogurt products and cheeses are imported into Ukraine. The main suppliers of dairy products to the domestic market are Poland, Germany, the Netherlands, France, Italy, and Belgium. [6].

Fermented cheeses are produced all over the world. Europe has the richest history of producing authentic cheese recipes, namely countries such as the Netherlands, Switzerland, Denmark, France, Italy and Spain. As defined by cheesemakers on the food market today, the most popular and included in the TOP-10 types of cheese in the world are Mozzarella, Emmentaler, Parmigiano reggiano, Brie, Chedar, Camembert, Gouda, Feta, Swiss cheese, and Grujere [7].

World production of cheese according to the International Dairy Federation — (International Dairy Federation (IDF)) in 2017 reached 20.5 million tons, in 2019 it was estimated at 25 million tons. The main producers of cheese in the world are the EU and the USA. Together, they produce about 16 million tons of cheese. The largest importers of cheese are Germany, Great Britain, Italy, France, and the United States, according to the agricultural information agency Agravery [8]. By 2020, over the past 50 years, the global cheese market has grown fourfold. The main consumers, in fact, as well as producers, remain European countries, although cheeses from the USA, Canada and Japan enter the market. There are currently no hard cheeses with bifidobacteria on the market.

The dairy industry of Ukraine is one of the leading ones in the agro-industrial complex, and cheese production is an important component of it. The production of hard cheeses by Ukrainian milk processors and transnational companies (Almira, Terafood, Milk Alliance, Como, Rud, Milkiland Ukraine, Lustdorf) with Ukrainian enterprises in Ukraine in 2015 decreased by a quarter (landlord.ua.). Until 2018, in Ukraine, 12 large enterprises produced 60% of cheese and cheese products (108 thousand tons), 87 dairy enterprises of medium capacity produced 50 thousand tons of cheese and cheese products, which was 28% of the total volume of production, and the rest — 22 thousand tons (12%), products are produced by small enterprises. Among the cheese-making enterprises there are large Ukrainian producers according to the Kompass Ukraine business directory (Kharkiv, info@kompass.ua) — Company ZhSZ —

Zhydachiv cheese factory, TDV (Zhydachiv, 1946, 1994); Company Buchatsky syrzavod, LLC (Buchach, Ternopilska, 2002); Company ZSK — Zvenigorod cheese factory, PrJSC of the SAVENCIA international group. They are such as Zvenigorodka, Cherkassy, 1931, TM Zveny Gora, 2001; Globinsky maslosirzavod, Globino Corporation LLC (Globino, Poltava, 1929, 2006); Cheese factories of the Lactalis company in Ukraine (Mykolaiv, 1996, Pavlograd, 2007, Shostka, 2021); Tulchyn cheese factory, branch of Terra Food (Tulchyn, Vinnytsia, 1999); Chertkiv cheese factory, company Chortkivsyr, PP (Chertkiv, 1967); Pyryatyn cheese factory, Milk Alliance holding LLC (Pyryatyn, Poltava, 1973); Okhtyr cheese factory, a branch of PP Ros of the Milkiland company (Okhtyrka, Sumy, 1988); Myrhorod Cheese Factory, LLC, a member of the State Enterprise "Milkyland-Ukraine" (Myrhorod, Poltava 1982, 2002); Andrushivsky maslosyrzavod, LLC (Andrushivka, Zhytomyrska, 2000); Novograd-Volyn cheese factory, PJSC Zhytomyrmoloko (Zhytomyrska, 1982); Losynivskyi maslosyrzavod, LLC (Nizhynskyi, Chernihivska, 1939, 1999); Euro-milk Joint Ukrainian-Czech Enterprise (Rohatyn, Ivano-Frankivsk, 1997, 2004); private cheese factories — "Forest goat", "Selyska cheese factory", "Farmer's dairy products", "Stanislavskaya cheese factory", "Obereg", "Stary Porytsk", "Cheeses from the farmer", "European cheese factory", "Chesnikov cheese factory", " Lviv Cheese Factory", and others [9].

In the structure of the country's dairy industry, cheese production makes up about 10%. More than 150 enterprises are engaged in the production of cheeses in Ukraine, 2/3 of which produce hard rennet cheeses, the rest — soft and processed (melted) [6].

The basis of the assortment of cheeses in Ukraine are hard cheeses that are pressed with a low temperature of the second heating. In August 2018, 17,125 tons were produced in Ukraine [10]. The output of cheese has increased significantly, but the further increase in the volume of its production is already in doubt. According to the data of the State Statistics Service of Ukraine for 2020. only 5-6 thousand tons of goods have been sold to foreign markets. With the total production of cheese in France of 1.8 million tons per year, one French person consumes 26.8 kg per person, and in Ukraine — 4.2 kg per person (the total volume of production is 140 thousand tons) according to the agricultural information agency Agravery [8].

In 2022, for the first time in the competition's 34 years of existence, the world cheese community, thanks to Ardis Group and ProCheese, got to know 39 Ukrainian cheeses with the slogan "Freedom tastes great" from 22 cheese factories at the World Cheese Awards 2022 in Great Britain, where 4,434 cheeses from 42 countries of the world. Thirteen Ukrainian cheeses received awards [11].

Classic fermented cheeses are classified by hardness into extra-hard, hard, se mi-hard and soft from the use of microcultures (0.5–10%) of starter and enzyme for curdling and curd formation, and also differ in the type of secondary microbiome — that is, those ripening with the help of lactic acid bacteria, propionic sourdough, blue and white mold. Hard cheeses are also divided by cooking temperature (second heating): high (thermophilic sourdough), medium (mixed sourdough), and low (mesophilic sourdough) [12].

Modeling of the general biotechnological system of the fermented cheese production

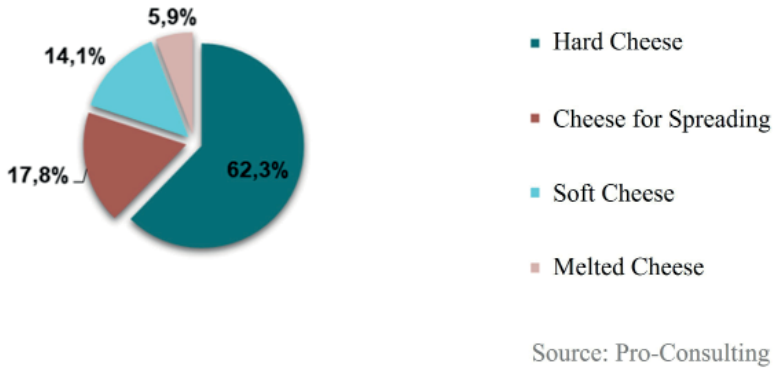


Fig. 1. **Segmentation of the cheese market by product types in Ukraine, 2021 according to Pro-Consulting** (hard cheese, pasty cheese, soft cheese, processed cheese)

process consists of the main stages: preliminary preparation of milk, fermentation, curdling, coagulation, grinding, cooking, washing, pressing, salting, forming, ripening, with the establishment and definition of technological parameters for each stage of production with specific modes the flow of physico-chemical and biochemical processes with the further search for opportunities for regulation and optimization of parameters, as well as the introduction of innovative methods of the technological process and successful entry into the commercial market of food products.

Global innovations in milk production are based on the tendency to increase the volume of milk production on the world market and promote the development of new functional preparations from live probiotic cultures in the field of biotechnology; products based on baking concentrates, including traditional fermented products, including cheeses and cheese products. Innovative methods of processing (ultrafiltration) of milk and cheese grain, processing time, coagulation and syneresis, etc. are increasingly used to standardize milk during cheese production.

The milk preparation process includes stages: selection, standardization, normalization, thermalization, pasteurization, microfiltration, ultrafiltration, bactofugation, homogenization. The quality of cheeses is determined, first of all, by the type and component composition of milk. The milk of cows, sheep, goats, and buffaloes is used for the production of cheese. Selection, standardization and pasteurization of milk is mandatory, because the sensory characteristics of cheese depend on the quality and freshness (acidity of 19 °T) of milk. Any milk consists of casein, whey proteins, lipids, minerals, lactose and other oligosaccharides.

The nature of cheeses depends on the content of milk fat (hydrolysis produces 41 fatty acids, including butyric, stearic, oleic, and glycerol) and milk proteins, which make up 3.3% of nitrogenous compounds of four fractions (casein — 2.8%; lactoalbumin — 0.43%, lactoglobulin — 0.06%, proteose peptone — 0.01%). The majority of proteins (85%) is casein (α — 60%, β — 25%, γ — 10% fractions), which is in the form of a calcium phosphate complex micelle and is released during milk acidification and coagulation of α and β fractions with rennet enzyme (pH = 4.6): the rest is albumin, which when heated turns into serum and precipitates (at 70–80 °C) and globulin, which also turns into serum and is dissolved in water and precipitates (at 75 °C) in an acidic environment [13].

The production of hard rennet cheeses is a complex multifunctional process, in which a change in the influence of even one of the technological factors can change the dynamics of biochemical, microbiological and physicochemical transformations of the cheese mass, which affects not only the organoleptic properties and biological value of the final product, but also its safety. During ripening, all components of the cheese mass undergo deep changes, because of which the specific taste, aroma of the cheese, its consistency and pattern are formed. An important feature of hard rennet cheeses made by traditional technology is their suitability for long-term storage. The formation of cheese quality is largely determined by the composition and properties of raw milk, microbiological and biochemical features of product maturation, and technological parameters of production [14].

Fermentation of milk is carried out with the help of lactic coagulation microbial producers of fermentation (lactic acid bacteria of the microflora of starter cultures), lactic acid coagulation enzymes (plant, animal, in particular, rennet, and microbial proteolytic enzymes, in particular renin) and for special cheeses — solid-phase ripening (propionic bacteria, yeast and molds). Therefore, milk may also contain other substances that affect the quality of milk and the final product — cheese: enzymes, in particular proteases (plasmin and cathepsin D), and lipases; antibiotics and mycotoxins. To prevent the growth of pathogenic microorganisms *Campylobacter, Staphylococcus, Salmonella spp., Clostridium tyrobutyricum,* thermal (thermalization (50–70 °C, 5–30 s) and pasteurization (72–75 °C, 20–15 s) and physical (microfiltration, homogenization and ultrafiltration are used to regulate the content of fats and proteins in milk.

During the acidification of milk, several phases of biochemical transformations take place, namely:

– bactericidal phase with the formation of natural milk antibiotics lactenins (*Streptococcus lactis* — nisin; *Streptococcus cremovis* — diplococcin; *Lactobacillus acidophilus* — acidophilin, lactocidin; *Lactobacillus plantarum* — lactolin; *Lactobacillus brevi* — brewin, lysozyme);

– the incubation phase with a decrease in acidity by the initial microflora of milk (at 37 °C for 6 hours);

– a mixed microbial phase with the decomposition of lactose into galactose and glucose by all present microflora, lactic acid with an increase in the biomass of *Lactobacillus* and a further decrease in pH;

– symbiotic phase with an increase in pH under the influence of yeast and mycelial fungi [15].

Sourdough starters are used for controlled fermentation. European cheese producers in the European Union most often choose mesophilic starters — in 53.3% of cases, thermophilic and mixed less often — in 20% of the analyzed cheeses. All the Ukrainian producers we studied use mesophilic starter cultures.

Selected starter cultures are used for milk fermentation: mesophilic (*Lactococcus lactis subsp. Lactis, Lactococcus lactis subsp. Cremoris, Lactococcus lactis subsp. Diacetylactis);* thermophilic *(Streptococcus thermophilus, Lactobacillus helveticus, Lactobacillus delbrueckii subsp. Lactis, Lactobacillus bulgaricus*); mixed [16].

The use of thermophilic lactic acid bacteria allows the use of a higher fermentation and coagulation temperature, and also shortens the ripening period of the cheese. It is also possible to directly acidify the addition of lactic acid and use natural starters from authorized farms for some cheeses. In this study, a comparative analysis of the qualitative composition of starters used in the recipes and production of various fermented cheeses, as well as the temperature and duration of action of lactic acid bacteria, was carried out.

Coagulation is carried out with the help of rennet of young herbivores — calves, lambs, goats, consisting of chymosin, pepsin in a small amount, lipase, and pregastric esterase. Beef pepsin, fungal and bacterial proteinase are also used in industry. The coagulation process is affected by acidity, calcium concentration, protein content, and temperature. The product of the coagulation process is the cheese mass, which is further crushed and subjected to further processing for the production of mature cheese. This study examines the use of enzymes and the temperature regime and duration of coagulation in various cheese productions.

By processing the curd, conditions are created for microbiological and enzymatic processes necessary for the production of cheese of the appropriate consistency. Shredding of cheese mass is carried out with special knives in horizontal cheese baths or vertical fermenters. The size of the granules depends on the technology of cheese production and can be from 2 to 30 mm. Grinding increases the surface area of the curd granules, mixes it well and improves further ripening.

Boiling (second heating) is carried out at a temperature of 33–55 °C, depending on the amount of moisture in the final product and the type of starter. Cheddarization is characteristic of some types of cheese, such as Cheddar and Cheshire. During cheddarization, the cheese mass is once again chopped into pieces of 5 kg each, salted and cheddarized to a pH of 5.4. During cheddarization, the cheese mass acquires a fibrous structure, like chicken breast meat, and becomes elastic.

The finished cheese mass is taken from the cheese vat and distributed among perforated molds of different sizes depending on the type of cheese. The size of the cheese head depends not only on the product appearance, but is also necessary for proper ripening. A cheese ripened by a surface microbiome must be small so that the enzymes secreted by the microorganisms growing on the surface can penetrate the entire volume of the cheese. However, cheeses that use propionic acid fermentation must be large enough to hold carbon dioxide in the middle to ripen the cells.

Pressing is used for extra-hard, hard and semi-hard cheeses. Mostly, the pressing takes place under a pressure of 1200 kg per 1 surface of the cheese head for hard cheeses and 2000 kg for extra hard cheeses. To improve the taste, table salt (sodium chloride) is added to some cheeses. The salt concentration depends on the type of cheese and can be from 0.3 to 10%, but most cheeses contain 2.5% salt. Pickling can be done in two ways: wet (17–30% salt solution) and dry (wrapping with dry salt).

Ripening of fermented cheese is necessary for the formation of its characteristic shape, texture, taste and aroma. Maturation involves enzymes (rennet, renin, plasmin, proteases, lipases, peptidases) and microorganisms of the secondary microbiome (bacteria *Propionibacterium freudenreichii*, *Propionibacterium acidipropionici*, mold fungi *Penicillium camemberti*, *Penicillium roqueforti*, yeast *Geotrichum candidum*) [17].

The chemical composition and sensory characteristics of cheese depend on: the chemical composition of the cheese mass; temperature; humidity; type of enzymes; origin of milk; species composition of the primary and secondary microbiome at each technological stage [18].

To conduct an analytical review of the technological process of production of various hard and semi-hard cheeses in the EU and Ukraine, as well as recipes for fermented cheeses for production at craft cheese factories and at home from the USA. Conduct an analysis of the conditions and terms of key stages of production, such as fermentation, coagulation, boiling, pressing, salting, ripening. To investigate the composition and type of lactic acid bacteria in starter cultures used in the production of fermented cheeses, as well as enzymes used for fermentolysis and coagulation of milk casein. To propose recommendations for the development of new types of hard and semi-hard cheeses in Ukraine, which would mainly be related to the regulation of the time and temperature of fermentation, cooking and ripening of cheeses, as well as the expansion of the biodiversity of the microbiome of primary lactic acid starters to improve the taste and aroma of ready-to-use product.

Materials and Methods

The object of the study is the technological process of production of various hard and semi-hard cheeses in the EU and Ukraine, as well as recipes for fermented cheeses for production at craft cheese factories and at home from the USA. Information from patents, technical conditions for the production of hard and semi-hard cheeses in the EU and Ukraine was used to evaluate the production technology of hard and semi-hard cheeses; DSTU 6003 (Solid cheeses); New England Cheesemaking Supply Company (NECSC) cheese recipes by Jim Wallace. The obtained data were processed by methods of statistical analysis, systematization, comparison and generalization of information.

Results and Discussion

In this study, hard and semi-hard cheeses were examined. An analysis of the market of hard cheeses and an overview of the biotechnological process of the production of fermented cheese and a comparative analysis of the key stages of production were completed. A study of the use of biodiversity of lactic acid bacteria in starters and enzymes used in the production of fermented cheeses at world productions, and an attempt to show prospects for the development and improvement of Ukrainian hard cheeses were done.

At the first stage of research, an analysis of the microbiome of lactic starters used in the production of fermented cheeses was carried out.

Milk fermentation is carried out using mesophilic and thermophilic yeasts, and it was established that in the recipes of the NECSC company, mesophilic yeasts are used in 37.3% of cases, thermophilic yeasts in 17.1%, and mixed yeasts in 45.7% (Fig. 2, *A*), (Table 1). Cheese producers in the European Union most often choose mesophilic starters — 53.3% of cases, thermophilic — 20.0% of cases, and mixed — 26.7% of cases (Fig. 2, *B*). In Ukraine, mesophilic starters are used in 100% of the analyzed cheeses (Fig. 2, *C*).

A comparative study of the species composition of lactic acid bacteria in starter cultures of lactic acid bacteria in NECSC company recipes by Jim Wallace was carried out, the results are presented in Table 1. According to the results of the study, mesophilic bacteria are most often used *Lactococcus lactis* subsp. *lactis*, *Lactococcus lactis* subsp. *cremoris,* in 29/35 spilled cheese recipes, in 22/35 analyzed cheeses, a thermophilic bacterium is used *Streptococcus thermophilus*, mesophilic bacterium *Lactococcus lactis subsp. diacetylactis* is used in 17/35 analyzed cheeses. Other thermophilic bacteria *Lactobacillus helveticus* are used less often in 8/35 cheeses, *Lactobacillus delbrueckii subsp. lactis* in 7/35 cheeses, Lactobacillus bulgaricus in 2/35 cheeses (Table 1, Fig. 3).

According to the conducted research, *Propionibacterium freudenreichii subsp. shermanii* was used as additional components of starter cultures and components of the secondary microbiome in 4/35 cheeses — a bacterium that carries out propionic acid fermentation, the yeast *Geotrichum candidum* and the brevibacterium *Brevibacterium linens* — in 2/35 cheeses and the fungus *Trichothecium domesticum* — in 2/35 cases (Table 1).

To study the technology of cheese production in the EU, the production technology of 30 cheeses was analyzed, the species composition of bacteria was described in 15 types of cheeses, and natural starters from certified farms were used in five types of analyzed cheeses. In the largest number of examined cheeses, 10/15, mesophilic bacteria *Lactococcus lactis subsp. lactis*, *Lactococcus lactis subsp. cremoris*, in 5/15 of the analyzed cheeses, the thermophilic bacterium *Streptococcus thermophilus*, the mesophilic bacterium *Lactococcus lactis subsp.*

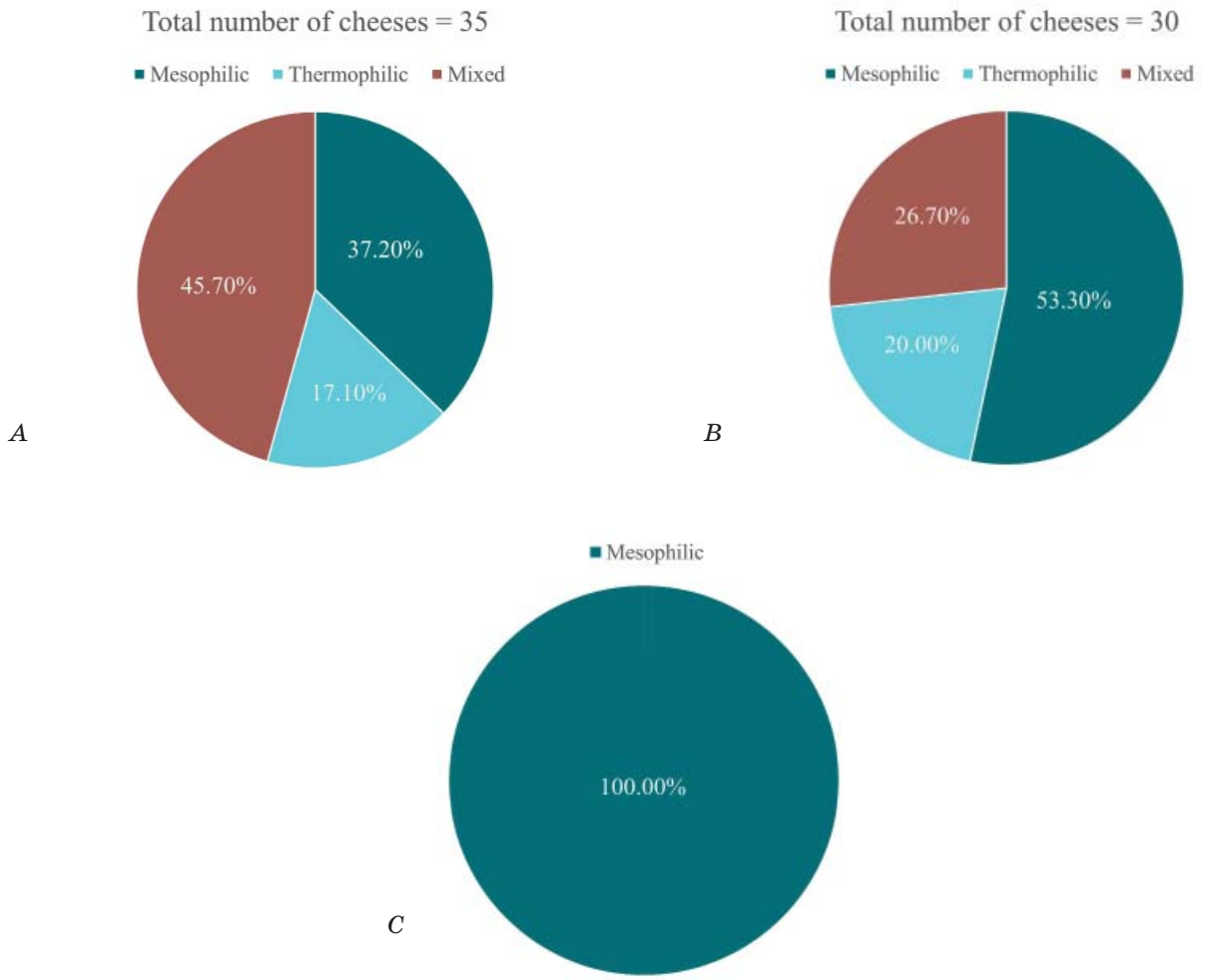


Fig. 2. **Percentage ratio of each type of lactic acid starters in NECSC company cheeses** (*A* — 35 сирів: мезофільних — 37,7%; thermophilic — 17,2%; mixed — 45,7%); cheeses produced in the EU (*B* — 30 сирів : мезофільних — 53,3%; thermophilic — 20,0%; mixed — 26,7%); in cheeses produced in Ukraine (*C* — 18 cheeses: mesophilic)

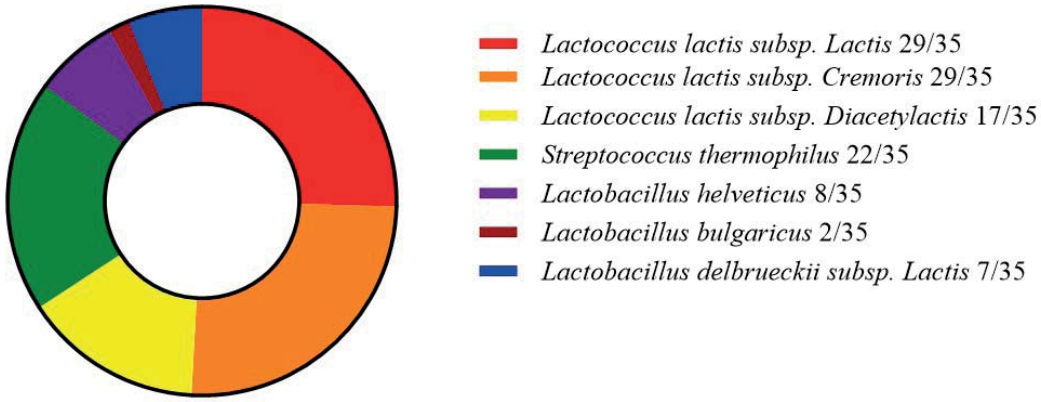


Fig. 3. **Ratio of lactic acid bacteria species in starters from NECSC recipes by Jim Wallace**

Table 1. **Comparative analysis of the species composition of lactic acid bacteria in starter cultures of lactic acid bacteria recipes of the NECSC company**

No.	The name of the cheese	Type of starter	Type of sourdough starter	Types of microorganisms
1	2	3	4	5
1	Alpine Style Cheese Альпійський сир	mixed	MA011	*Lactococcus lactis* subsp. *lactis*, *Lactococcus lactis* subsp. *cremoris*
			TA61	*Streptococcus thermophilus*
			FLAV54	*Lactobacillus helveticus*
2	Alpine Tomme Альпійський порожній	thermophilic	C201	*Streptococcus thermophilus*, *Lactobacillus helveticus*, *Lactobacillus delbrueckii* subsp. *lactis*
2	Alpine Washed Rind Альпійський з промитою шкіркою	mixed	MA 4002	*Lactococcus lactis* subsp. *lactis*, *Lactococcus lactis* subsp. *cremoris*, *Lactococcus lactis* subsp. *diacetylactis*, *Streptococcus thermophilus*
		propionic	Propionic Shermanii	*Propionibacterium freudenreichii* subsp. *shermanii*
			GEO17	*Geotrichum candidum*
			Bacteria Linens	*Brevibacterium linens*
3	Appenzeller Апенцеллер	thermophilic	Thermo B	*Streptococcus thermophilus*, *Lactobacillus delbrueckii* subsp. *bulgaricus*
4	Asiago Азіаго	thermophilic	C201	*Streptococcus thermophilus*, *Lactobacillus helveticus*, *Lactobacillus delbrueckii* subsp. *lactis*
			LH100	*Lactobacillus helveticus*, *Lactobacillus lactis*
5	Beaufort Бофор	mixed	MA011	*Lactococcus lactis* subsp. *lactis*, *Lactococcus lactis* subsp. *cremoris*
			TA61	*Streptococcus thermophilus*
			LH100	*Lactobacillus helveticus*, *Lactobacillus lactis*
6	Brick Брусковий	mesophilic	MM100	*Lactococcus lactis* subsp. *lactis*, *Lactococcus lactis* subsp. *cremoris*, *Lactococcus lactis* subsp. *lactis biovar diacetylactis*
			Bacteria Linens	*Brevibacterium linens*
7	Cabra al Vino П'яна коза	mixed	MA 4002	*Lactococcus lactis* subsp. *lactis*, *Lactococcus lactis* subsp. *cremoris*, *Lactococcus lactis* subsp. *diacetylactis*, *Streptococcus thermophilus*
8	Caerphilly Карфіллі	mixed	MA 4002	*Lactococcus lactis* subsp. *lactis*, *Lactococcus lactis* subsp. *cremoris*, *Lactococcus lactis* subsp. *diacetylactis*, *Streptococcus thermophilus*
		fungal	Mycodore	*Trichothecium domesticum*
9	Canestrato Italian Basket Канестрато	mixed	C101	*Lactococcus lactis* subsp. *lactis*, *Lactococcus lactis* subsp. *cremoris*
			Y1	*Streptococcus thermophilus*, *Lactobacillus bulgaricus*, *Bifidobacterium lactis*, *Lactobacillus rhamnosus*

Table 3 (continued)

1	2	3	4	5
10	Cantal Канталь	mixed	MA 4002	*Lactococcus lactis* subsp. *lactis*, *Lactococcus lactis* subsp. *cremoris*, *Lactococcus lactis* subsp. *diacetylactis*, *Streptococcus thermophilus*
11	Cheddar Чеддер	mesophilic	C101	*Lactococcus lactis* subsp. *lactis*, *Lactococcus lactis* subsp. *cremoris*
12	Colby Колбі	mesophilic	C101	*Lactococcus lactis* subsp. *lactis*, *Lactococcus lactis* subsp. *cremoris*
13	Cheshire Чешир	mesophilic	C101	*Lactococcus lactis* subsp. *lactis*, *Lactococcus lactis* subsp. *cremoris*
14	Dunlop Данлоп	mesophilic	C101	*Lactococcus lactis* subsp. *lactis*, *Lactococcus lactis* subsp. *cremoris*
15	Edam Едам	mesophilic	MM 100	*Lactococcus lactis* subsp. *lactis*, *Lactococcus lactis* subsp. *cremoris* *Lactococcus lactis* subsp. *lactis biovar diacetylactis*
16	Farmstead Фермерський	mesophilic	C101	*Lactococcus lactis* subsp. *lactis*, *Lactococcus lactis* subsp. *cremoris*
17	Gouda Гауда	mesophilic	MM 100	*Lactococcus lactis* subsp. *lactis*, *Lactococcus lactis* subsp. *cremoris* *Lactococcus lactis* subsp. *lactis biovar diacetylactis*
18	Sweet Gouda Солодка Гауда	mesophilic	MM 100	*Lactococcus lactis* subsp. *lactis*, *Lactococcus lactis* subsp. *cremoris* *Lactococcus lactis* subsp. *lactis biovar diacetylactis*
19	Gruyere Грюєр	thermophilic	TA61	*Streptococcus thermophilus*
		propionic	C6	*Propionibacterium freudenreichii* subsp. *shermanii*
20	Hispanico Іспанський	mixed	MA 4002	*Lactococcus lactis* subsp. *lactis*, *Lactococcus lactis* subsp. *cremoris* *Lactococcus lactis* subsp. *diacetylactis*, *Streptococcus thermophilus*
21	Ibores Іборес	mixed	MA 4002	*Lactococcus lactis* subsp. *lactis*, *Lactococcus lactis* subsp. *cremoris* *Lactococcus lactis* subsp. *diacetylactis*, *Streptococcus thermophilus*
22	Jack Джек	mesophilic	MA011	*Lactococcus lactis* subsp. *lactis*, *Lactococcus lactis* subsp. *cremoris*
23	Jarlsberg Ярлсберг	mesophilic	Flora Danica	*Lactococcus lactis* subsp. *lactis*, *Lactococcus lactis* subsp. *cremoris* *Lactococcus lactis* subsp. *diacetylactis*
24	Lancashire Ланкашир	mixed	MA 4002	*Lactococcus lactis* subsp. *lactis*, *Lactococcus lactis* subsp. *cremoris* *Lactococcus lactis* subsp. *Diacetylactis*. *Streptococcus thermophilus*
25	Maasdam Мааздам	mixed	Su Casu	*Streptococcus thermophilus*, *Lactobacillus delbrueckii* subsp. *lactis*, *Lactobacillus helveticus*
		propionic	C6	*Propionibacterium freudenreichii* subsp. *shermanii*
			MM 100	*Lactococcus lactis* subsp. *lactis*, *Lactococcus lactis* subsp. *cremoris*, *Lactococcus lactis* subsp. *lactis biovar diacetylactis*

Table 3 (end)

1	2	3	4	5
26	Mutschli Herdsman's Маленький пастуший сир	thermophilic	C201	*Streptococcus thermophilus*, *Lactobacillus helveticus*, *Lactobacillus delbrueckii* subsp. *lactis*
		propionic	C6	*Propionibacterium freudenreichii subsp. Shermanii*
			GEO17	*Geotrichum candidum*
			Bacteria Linens	*Brevibacterium linens*
27	Red Leicester Червоний Лестер	mesophilic	C101	*Lactococcus lactis* subsp. *lactis*, *Lactococcus lactis* subsp. *cremoris*
28	Romano Романо	thermophilic	C201	*Streptococcus thermophilus*, *Lactobacillus helveticus*, *Lactobacillus delbrueckii* subsp. *lactis*
29	Sao Jorge Сан-Жоржі	mixed	MA 4002	*Lactococcus lactis* subsp. *lactis*, *Lactococcus lactis* subsp. *cremoris*, *Lactococcus lactis* subsp. *diacetylactis*, *Streptococcus thermophilus*
30	Toma Ossolano Тома	mixed	MA 4002	*Lactococcus lactis* subsp. *lactis*, *Lactococcus lactis* subsp. *cremoris*, *Lactococcus lactis* subsp. *diacetylactis*, *Streptococcus thermophilus*
			GEO17	*Geotrichum candidum*
			Bacteria Linens	*Brevibacterium linens*
31	Tomme Томе	mixed	MA 4002	*Lactococcus lactis* subsp. *lactis*, *Lactococcus lactis* subsp. *cremoris*, *Lactococcus lactis* subsp. *diacetylactis*, *Streptococcus thermophilus*
			LH100	*Lactobacillus helveticus*, *Lactobacillus lactis*
			Mycodore	*Trichothecium domesticum*
32	Toscano Pepato Тосканський	mixed	C101	*Lactococcus lactis* subsp. *lactis*, *Lactococcus lactis* subsp. *cremoris*
			C201	*Streptococcus thermophilus*, *Lactobacillus helveticus*, *Lactobacillus delbrueckii* subsp. *lactis*
33	Yorkshire Йоркшир	mesophilic	C101	*Lactococcus lactis* subsp. *lactis*, *Lactococcus lactis* subsp. *cremoris*
34	Queso de Mah n Mao	mixed	MA 4002	*Lactococcus lactis* subsp. *lactis*, *Lactococcus lactis* subsp. *cremoris*, *Lactococcus lactis* subsp. *diacetylactis*, *Streptococcus thermophilus*

diacetylactis is used in 9/15 analyzed cheeses. Other thermophilic bacteria *Lactobacillus helveticus* are used less often in 6/15 cheeses, *Lactobacillus delbrueckii subsp. lactis* in 3/15 cheeses, *Lactobacillus bulgari*cus in 3/15 cheeses, the bacterium *Leuconostoc mesenteroides* was also used in production in 4/15 analyzed cheeses (Table 2).

According to the study, *Propionibacterium freudenreichii subsp. shermanii* was used as an additional leavening component in 3/30 cheeses — a bacterium that carries out propionic acid fermentation.

Because of the research, 8 types of hard cheeses of Ukrainian producers were analyzed. Mesophilic bacteria *Lactococcus lactis subsp. lactis* were used in all types of examined cheeses, *Lactococcus lactis subsp. cremoris*, *Lactococcus lactis subsp. diacetylactis* (Table 3).

Hard rennet cheeses are evaluated according to organoleptic indicators according to a 100-point system. Depending on the amount of points scored, the grade of cheese is determined: higher or first. Hard rennet cheeses "Russian", "Poshekhonsky",

Table 2. **Comparative analysis of the species composition of lactic acid bacteria in starter cultures of lactic acid bacteria in cheeses produced in the EU**

No.	The name of the cheese	Producer/ Patent owner/ Region	Type of starter	Types of microorganisms
1	Gouda Гауда	BC2 Friesland B.V.	mixed	*Lactococcus lactis* subsp. *lactis & cremoris*, *Lactococcus lactis* subsp. *lactis* subsp. *diacetylactis*, *Lactobacillus helveticus*, *Lactobacillus lactis*
2	Gouda Гауда	DSM IP Assets B.V	mesophilic	*Lactococcus lactis* subsp. *lactis & cremoris* *Lactobacillus helveticus*, *Lactobacillus lactis*
3	Swiss-type Швейцарський	CSK Food Enrichment B.V.	thermophilic	*Streptococcus thermophilus*, *Lactobacillus delbrueckii* subsp. *lactis*, *Lactobacillus helveticus*
			propionic	*Propionibacterium freudenreichii* subsp. *shermanii*
4	Berner Alpk se Бернський	Berner Hobelk se PDO	thermophilic	*Streptococcus thermophilus*, *Lactobacillus delbrueckii* subsp. *lactis*, *Lactobacillus helveticus*
5	Graviera Kritis Грав'єра	Crete, Greece	thermophilic	*Streptococcus thermophilus*, *Lactobacillus delbrueckii* subsp *bulgaricus*, *Lactobacillus helveticus*
6	Kefalotyri Кефалотирі	Greece	mixed	*Streptococcus thermophilus*, *Lactobacillus* subsp *delbrueckii bulgaricus*, *Lactobacillus helveticus*, *Lactococcus lactis* subsp. *lactis & cremoris*
7	Le Gruy re Грюєр	La Neuveville, Switzerland	thermophilic	*Streptococcus thermophilus*, *Lactobacillus delbrueckii* subsp *lactis* *Lactobacillus helveticus*
8	V sterbottensost Вестерботтен	V sterbotten, Sweden	mesophilic	*Lactococcus lactis* subsp. *lactis & cremoris*, *Lactococcus lactis* subsp. *lactis* biovar. *diacetylactis*, *Leuconostoc mesenteroides*
9	Appenzeller Апенцеллер	Appenzell, Switzerland	thermophilic	*Streptococcus thermophilus*, *Lactobacillus. delbrueckii* subsp *bulgaricus*, *Lactobacillus casei*
10	Havarti Хавартi	Denmark	mesophilic	*Lactococcus lactis* subsp. *lactis & cremoris*, *Lactococcus lactis* subsp. *lactis* biovar. *diacetylactis*, *Leuconostoc mesenteroides*.
11	Herrgard Шведський	Sweden	mesophilic	*Lactococcus lactis* subsp. *lactis & cremoris*, *Lactococcus lactis* subsp. *lactis* biovar. *diacetylactis*, *Leuconostoc mesenteroides*
12	Pr st Прастост	Sweden	mesophilic	*Lactococcus lactis* subsp. *lactis & cremoris*, *Lactococcus lactis* subsp. *lactis* biovar. *diacetylactis*, *Leuconostoc mesenteroides*.
13	San Sim n Сан Симон	Galicia, Spain	mesophilic	*Lactococcus lactis* subsp. *lactis & cremoris*
14	Svecia Швеція	Sweden	mesophilic	*Lactococcus lactis* subsp. *lactis & cremoris*, *Lactococcus lactis* subsp. *lactis* biovar. *diacetylactis*, *Leuconostoc mesenteroides*
15	Gouda Гауда	Netherlands	mesophilic	*Lactococcus lactis* subsp. *lactis & cremoris*, *Lactococcus lactis* subsp. *lactis* biovar. *diacetylactis*

Table 3. **Comparative analysis of the species composition of lactic acid bacteria in starter cultures of lactic acid bacteria in cheeses produced in Ukraine**

No.	The name of the cheese	Producer/ Patent owner	Type of starter	Types of microorganisms
1	Костромський Kostroma	«Жидачівський сирзавод» «Zhydachiv cheese factory»	mesophilic	*Lactococcus lactis* subsp. *lactis*, *Lactococcus lactis* subsp. *cremoris*, *Lactococcus lactis* subsp. *diacetylactis*
2	Голландський круглий Dutch round	«Жидачівський сирзавод» «Zhydachiv cheese factory»	mesophilic	*Lactococcus lactis* subsp. *lactis*, *Lactococcus lactis* subsp. *cremoris*, *Lactococcus lactis* subsp. *diacetylactis*
3	Голландський брусковий Dutch bar	«Жидачівський сирзавод» «Zhydachiv cheese factory»	mesophilic	*Lactococcus lactis* subsp. *lactis*, *Lactococcus lactis* subsp. *cremoris*, *Lactococcus lactis* subsp. *diacetylactis*
4	Голландський Dutch	Бучацький сирзавод Buchatsky cheese factory	mesophilic	*Lactococcus lactis* subsp. *lactis*, *Lactococcus lactis* subsp. *cremoris*, *Lactococcus lactis* subsp. *diacetylactis*
5	Ейдам Aidam	«Євро-мілк» «Euro-milk»	mesophilic	*Lactococcus lactis* subsp. *lactis*, *Lactococcus lactis* subsp. *cremoris*, *Lactococcus lactis* subsp. *diacetylactis*
6	Рогатинський Rohatynskyi	«Євро-мілк» «Euro-milk»	mesophilic	*Lactococcus lactis* subsp. *lactis*, *Lactococcus lactis* subsp. *cremoris*, *Lactococcus lactis* subsp. *diacetylactis*
7	Прикарпатський Carpathian	«Євро-мілк» «Euro-milk»	mesophilic	*Lactococcus lactis* subsp. *lactis*, *Lactococcus lactis* subsp. *cremoris*, *Lactococcus lactis* subsp. *diacetylactis*
8	Явір Sycamore	«Євро-мілк» «Euro-milk»	mesophilic	*Lactococcus lactis* subsp. *lactis*, *Lactococcus lactis* subsp. *cremoris*, *Lactococcus lactis* subsp. *diacetylactis*

"Picantny", reduced fat and accelerated ripening (1 month), as well as soft and processed cheeses are not divided into varieties. Their quality is determined by the compliance of their indicators with the requirements of standards or technical conditions.

During the certification of cheeses, in addition to organoleptic ones, the main physico-chemical indicators characterizing the nutritional value of cheeses are determined, including the mass fraction of fat, salt, moisture, titrated and active acidity, sodium nitrate content.

At the second stage of the research, an analysis of production conditions and the duration of the key stages of production of fermented cheeses was carried out. The recipes of the NECSC company, authored by Jim Wallace, and the technical conditions of production in Ukraine and the EU were also analyzed. 35 types of fermented cheeses were analyzed according to the temperature of cheese fermentation and coagulation; cooking (second heating); maturation.

Based on the analysis of the obtained data, it was found that the temperature of fermentation and coagulation is in the range from 29 °C to 37 °C. A lower temperature of 29–33 °C is used for cheeses with a mesophilic type of sourdough, the highest temperature of 35–37 °C is used for cheeses with thermophilic sourdoughs, in recipes for cheeses with mixed sourdoughs, an intermediate temperature regime is used (Fig. 4, *A*). The temperature of the second heating depends on the desired moisture content in the final product and the type of starter used and is 30–55 °C. For semi-hard cheeses with mesophilic sourdough, a temperature of 30–35 °C is used, for hard cheeses with thermophilic sourdough, a temperature of 45–55 °C is used. The ripening temperature is low and is set at 12–14 °C, only for 3 cheese recipes the recommended temperature is 16–21 °C (Fig. 4, *A*).

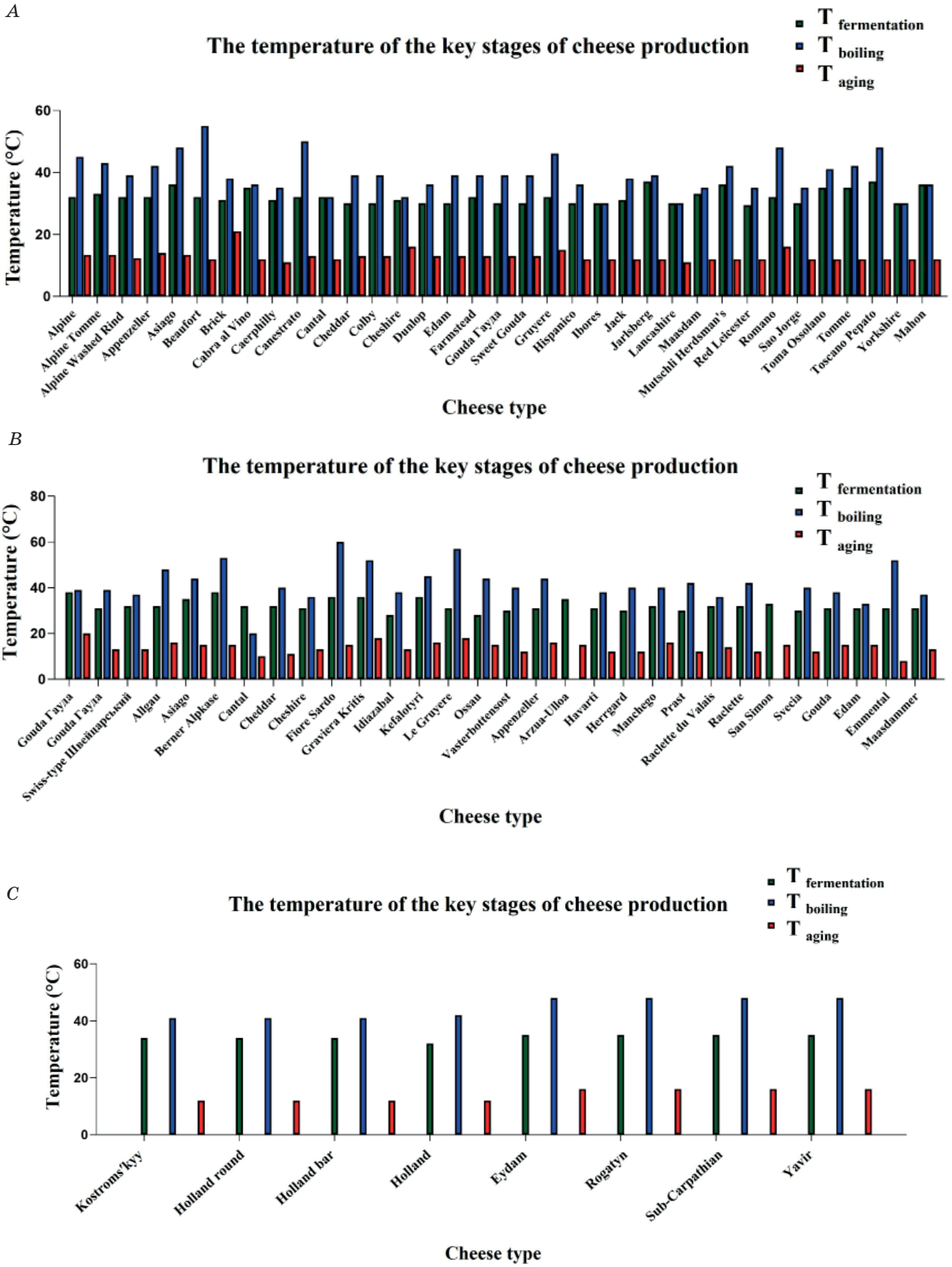


Fig. 4. **Quantitative ratio of the temperature of the key stages of cheese production in the recipes of the NCSC company (*A*), produced in the EU (*B*), produced in Ukraine (*C*)**

An analysis of 30 types of fermented cheeses produced in the EU was carried out according to the temperature of cheese fermentation and coagulation; cooking (second heating); maturation. Based on the analysis of the obtained data, it was found that the temperature of fermentation and coagulation is in the range from 30 °C to 38 °C. A lower temperature of 30–33 °C is used for cheeses with a mesophilic type of sourdough, the highest temperature of 35–38 °C is used for cheeses with thermophilic sourdoughs, in recipes for cheeses with mixed sourdoughs, an intermediate temperature regime is used (Fig. 4, *B*). The temperature of the second heating depends on the desired moisture content of the final product and the type of starter used, and is higher in large-scale production than in recipes for craft cheese factories and is 33–60 °C. For semi-hard cheeses with mesophilic sourdough, a temperature of 33–40 °C is used, for hard cheeses with thermophilic sourdough, a temperature of 45–60 °C is used. The ripening temperature in production varies more among different cheeses than in recipes, because in large enterprises it is possible to build several types of thermostatic shops with a temperature characteristic for a certain type of cheese, ranging from 8–20 °C and humidity. A higher ripening temperature reduces its duration and improves the taste characteristics of cheese due to the higher activity of enzymes and microorganisms of the secondary microbiome (Fig. 4, *B*).

8 types of fermented cheeses produced in Ukraine were studied. For Ukrainian cheeses, the fermentation temperature is approximately the same, since only mesophilic sourdoughs are used and is 34 °C for cheeses produced by the Zhydachiv cheese plant and 35 °C for cheeses from the Evro-milk enterprise. The cooking temperature is 41 °C for cheeses produced by "Zhydachiv cheese plant" and 48 °C for cheeses from the "Euro-Milk" enterprise. The ripening temperature is 12 °C for cheeses produced by the "Zhydachiv cheese factory" and 16 °C for cheeses from the "Euro-milk" enterprise (Fig. 3, *C*). At the third stage of research, an analysis of the duration of the stages of fermentation and coagulation, as well as ripening, was carried out. All NECSC recipes by Jim Wallace use fermentation before coagulation. That is, lactic acid starter is first added to milk heated to the appropriate temperature, and only after 30–60 minutes rennet is added, and in large-scale production, lactic acid starter and enzyme are added at the same time, pre-fermentation is characteristic only for 5 types of European and none of the analyzed Ukrainian cheeses.

Therefore, as a result of the study, two comparative analyzes were made: the first includes the time of preliminary fermentation and coagulation, which is presented in the graph as the time of fermentation with the help of lactic acid bacteria (Fig. 5), and the second — the time of coagulation with the help of rennet (Fig. 6).

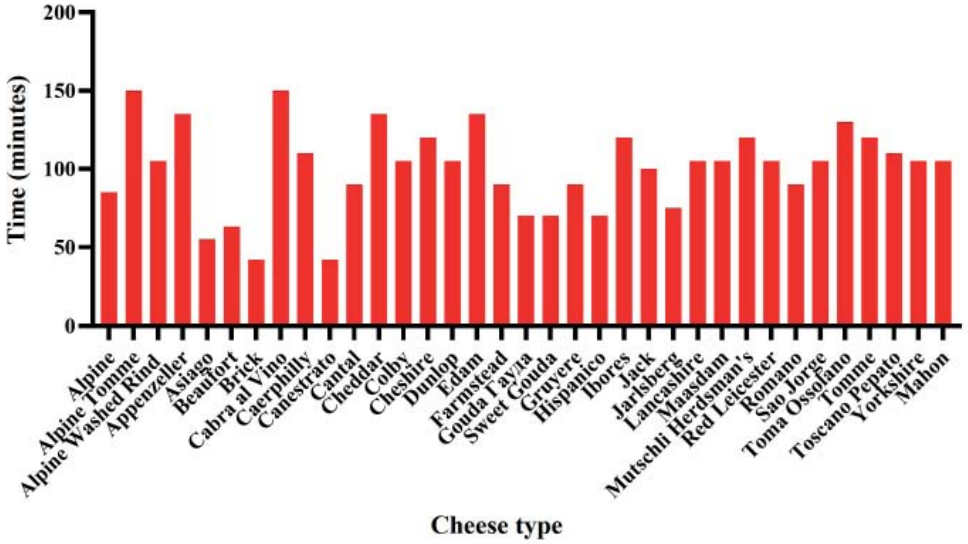
In the NECSC recipes by Jim Wallace, the fermentation time with lactic acid bacteria ranges from 40 to 140 min (Fig. 5, *A*), in the technical conditions of EU productions from 25 to 75 min (Fig. 5, *B*), in Ukrainian technical conditions, lactic acid fermentation lasts 25–35 min (Fig. 5, *C*).

In the recipes of the NECSC company by the authorship of Jim Wallace, the time of hydrolysis with the help of rennet was from 30 to 60 min (Fig. 6, *A*), in the technical conditions of EU productions from 25 to 60 min (Fig. 6, *B*), in the Ukrainian technical conditions, lactic acid fermentation lasts 25 –35 min (Fig. 6, *C*). Because of the comparative analysis, the type of rennet used for coagulation was also investigated. In all NECSC recipes, calf rennet is recommended, but microbial rennin can be used, which does not affect the structure and taste characteristics of the cheese, as noted by the author. In the technical conditions of EU productions, calf rennet is used in most cases, if sheep milk is used, the technology requires the use of lamb rennet, and beef rennet occurs with a small frequency, for most cheeses produced in large volumes, there are analogues with microbial renin. In the studied technical conditions, beef rennet is used for most cheeses of Ukrainian producers, but microbial rennin occurs with a small frequency.

According to the analysis of the duration of ripening, it was established that the duration of ripening of hard and semi-hard cheeses in the recipes of the NECSC Company ranges from 70 to 730 days. The exception is only some cheeses with propionic leavens (Edam, Ibores, Farmersky), the duration of which is less than 45 days of ripening (Fig. 7, *A*). In cheeses produced in the EU, the duration of ripening ranges from 14 to 730 days. The average duration of maturation of European cheeses is 223 days. Cheeses with the addition of microorganisms of the secondary microbiome described in the introduction: propioic acid bacteria, yeast, and mold fungi have a short ripening period of up to 40 days (Fig. 7, *B*). In the studied cheeses of Ukrainian producers, the duration of ripening ranges from 35 to 60 days (Fig. 7, *C*).

As the study of technological schemes for the production of hard cheeses shows, the

A



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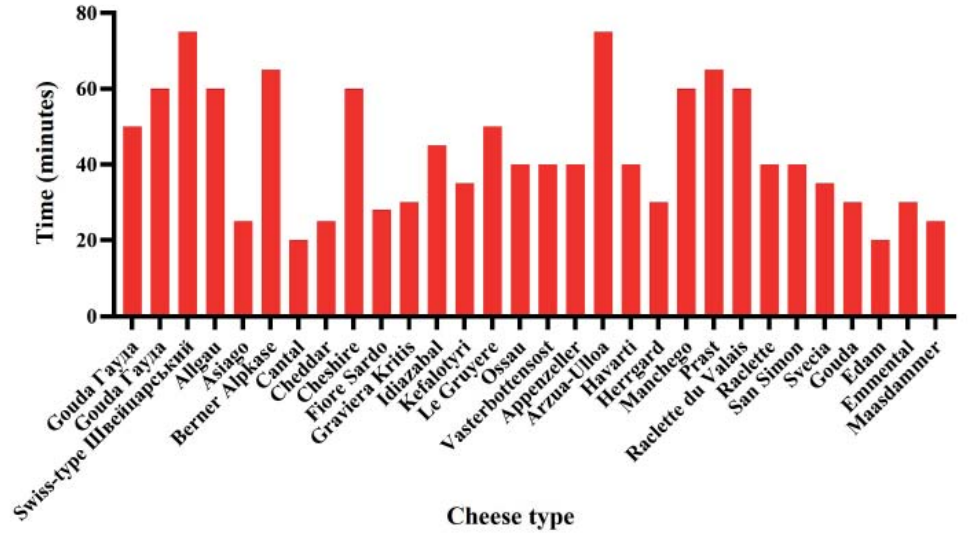

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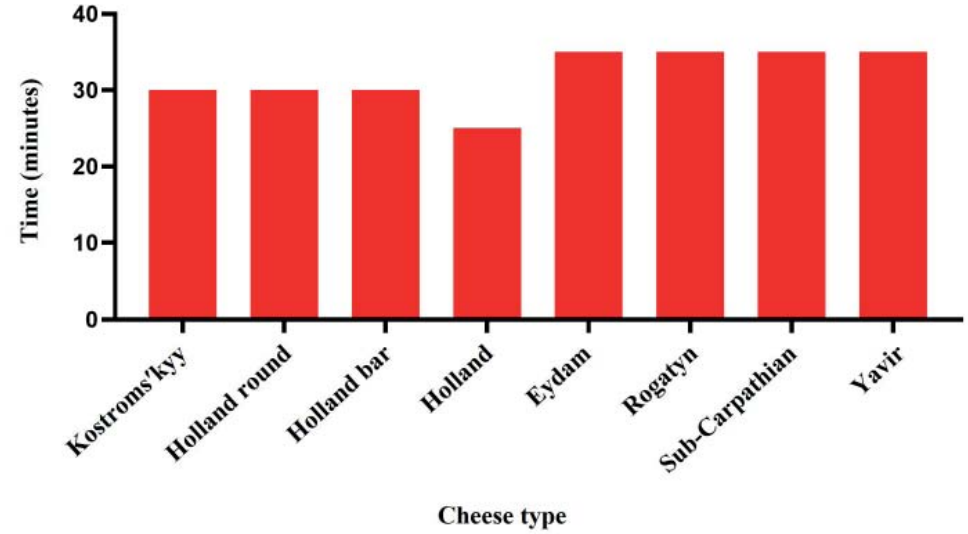
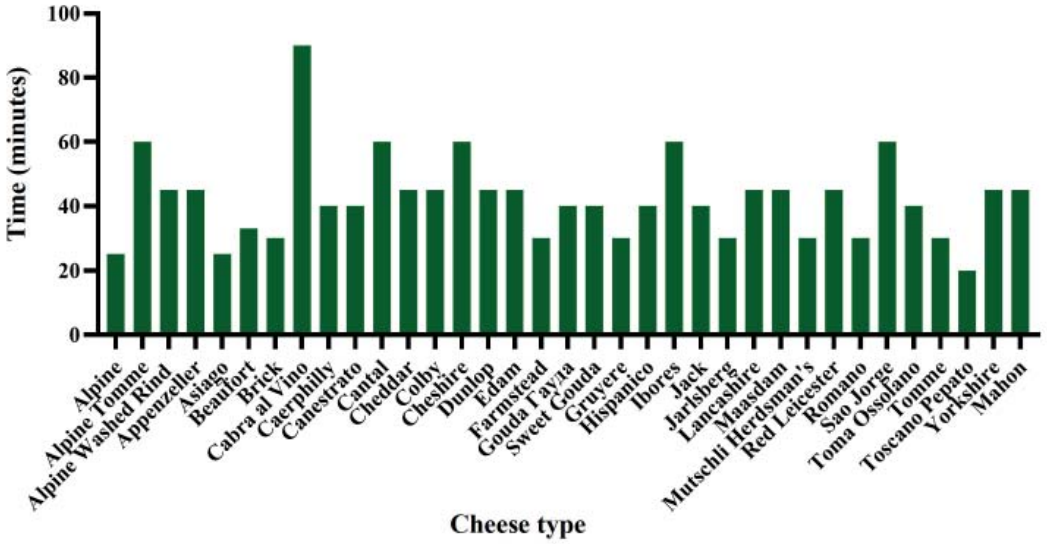


Fig. 5. **Quantitative ratio of the duration of lactic acid fermentation in the technical conditions of cheeses, in the recipes of the NCSC company (*A*), produced in the EU (*B*), produced in Ukraine (*C*)**

A



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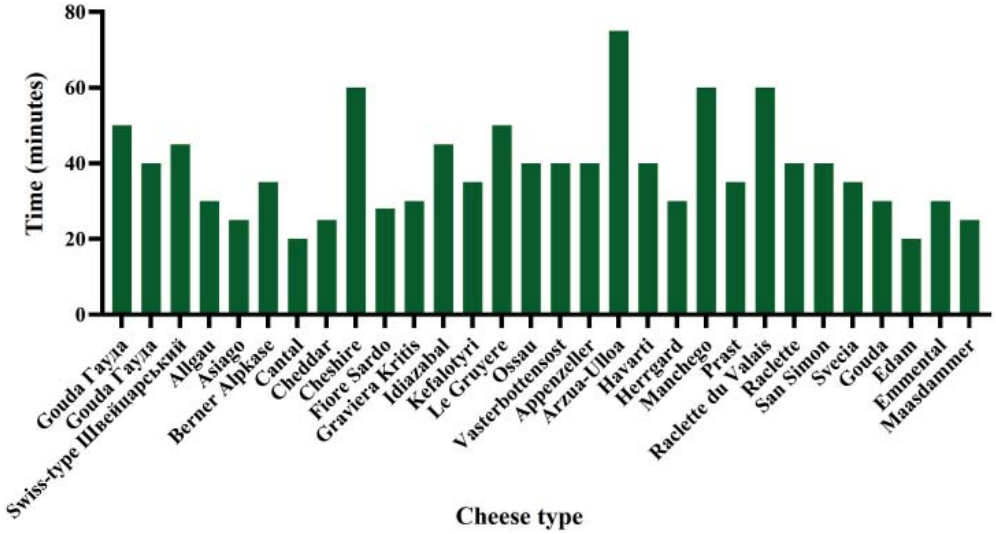

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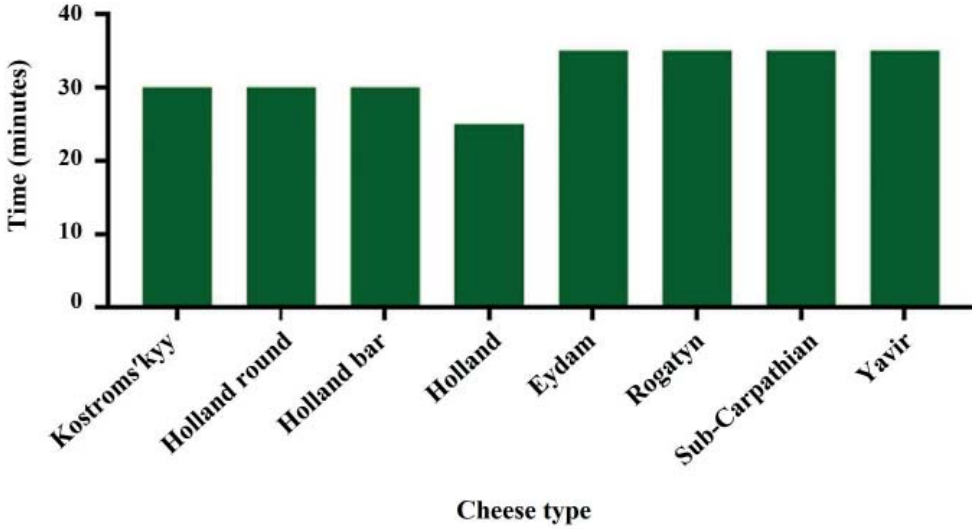
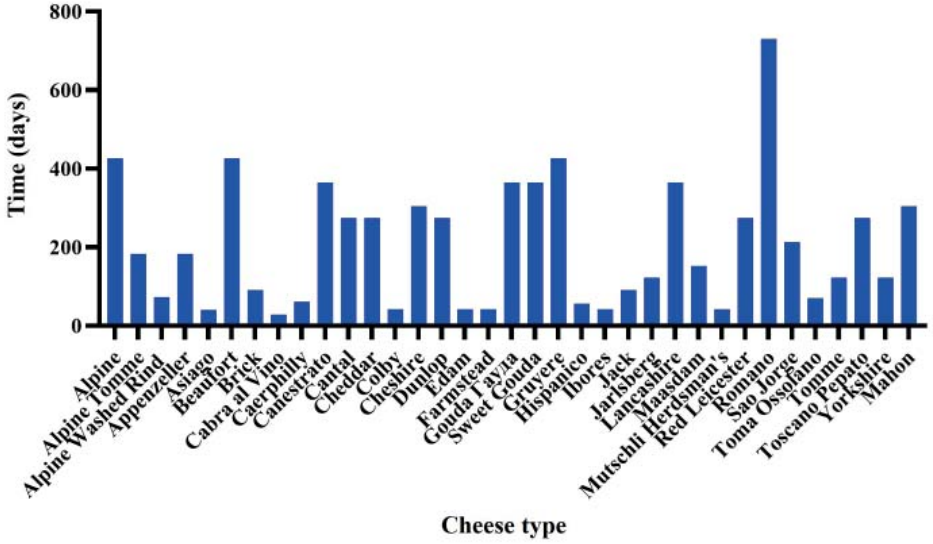
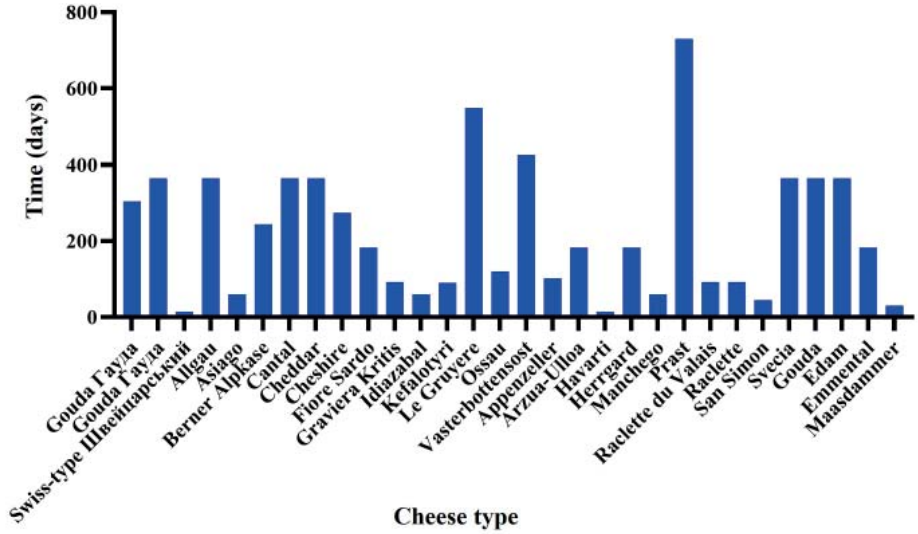


Fig. 6. **Quantitative ratio of duration of coagulation in technical conditions of cheeses, NCSC (*A*), produced in the EU (*B*), produced in Ukraine (*C*)**

A



B



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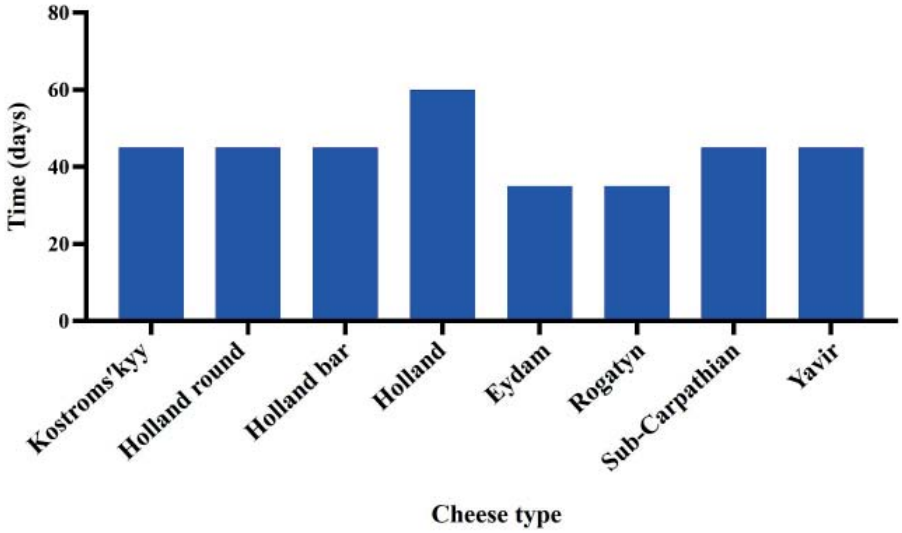

Fig. 7. **Quantitative ratio of ripening duration in the technical conditions of cheeses of the NCSC company (*A*), produced in the EU (*B*), produced in Ukraine (*C*)**

general principle stages are mostly similar and involve the implementation of optimization changes and innovative methods of improving production in terms of product quality and obtaining commercial benefits.

Conclusions

As a result of a comprehensive study of the technological process of the production of various hard and semi-hard cheeses in the EU and Ukraine, as well as recipes of fermented cheeses for production at craft cheese factories and at home from the USA, a total of 73 types of hard and semi-hard cheeses were analyzed: 35 recipes of the New England Cheesemaking Supply Company by Jim Wallace; 30 production processes of hard and semi-hard cheeses from the EU; 8 technical conditions of hard cheeses of Ukrainian producers.

It is shown that the prospects for the development of new types of hard and semi-hard cheeses in Ukraine should include the following recommendations: if possible, apply short-term fermentation with the help of lactic acid starters before adding rennet, which will increase the activity of rennet, since it will be immediately added to milk with a pH of 4.6; the use of thermophilic bacteria in the composition of primary starters shortens the ripening period of cheese and improves its taste characteristics; when using thermophilic or mixed leavens, it is necessary to increase the temperature of fermentation and cooking; the use of rennet from young animals is only advisable, because beef enzyme contains little chymosin and a lot of pepsin and has a lower optimal pH of 2, it is only advisable to replace calf rennet with microbial renin; for better fermentation with the help of microorganisms of the secondary microbiome and enzymes, it is necessary to increase the ripening period to at least 180 days for classic hard cheese, the ripening period can be shortened by adding propionic acid bacteria, yeast and mold fungi as additional components of starter cultures, as well as by increasing the ripening temperature.

By applying the recommendations for optimization of fermentation processes, introduction of innovative biotechnologies, with an assessment of the prospects for the development of new biotechnological methods in Ukraine, it is possible to produce the final product of Ukrainian good-quality and safe hard cheeses for healthy eating, which, in terms of sensory characteristics and texture, will obviously not differ from cheeses produced in EU countries.

«This article contains no human or animal research conducted by any of the authors.»

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АНАЛІЗ ТЕХНОЛОГІЧНОГО ПРОЦЕСУ ВИРОБНИЦТВА ТВЕРДИХ ТА НАПІВТВЕРДИХ СИРІВ В ЄС ТА УКРАЇНІ

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Проведено аналітичний огляд технологічного процесу виробництва різноманітних твердих і напівтвердих сирів в ЄС та Україні, а також вітчизняних рецептур ферментних сирів для виробництва на крафтових сироварнях та у домашніх умовах. Здійснено аналіз умов ключових стадій виробництва, зокрема сквашування, коагуляція та дозрівання. Досліджено склад та тип молочнокислих бактерій у закваскаx для ферментних сирів, а також ферментів для ферментолізу та коагуляції казеїну молока. Показано, що перспективи оптимізації розроблення нових видів твердих та напівтвердих сирів в Україні переважно пов'язані з регулюванням часу та температури сквашування, варіння та дозрівання сирів, а також розширенням біорізноманіття первинного та вторинного мікробіому заквасок для покращення смаку та аромату готового до вживання продукту.

Мета — проаналізувати ферментаційний процес та рецептури виробництва твердих сирів в Україні з основними світовими зразками, порівняти склад та тип молочнокислих бактерій у промислових та крафтових закваскаx, також типи ферментів для ферментолізу та коагуляції казеїну молока, щодо оптимізації виробництв для покращення смаку та аромату готового до вживання продукту.

Матеріали та методи. Методичний аналіз й абстрактно-логічний метод для узагальнення критеріїв оцінки біотехнологічного процесу різноманітних твердих і напівтвердих вітчизняних сирів зі світовими зразками за рекомедаціями, вимогами та стандартами з опрацюванням патентів, технічних умов їх виробництва в ЄС та Україні; ДСТУ 6003 (Сири Тверді); рецепти сирів компанії New England Cheesemaking Supply Company за авторством Джима Валласа. Отримані дані опрацьовані методами статистичного аналізу, систематизації, порівняння та узагальнення інформації.

Результати. У даному досліджені розглянуто докумендацію та проаналізовано дані стандартів, положень, вимог та рекомедації щодо біотехнології твердих та напівтвердих сирів, проведено аналіз ринку твердих сирів та особливостей ферментаційного процесу їх виробництва з визначенням критичних точок та ключових стадій виробництва з використанням промислових депонованих продуцентів сквашування та ферментів білкової коагуляції та біорізноманіття молочнокислих бактерій у закваскаx для ферментованих сирів на світових виробництвах, з оціненням можливостей перспективи розроблення нових та удосконалення біотехнології українських доброякісних та безпечних твердих сирів для здорового харчування.

Ключові слова: твердий сир; напівтвердий сир; сквашування; мезофільні та термофільні молочнокислі бактерії; коагуляція; сичужний фермент; хімозин; ренін; пепсин; дозрівання ферментного сиру; мікробіом.

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RADIATION PHENOMENA: SOME NATURAL SOURCES, MECHANISMS OF EFFECTS, WAYS OF BIOLOGICAL ORGANISMS PROTECTION AND REHABILITATION

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Radiation is an important and dangerous factor in contemporary reality in some regions of industrial countries, after technological accidents at nuclear objects, chemical enterprises, etc. This is also the reality of some contemporary military activities and armed conflicts. Radiation damages of organisms can arise also due to the natural reasons — aviation or space flights at high altitudes or even long stay on mountain heights. Natural reasons of such effects have been studied insufficiently for today.

Purpose. To outline briefly some results of studies of the characteristics of ionizing radiation at different heights above the Earth. To describe briefly the influence of radiation factors on biological organisms and main mechanisms of these effects. To describe effects that cause pathological changes in organisms of people exposed to the low doses of radiation for a long time and methods of post-radiation rehabilitation of affected people in highlands conditions.

Methods. Space satellite exploring of the Earth atmosphere at different altitudes above sea level with measurements of different characteristics of solar and galactic radiation (mainly X-ray, gamma radiation, as well as other types of ionizing radiation in some other ranges). Comparative analysis of the results of long-term observation of patients in hospital conditions using many standard laboratory methods of their states examinations. The conducted scientific research consisted of a complex of methodological techniques and approaches: clinical and physiological studies of respiratory and cardiovascular systems, hematological and immunological states, and functional state of higher nervous activity, mental and neurotic state; administration of antihypoxants, histochemical, biophysical and other methods were used to evaluate oxybiotic processes. Mathematical processing of the results, as well as methods of mathematical modeling were applied.

Results. The results of the measurements of ionizing radiation levels during the satellite exploring of the Earth atmosphere at different altitudes were analyzed and presented in schemes. The mechanisms of damaging radiation effects in organisms at nano level were described: water radiolysis, "oxygen effect" as radio sensitizer, formation of various types of free radicals and peroxides with future consequences for organic compounds, cells, tissues, organs, and organisms. The results of medical treatment and rehabilitation at the EMBS of the persons irradiated by the low doses of radiation were presented, observed and discussed. Many of represented results were obtained thanks to the collective work of the great commands of our predecessors in science who searched for the possibilities of medical treatment and rehabilitation of patients who obtained low doses of radiation during long time. The contemporary results of possibilities of some developed pathological states pharmacological corrections were discussed; practical recommendations were done.

Conclusions. Some of results of fulfilled works, which can be valuable in the treatment and rehabilitation of people of various contingents exposed to low doses of radiation of various natures for a long time, were presented. The outlined recommendations can be offered to persons of various radiation risk contingents for the purposes of their rehabilitation, in practice of health care, etc.

Key words: radiation damage to organisms, high altitudes, adaptation, radioprotectors, correction.

Increased level of radiation is an important and dangerous factor of contemporary reality in some regions of industrial countries, after technological accidents at nuclear objects, as well as reality of some contemporary military activity and armed conflicts. Radiation damages of organisms can arise also due to the natural reasons — aviation or space flights at high altitudes or long stay in mountain conditions. Natural reasons of these effects have been studied insufficiently for today. But it is known that the high level of radiation in mountain conditions is formed due to two main reasons: 1 — high level of space radiation due to a smaller (compared to sea level) thickness of the residual layer of the atmosphere and 2 — the higher radiation of granite rocks in comparison with sedimentary rocks. People are also exposed to small doses of radiation during flights at high altitudes, primarily pilots, but the effect of this factor on their organisms has not yet been studied in detail. It should be noted that common features characterize many mechanisms of the influence of ionizing radiation on biological organisms. Accordingly, some individual methods for correcting radiation damage, studied on one of the models, can be applied (after the appropriate studies) on another model.

Our studies of these phenomena were based on the results of the work of the groups of our predecessors in science, who worked at Elbrus Medical and Biological Station (EMBS) under the leadership of its Director Prof. Pavel Beloshitsky for many years, and under whose supervision Dr. Klyuchko Olena performed numerous scientific works. Results of numerous contemporary investigations in these directions we have accumulated in present observation [1–65]. Among them there are our personal publications linked with these items [1–3, 64, 65]. Studies of the influence of radiation factors under the high-altitude meteorological conditions, adverse environmental conditions (for example, as a result of Chernobyl tragedy) and the possibility of subsequent rehabilitation of the people exposed to these factors were the subjects of research at the EMBS for a number of years. Organization EMBS was subordinated to the National Academy of Sciences of Ukraine during long years, and it was located in Caucasus Mountains on the territory of Kabardino-Balkarian Republic (now — territory of Russia). This station was located in the highlands of the Caucasus on the slopes of Elbrus, the highest mountain in Europe — 5,641 m above sea level (a.s.l.). EMBS was the site of many years of research by Ukrainian scientists, as well as scientists of other multinational teams. They studied and solved topical problems in biology and medicine using the latest methods at every stage. Research works were started here by Academician M. M. Sirotinin in 1929 and continued by his students [1, 64, 65]. A number of obtained here results (often unique) in various fields of knowledge have already been observed in our previous publications. There were represented our results, obtained in various directions of investigations at EMBS and later, in process of works with the results previously obtained at EMBS [66–75]. Important results of the study of radiation effects on living organisms were obtained and accumulated at EMBS long before 1986. Also there were obtained numerous results and suggested the ways of rehabilitation of irradiated persons in highlands conditions. So, with all this potential (intellectual, laboratory, hospital conditions, others), already in the first days after the Chernobyl accident, EMBS Director at these times, Doctor of Medical Sciences Pavel Beloshitsky and the staff of the station were suggested to start post-radiation rehabilitation of affected people in the conditions of the Caucasian mountains as well as the search of new ways for their treatment. This program was successfully implemented at EMBS, as a result of which significant number of the people obtained here their treatment and were successfully recovered ("chernobyltsy" — Chernobyl residents as well as "liquidators" of the consequences of the Chernobyl accident — people who fought with the consequences of that accident) [64, 65].

Works on the rehabilitation of "chernobyltsy" and "liquidators" were started at EMBS on May 1986 [64, 65]. This happened after the approval of the information sheet on the use of the methods of treatment and rehabilitation of irradiated persons in mountain climate conditions developed at EMBS in combination with taking antioxidants, vitamins and other medical substances; document is by Academic Councils of O.O. Bogomoletz Institute of Physiology of the National Academy of Sciences of Ukraine and the Institute of Oncology and Radiology of the Ministry of Health of Ukraine (May, 1986). Below there are some of results of these works, which can be valuable in the treatment and rehabilitation of people of various contingents exposed to low doses of radiation of various nature for a long time.

To outline briefly some results of the radiation researches in space physics and geophysics; influence of these factors on

biological organisms and main mechanisms of these effects. To describe influences that cause pathological changes in organisms of people exposed to the low doses of radiation for a long time and methods of post-radiation rehabilitation of affected people in highlands conditions, including pharmacological corrections of some damages. We would like to apply further such methods in health care practice, for students' education, and to continue these researches.

Radiation effects at different altitude levels above the Earth surface up to the ionosphere: brief analysis. In some our preliminary works we had observed the influence of ionizing radiation at different altitudes above the Earth surface on various substances and objects in the atmosphere [1]. The necessity of such works was caused by the high radiation doses, obtained by the pilots and passengers during the flight, radiation influences on the surfaces of the aircrafts such as airplanes, satellites, etc. Continuing this theme in present work, we would like to observe the specificity of some factors that cause such effects. According to contemporary imaginations, total radiation above the Earth can be subdivided mainly onto two main components – solar radiation and galactic radiation, both have different origin and characterized by different physical characteristics (sometimes other components are distinguished too). Contacting with the atmosphere, they cause such phenomena, as ionosphere. The ionosphere could be called "plasma covering membrane of the Earth" [2–9, 19], and its properties had been studied deeply and reflected in numerous publications [2–64]. The manuals specify that the ionosphere covers the region of near-Earth space in the height range from 50 km, where the presence of free electrons already noticeably affects the propagation of radio waves, up to a conditional 1000 km, where the ionosphere continuously transitions into the magnetosphere. The ionosphere includes regions D (50–90 km), E (90–140 km), F1 (140–200 km), and F2 (above 200 km). This division is not only traditional, but also reflects the difference in physical processes that define the state of plasma at different altitudes. Fig. 1 demonstrates the standard profile of the ionosphere — the dependence of concentrations of free electrons on different heights. A logarithmic scale is applied to the horizontal axis, which visually smooths the graph; in fact, the plasma concentration at the altitude of, f.e., 300 km is 5–10 times higher than at 100 km. Thus, the profile of the ionosphere is stretched in vertical direction. The main mass of the plasma is enclosed in layers at altitudes of 200–600 km. Unlike the neutral atmosphere, the ionospheric layer does not adhere to the Earth's surface, but, as we can see, floats above it.

The ionosphere is formed as a result of atmospheric absorption of solar radiation at altitudes of 100–200 km. In the range of wavelengths less than 1000 (extreme ultraviolet and X-ray), the energy of photons exceeds the thresholds of dissociation and ionization of atmospheric gases, which initiates chains of photochemical reactions in the atmosphere, and this radically changes the properties of the atmosphere.

The ionosphere is not a static object, but a stationary process of circulation of neutral and charged particles. Arising under the action of solar ionizing radiation, charged particles partially recombine with each other, returning to the mother's neutral atmosphere, and partially flow along the lines of force of the Earth's magnetic field upwards into the magnetosphere. At night, the plasma stored in the magnetosphere descends to the heights of its birth and recombines. So, if to speak about such effects on living organisms at different altitudes, we have to subdivide two main factors of influences: 1) the radiation by itself (solar, galactic, or other types) (Figs. 1, 2); and 2) influences of charged atmospheric particles (Fig. 3). These effects were already studied and observed, the results were presented on Figs.1, 2, 3 [26] and Fig. 4.

The spectrum of solar radiation includes a powerful and very stable optical part (visible part of the spectrum, the "solar constant"), as well as weak and variable short-wave and long-wave components (solar activity). The

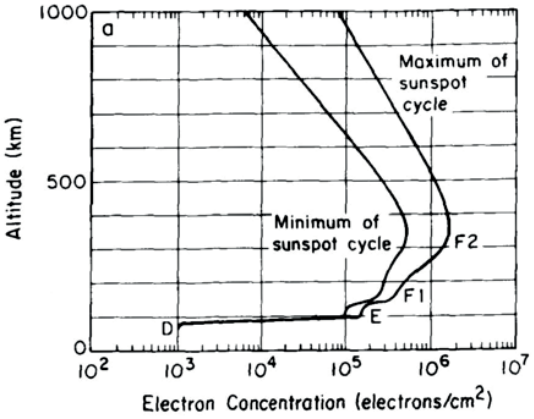

Fig. 1. **Dependence of ionospheric plasma concentration (horizontal axis) on height (vertical axis) in conditions of low and high solar activity [1, 2, 19, 26]**

upper atmosphere is a chemically active environment under the aggressive influence of solar radiation. The upper atmosphere is a "photochemical boiler". Plasma is born, drifts up and down and recombines. In such a way the ionosphere is formed. The spectrum of solar radiation is represented on Fig. 2. Radiation in the ranges of this spectrum causes its effects on the biological organisms at different altitudes in the Earth atmosphere. These various effects associated with high-energy, radiative effects on substances in the atmosphere and at the surface of the Earth's, as well as on living organisms at different heights above the Earth's surface. According to the known regularities of the Nature, the part of the cosmic and solar radiation that enters the atmosphere dissipates, other part is absorbed — most strongly in the upper layers of the atmosphere — the ionosphere, and part of the component is reflected from the upper layers of the ionosphere, as from a mirror. In other words, due to all the above effects, the Earth's atmosphere protects biological beings at the bottom of this "atmospheric ocean" (the Earth surface) from the harmful effects of solar ionizing radiation. However, the intensity of both cosmic and solar radiation fluxes to the Earth is very high. Therefore, a certain part of the components of this ionizing radiation flux still "breaks through" the atmospheric shell and reaches the Earth's surface. Studying dangerous influence of observed kinds of radiation on biological organisms we have to take into account all these phenomena.

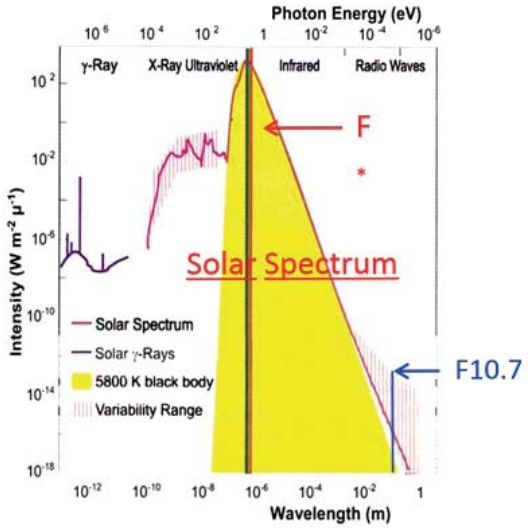

Fig. 2. **Solar spectrum — spectrum of solar radiation that penetrates the Earth atmosphere from the upper layers of the ionosphere down to the surface of the Earth (explanations see in text) [1, 2, 19, 26]**

We had mentioned above about the influence of solar and galactic radiation on particles in atmosphere. When particles in the upper atmosphere are exposed to solar radiation, energy is transferred to them, which transfers the simplest molecules and atoms of elements in the gases of the atmosphere into an excited state, which leads to their transformation. It causes the formation of such forms as ions, free radicals, various other charged particles with high energies that are able to damage biological organisms. Space is not empty! At an altitude of 600 km, the concentration of particles is ~106 cm^{-3}, and far in interplanetary space is ~10 cm^{-3}. But for understanding the properties of the space environment, it is not so much the concentration that matters, but the length (distance) of the free path of the particles. The closer to the Earth's surface, the shorter the free distances (paths) between the particles of the atmosphere. And vice versa — the higher above the Earth's surface, the greater the free path between these particles. This dependence is shown in Fig. 3.

The direction of the arrows coincides with the direction of the ionizing radiation flux vectors — i.e. to the surface of the Earth. When considering the physical and physio-chemical effects, it is indicated whether they increase or decrease in this direction (explanations see in text).

Studying various natural phenomena that cause numerous effects associated with high-energy, radiative effects on substances in the atmosphere and at the surface of the Earth

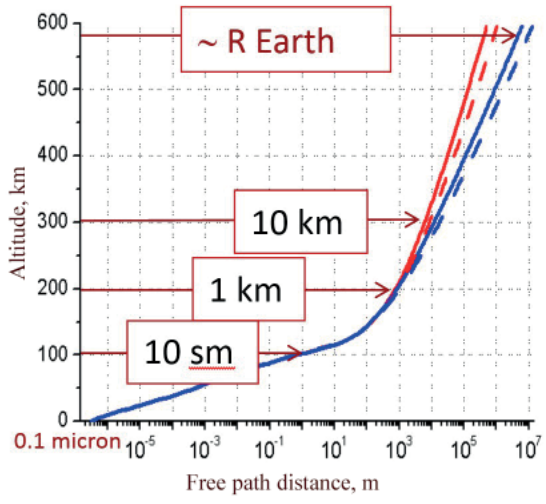

Fig. 3. **Various densities of matter particles (gases, microscopic dust particles other) at different altitudes above the Earth surface (explanations see in text) [1, 2, 19, 26]**

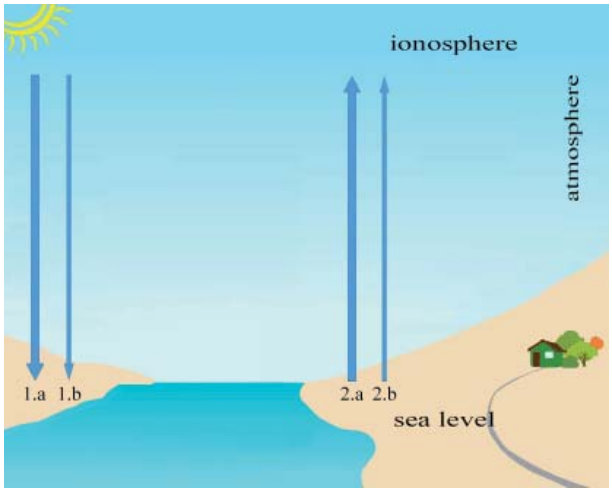

Fig. 4. **Various natural phenomena that cause numerous effects associated with high-energy, radiative effects on substances in the atmosphere and at the surface of the Earth**

as well as biological objects, we distinguished four groups of effects; they are listed below (see also Fig. 4). These effects, their changes in time and interrelations are on the Fig. 4; they all are given below.

1.a. Increasing of effects along the vertical "ionosphere — Earth surface"

1) The densities of matter particles at different altitudes above the Earth surface increase (gases, microscopic dust particles others – up to biomolecules and solid matters). 2) Number of neutral particles in atmosphere increases. 3) Protective properties of the atmosphere increase.

1.b. Decreasing of effects along the vertical "ionosphere — Earth surface"

1) Radiation in the narrower ranges of spectrum is registered close to the Earth surface. 2) High-energy, high intensity radiation is registered in ionosphere; it decreases in direction to the Earth surface. 3) Various effects associated with high-energy, radiative effects on substances decrease. 4) The closer to the Earth's surface, the shorter the free distances (paths) between atmospheric particles. 5) Radiation doses, obtained by persons during the flight. 6) Radiation influences on the surfaces of the aircrafts. 7) Number of charged particles and free radicals in atmosphere decreases. 8) Aggressive influence of solar radiation decreases.

2.a. Increasing of effects along the vertical "Earth surface – ionosphere"

1) Radiation in the widest ranges of spectrum is registered in ionosphere. 2) High-energy, high intensity radiation is registered in the ionosphere; it increases in direction to the ionosphere. 3) Various effects associated with high-energy, radiative effects on substances increase. 4) The further from the Earth's surface, the longer the free distances (paths) between atmospheric particles. 5) Radiation doses, obtained by persons during the flight. 6) Radiation influences on the surfaces of the aircrafts. 7) Number of charged particles and free radicals in atmosphere increases. 8) Aggressive influence of solar radiation increases.

2.b. Decreasing of effects along the vertical "Earth surface – ionosphere"

1) The densities of matter particles at different altitudes above the Earth surface decrease (from biomolecules and solid matters — to gases, microscopic dust particles others). 2) Number of neutral particles in atmosphere decreases. 3) Protective properties of the atmosphere decrease.

So, in this chapter, we considered various natural phenomena that cause various effects associated with high-energy, radiative effects on substances in the atmosphere and at the surface of the Earth's, as well as on living organisms at different heights above the Earth's surface. In the chapter below the mechanisms of the radiation influence on atoms and molecules in living organisms will be explained in details.

Mechanisms radiation influences on organisms. Organisms irradiation in conditions of high-altitude flights and high altitudes. In this chapter, we will consider how those components of ionizing radiation that enter the lower layers of the atmosphere affect water molecules, biological macromolecules, living organisms in these layers and on the surface of the Earth. The materials of this chapter are based on long-term studies of teams of Ukrainian scientists at the EMBS of the National Academy of Sciences of Ukraine, some of which were included in the book [65]. So, these studies were carried out at altitudes comparable to the height of Mount Elbrus (5642 m a.s.l., Caucasus Mountains).

The problem of biological effects of ionizing radiation is, actually, the problem of the excess of radiant energy transfer to a living system, biological substrate and the subsequent destiny of this energy in cell, or in organism. Ionizing radiation is high energy radiation; its carriers — ionizing particles and quanta (photons) carry such significant energy that during primary or secondary (neutron fluxes) interaction with atoms (molecules) of

substances they cause ionization (they pull out individual electrons from an electrically neutral atom or turn its nucleus into an ionizing particle).

For the destiny of irradiated cell, organism, the fact that the primary and secondary products of interaction of high-energy radiation with a living system have a powerful chemical and biological activity, start, initiate a whole chain of successive chemical (biochemical) reactions. It is these chemical reactions transformations, reaching vital cellular structures (DNA, nuclear chromatin, systems of biological membranes, mitochondria) cause their more or less deep, up to irreversible damage, lead to the death of some of the most radiosensitive cells or the entire organism [65].

It is especially important that the radiation death of organism, including such perfect one as human organism, occurs at doses of ionizing radiation that do not cause noticeable increase of living system temperature. This so-called "radiobiological paradox" is known for a long time. It means that it is not the amount of absorbed energy that is decisive for the destiny of the irradiated organism, but the number and chemical (biochemical) activity of those active products that are formed after the effect of primary ionization, as well as electronic excitation of atoms in living system [65].

What are these active products?

The knocked-out electrons can move freely between atoms for some time until they join neutral particles (atoms, molecules), forming negatively charged ions. Consequently, an electronic vacancy, a "hole" in the place of ejected electron, turns the corresponding atom (molecule) into a (+)-ion. Along with (+)- and (-)-ions, excited atoms also can be formed. In such atoms the electron is not knocked out of the atom, but only moved to a higher electronic level. Such atom also has reserve of additional energy and therefore it is capable to be involved into more diverse chemical reactions than an unexcited atom.

Since The tissues of the human body consist on 65–70% of water, the primary radiation-chemical reactions develop primarily in the aqueous phases. In this case, the ejected electrons in the aquatic medium acquire additional stability, each being surrounded by a kind of envelope of water molecules (hydrated electron, e'_{hydr}). Ions H_2O^+ and H_2O^-, as well as excited H_2O^* molecules decompose easily with the formation of H· protons and molecules of hydrogen H_2, free radicals O_{2-}·, HO_2, ·OH and –OH·. In this case, the initial amount of reducing (e^-_{hydr}, H·, H_2) and oxidizing (O_2·, ·OH, H_2O_2) products is the same [65].

However, further, already at the second stage of transformations of products of water radiolysis, living tissues, oxygen starts to play its role. Being a necessary component of living tissues, it is present in the extracellular and intracellular environment, in all liquid media of organism, oxygen directly reacts with primary products of irradiation. Following reactions are going [65]:

$$e^-_{hydr} + O_2 \rightarrow HO^{\cdot}_{2-} \quad (1)$$

$$H\cdot + O_2 \rightarrow HO^{\cdot}_2 \quad (2)$$

As a result, all elementary products after interaction with O_2 acquire oxidative characteristics. Indeed, the radiation injury, radiation sickness is the result of the attacks of vital cellular structures by active oxidative products of radiation. Among them there are following free oxidizing radicals: superoxide O_{2-}·, hydroperoxide HO_2·, hydroxyl ·OH, as well as the so-called singlet oxygen $^1O_2$, hydrogen peroxide H_2O_2. When oxygen and oxidizing water radicals interact with organic radicals (R·), hydroperoxide radicals and hydroperoxides can be formed easily [65]:

$$R^{\cdot} + O_2 \rightarrow ROO^{\cdot} \rightarrow ROOH \quad (3)$$

$$R^{\cdot} + HO^{\cdot}_2 \rightarrow ROOH \quad (4)$$

After the interaction with molecular oxygen, all active irradiation products not only become strong oxidizing agents, but also acquire a longer life time. So, after this they obtain a longer time of life and, consequently, a greater radius of action on biologically important cellular structures. Therefore, the presence of oxygen enhances significantly the damaging effect of ionizing radiation — 3 times more by maximum! In other words, the oxygen which presents in the tissues at the time of irradiation behaves as radiosensitizer, as a substance that increases the sensitivity of organic comcalled "oxygen effect" characterizes the most important role of oxygen in the biological effect of radiation [64, 65].

Radiation disorganization of oxidative and energy metabolism makes an additional contribution to radiation damage of organism.

Finally, the process of post-radiation restoring of biological structures, return of irradiated organism to a more or less normal life needs energy supply, biosynthetic processes and, consequently, the participation of oxygen, the activation of oxidative

phosphorylation. So, oxygen is an active and necessary participant in the process of reparation of structures affected by irradiation and, consequently, acts in an opposite way to the primary oxygen effect.

Therefore, approaching theoretically, by increasing and decreasing O_2 content in body cells, one can try to correct the effects of ionizing radiation within certain limits. Based on these considerations, EMBS scientists proposed an effective way of post-radiation recovery of cells, tissues, and organisms on 1986 [64, 65]. It became possible to develop these methods as a result of long-term works at EMBS. It had happened due to the fact that for a number of years, a lot of attention at EMBS was paid to the study of O_2 role in organism and the effect of its lack (hypoxia). Consequently, a large amount of results, various materials were developed [1, 64, 65]. It was shown that the dosed effect of hypoxic hypoxia, primarily the factors of high mountains (among which mountain hypoxia played the leading role) stimulated the vital forces of irradiated organism, increased its non-specific resistance, accelerated the medical and social rehabilitation of people from the radiation risk contingent [1, 64, 65]. The use of developed approaches helped to restore the health of significant number of affected Chernobyl residents who underwent treatment and rehabilitation at the EMBS.

The influence of adaptation to the factors of mountain conditions on the state of irradiated organism. The analysis of accumulated experimental data makes it possible to give a comparative estimation of the effectiveness of existing means of increasing of organism's resistance to extreme factors [1, 64, 65], including radiation factors. Based on the researches at EMBS, the use of active adaptation to the high-altitude climate for this purpose is recognized as the most promising [64, 65]. Accordingly, among the various methods of training and adaptation to high altitudes, the most effective regimen for increasing the organism's resistance is recognized as the stepwise high altitude adaptation. In animal experiments, it was shown that primary adaptation to high-altitude hypoxia (adaptation to high-altitude conditions) significantly increases animals' resistance to cold, ionizing radiation, blood loss, and lateral accelerations; to cyanides and factors that cause myocardial necrosis, hypertension, etc. In addition, numerous results of empirical observations are known, which indicate that patients with anemia, respiratory allergies and other diseases can be recovered quite quickly in mountain conditions.

The transient processes occur that disrupt the activity of antiradical and antioxidant protection with the changes in the external environment, complex action of various adverse factors, action of large doses of high-frequency radiation waves, ultraviolet and ionizing radiation. In this case, a side effect of excess O_2 is revealed, this is so-called oxygen effect [65]. At the same time, the presence of ions of variable valence (including calcium of the endoplasmic reticulum, iron, which is a part of the heme released during the breakdown of methemoglobin), activates peroxide and radical processes. Therefore, the facts of the increase of the number of organic lesions and encephalopathies in liquidators of the Chernobyl accident can be explained by the activation of peroxide processes and closely related phenomena of intercellular interactions in blood capillaries, membrane permeability disorders, and development of circulatory and tissue hypoxia [64, 65].

Many researchers recommend using the climate of mountain heights for the rehabilitation of such pathological conditions as anemia of various etiologies: hemolytic, post-hemorrhagic, post-radiation, hypoplastic, as well as caused by the action of chemical substances. Recommendations were based on positive morpho-functional and biochemical dynamics of blood indicators: increase in hemoglobin content, disappearance of metabolic acidosis, normalization of glucocorticoid supply of organism and content of ovarian steroids. The success of the treatment of iron-deficiency anemias in mountains should be accompanied by obvious intake of iron-containing medical preparations in combination with vitamin and antioxidant therapy both before arriving in the mountains and during the stay in mountains [64, 65].

Obtained data form theoretical basis for the researches of possibilities of using the mountain climate for the purpose of irradiated patients' medical treatment, for the development of appropriate indicators, contraindications and recommendations. Several hundred people were examined on the basis of EMBS — sick and healthy residents of Kyiv, Slavutych, Borodyanka in the 1990s of the XX century. A comprehensive estimations of the state of health of examined persons were carried out primarily in Kyiv, and later in the mountains at EMBS: on the 2^{nd}-3^{rd} day after arrival in Terskol (2100 m), and on the 22^{nd}-23^{rd} day after active gradual acclimatization.

Complex method of rehabilitation included climate treatment in mountain conditions for 24 days, a dosed movement regime, ascents on a cable car to altitudes of 2750–3700 m a.s.l., dietary, phyto-, narzanotherapy, thermobarotherapy, etc. [64, 65].

The conducted scientific research consisted of a complex of methodological techniques and approaches: clinical and physiological studies of respiratory and cardiovascular systems, hematological and immunological states, and functional state of higher nervous activity, mental and neurotic state; histochemical, biophysical and other methods were used to evaluate oxybiotic processes. Some data are represented in the Tables 1, 2 [1, 64, 65].

During the studies of the processes of transport and utilization of oxygen at the systemic and tissue levels in irradiated persons during rehabilitation in the conditions of mountain heights the following results were

Table 1

Indicators of the efficiency of respiratory system, obtained in Terskol (2100 m a.s.l.) during patients examination on the second (1) and twenty-second (2) days of adaptation [64, 65]

Name	Minute volume of respiration (MVR), $l.min^{-1}$		Alveolar ventilation (AV), $l.min^{-1}$	Respiratory volume (RV), ml	Respiratory rate (RR)	Ventilation rate (VR)	Oxygen effect of respiratory cycle (OERC), ml/r.c.
1-st	1	7.0	4.4	500	0.77	40	13.0
	2	6.5	3.5	440	1.00	46	16.0
2-d	1	5.9	4.6	530	0.89	32	16.0
	2	5.3	2.9	670	0.82	47	14.0
3-d	1	6.6	4.4	410	0.84	35	12.0
	2	6.7	5.3	560	0.79	39	14.0
4-th	1	5.3	2.5	300	0.82	55	5.4
	2	6.2	3.8	410	0.73	43	9.4
5-th	1	5.0	3.7	360	0.77	40	9.1
	2	4.6	3.4	420	0.79	44	9.1
6-th	1	5.2	3.5	330	0.83	44	9.5
	2	5.1	3.6	430	0.73	38	11.0
7-th	1	3.6	2.2	210	0.78	41	5.2
	2	3.5	3.1	350	0.85	32	11.0
8-th	1	7.3	3.9	610	0.92	44	14.0
	2	6.7	4.7	670	0.90	41	16.0
9-th	1	4.9	2.9	450	0.75	30	15.0
	2	5.0	3.1	420	0.76	35	12.0
10-th	1	462	3.0	720	0.97	40	9.4
	2	4.8	3.3	440	0.80	45	9.8
11-th	1	5.8	4.4	440	0.84	35	13.0
	2	6.0	5.0	500	0.75	32	16.0
12-th	1	3.7	2.1	270	0.95	43	6.2
	2	4.2	2.6	260	0.90	44	6.3
13-th	2	7.6	4.9	630	0.90	43	15.0
M	1	5.3	3.4	425	0.85	39.9	10.6
	2	5.5	3.8	477	0.82	40.1	12.3
m	1	0.3	0.3	42	0.02	1.9	1.08
	2	0.3	0.2	34	0.02	1.4	0.86

Comments. The leftmost column shows the order number of the individual examined patients (names are not given in accordance with medical ethics).

registered. After twenty days of adaptation, the respiratory and hemodynamic systems of the examined people began to work more economically, the O_2 content in the arterial blood increased in almost all the observed persons. Such an increase allowed organism to provide the necessary rate of O_2 delivery by arterial blood at a lower minute volume of blood. An increase in O_2 content in mixed venous blood was also registered.

Anti-radiation effect of medical preparations that are capable to increase the level of physiological antioxidant system. As it was mentioned above, under the conditions of exposure to low-intensity radiation for significant periods of time, a state of oxidative stress occurs in organism. A long-term state of oxidative stress leads to a gradual "burnout" of endogenous antioxidants (AO), to a decrease in the efficiency of the AO enzyme systems. As a result, the insufficient power and efficiency of the physiological antioxidant system (FAOS), its depletion under the action of low-intensity irradiation, is the limiting factor determining the organism's stability.

In these conditions, the most effective against the consequences of organism irradiation is the use of those medical preparations that can increase the power of the PAOS, namely antioxidants, adaptogenes, immunomodulators. The authors have already published descriptions of some experiments of the study of effects of some substances capable of such functions performing [1]. Reducing the intensity of lipid peroxidation (LPO) and replenishing endogenous antioxidant (AO)-resources, having low toxicity, AO were able to counteract effectively the negative effects of long-term low-intensity irradiation. Since LPO activation is a necessary molecular link of the mechanism of stress, including one of non-radiational nature, the use of AO is able to counteract the undesirable effects of environmental and psychoemotional stress. On the other hand, adaptogens and anti-stressor medical preparations are effective even with long-term exposure. Finally, immunomodulators, under the condition of very careful and dosed use, are capable of mitigating the effects of post-radiation immunodeficiency, which is inherent to contingents of radiation risk from the Chernobyl zone [63–65].

All medical preparations that can be assigned to the specified group are actually different from both radioprotectors and means of treatment of radiation injuries. First, they were introduced, mainly, against the background of exposure continuation, but not before and not after its finishing. Secondly, they are united by a general focus on overcoming (mitigating) of early post-radiation processes, such as LPO activation, stress response. Therefore, EMBS scientists proposed to allocate them to a special third group of anti-radiation substances — means of early pathogenetic therapy of radiation damage [63–65].

The substances and preparations for some pathological states pharmacological corrections we had suggested already on the base of the works, done by Dr O. Gonchar and her colleagues [114–123]. For pharmacological correction of disorders caused by radiation, following substances and preparations as fullerene C60, yackton, sufan, splenozide, others can be used; they primarily were studied as antioxidants and suggested for hypoxia disorders corrections [1, 85, 116–118].

The effectiveness of mountain climate therapy. The state of oxygen deficiency is pathogenetically related closely to the main clinical syndromes characteristic of irradiated people ("liquidators", residents of the Chernobyl zone, others). These clinical manifestations include iron-deficiency anemia, encephalopathies, vegetative-vascular dystonias, as well as secondary immunodeficiencies, which are accompanied by an increase in the frequency of acute inflammatory diseases (primarily respiratory), exacerbations of chronic diseases, and an increase in their duration and frequency of complications. Therefore, the correction of hypoxic conditions in various categories of radiation-affected persons, their treatment with the use of healing factors, including mountain ones, are relevant and promising. One of the effective means of increasing the organism's defenses is adaptation to hypoxybaria. The results of the effectiveness of mountain climate therapy are shown in the Table 3.

Some key results, which can be used for treatment, rehabilitation of the people of various contingents exposed to low doses of radiation (EMBS experience). Here are some conclusions made on the basis of works on the rehabilitation of the people that were exposed to the consequences of the Chernobyl accident "chernobyltsy". We note that they can be valuable for carrying out work on the treatment and rehabilitation of people from other contingents of radiation risk. These recommendations previously were published in Ukrainian [64].

Table 2

Estimated indicators of hemodynamic system and hypoxic state obtained in Terskol (2100 m a.s.l.) during patients examination on the third (1) and twentieth (2) days of adaptation [64, 65]

Patient	Day of adaptation	Minute blood volume (MBV), $l.min^{-1}$	hemodynamic equivalent (HE)	oxygen effect of the cardiac cycle (OECC). ml/beat	blood oxygen capacity (BOC).	oxygen content		saturation of mixed venous blood with oxygen. ($S\bar{v}O_2$)
						in arterial blood (CaO_2)	in mixed venous blood ($C\bar{v}O_2$)	
1-st	3-d	3.5	20.0	2.6	19	16.25	11.10	58.4
	20-th	3.3	23.0	2.2	20	18.84	14.59	72.9
2-d	3-d	4.1	23.0	2.4	18	15.21	10.82	60.1
	20-th	2.6	23.0	1.9	21	14.70	10.46	49.9
3-d	3-d	4.3	23.0	3.1	21	18.06	13.64	71.8
	20-th	3.1	18.0	3.1	19	13.33	7.84	71.2
4-th	3-d	4.3	44.0	3.2	19	16.82	14.56	44.2
	20-th	4.2	29.0	2.2	19	15.96	12.39	65.2
5-th	3-d	3.5	28.0	2.4	19	14.15	11.20	65.2
	20-th	3.8	37.0	1.5	19	17.48	13.57	78.2
6-th	3-d	4.4	32.0	2.0	18	15.84	12.66	70.3
	20-th	4.4	32.0	2.0	19	16.15	12.97	68.3
7-th	3-d	3.0	34.0	1.5	14	11.90	8.97	49.8
	20-th	2.7	24.0	2.0	16	13.60	9.53	59.5
8-th	3-d	4.7	28.0	2.4	18	15.48	11.86	65.9
	20-th	3.8	23.0	2.4	18	16.20	11.99	47.4
9-th	3-d	4.8	29.0	2.4	14	13.13	9.80	70.0
	20-th	3.4	24.0	2.1	16	13.60	9.19	57.4
10-th	3-d	4.2	23.0	3.0	15	12.00	9.62	64.1
	20-th	4.3	40.0	1.5	18	14.49	11.93	66.3
11-th	3-d	3.6	22.0	2.6	16	14.16	9.44	59.0
	20-th	4.6	24.0	2.8	19	16.91	12.78	67.3
12-th	3-d	3.9	46.0	1.4	16	13.84	11.63	72.7
	20-th	3.6	38.0	1.6	18	14.40	11.76	65.3
13-th	20-th	2.8	21.0	1.9	19	17.77	11.34	59.7
M	3-d	4.03	29.3	2.4	17.3	14.73	11.27	62.3
	20-th	3.58	27.3	2.1	18.5	15.65	11.56	63.7
m	3-d	0.15	2.4	0.2	0.64	0.54	0.50	2.5
	20-th	0.18	1.9	0.1	0.38	0.49	0.52	2.4

Comments. The leftmost column shows the order number of the individual examined patients (names are not given in accordance with medical ethics).

Table 3

Symptoms of diseases of liquidators of the Chernobyl accident according to the data of Ministry of Health of Ukraine (I), authors' data (EMBS) (II), and (III) demonstration of effectiveness of mountain climate-therapy [64, 65]

Symptoms	Frequency of symptoms, %			
	I	II	III-a	III-b
Headaches	82	100	52	48
Vertigo	91	72	48	20
Nausea	98	80	48	32
Vomiting	–	28	–	–
Pain in the eyeballs	–	48	95	20
Noise in head	36	36	16	20
Disequilibrium	81	76	52	24
Increased sweating	62	76	48	24
Astringency in throat	82	72	64	8
Sleep disturbance	64	40	32	8
Short-term loss of consciousness	37	32	28	4
Pain in area of heart	–	80	44	32
Vegetative crises	–	76	48	21
Trunk seizures	–	24	24	–
Asthenic syndrome	58	20	20	–
Decrease in working capacity	53	–	–	–
General weakness	96	–	–	–
Hoarseness of voice	64	–	–	–
Hearing loss	42	–	–	–
Motion sickness in transport	52	–	–	–
Pains in joints	–	55	8	12
Increased fatigue	–	88	72	16
Seizures of "cramp" type	–	28	20	8
Asthenic syndrome	–	20	12	8

Comments. Symptoms: a — disappeared completely, b — disappeared partially, "–" — the data were not presented.

1. The symptoms of diseases of the liquidators were determined (Table 3). Also the structure of radiation-induced morbidity of children from the 4th zone was determined: gastrointestinal diseases 78.6%, respiratory diseases 58.9%, thyroid gland 57.1%, vegetative-vascular dystonia 19%.

2. Approximately ten the most informative criteria for evaluation of "mountain-therapy" were defined. These are the criteria of the organism's oxygen balance, the efficiency of processes of oxygen transport and utilization, the degree of progressive action of hypoxia, adaptability and level of adaptation, physical and mental capacity, etc.

3. It was revealed that in the genesis of the "Chernobyl syndrome" of irradiated people, the polyfunctional disorders in the systems of transport and utilization of oxygen, which leads to the development of hypoxic conditions, are of primary importance. These states of organisms were revealed through the clinical pictures of vegetative-vascular dystonias, anemias, respiratory allergies, dyscirculatory encephalopathies, etc.

4. It was revealed that in persons chronically irradiated with small doses of radiation, the organism's reserve capacities were reduced. There are: indicators of oxygen consumption, efficiency of oxygen transport systems, and activity of respiratory enzymes responsible for urgent adaptation.

5. It was established that in the process of adaptation to the mountain climate in the specified contingent of peoples numerous characteristics were changed. There were:

psycho-emotional state and regulation of vegetative functions were improved, the parameters of respiration, hemodynamics, and the immune status of the blood were normalized, the mode and electrical activity of the heart were stabilized, degenerative changes on the part of blood cells were reduced, regeneration processes were activated, oxygen content in arterial blood was increased, lysosomal activity of white blood cells was increased, DNA synthesis was increased, activity of respiratory enzymes responsible for urgent adaptation was increased, aerobic and anaerobic tissue enzymes were activated, oxygen transport systems were economized, indicators of the level of functional mobility, dynamism of nervous processes were improved. So, in patients with anemia, "mountain-treatment" caused an improvement in the general condition and well-being, increase in the adaptation reserve, transition to a new level of regulation, improvement in the quality indicators of erythrocytes, platelets; increase in the number of erythrocytes, leukocytes, rod-nuclear neutrophils, and plasma lymphocytes; decrease in values of "ventilatory" and hemodynamic equivalents; improvement of indicators of the dynamics of nervous system, attention, ability to direct reproduce the test material, and etc.

6. For the first time, a comprehensive method of rehabilitation of "Chernobyl" residents was developed and used with high efficiency in the conditions of stepwise mountain adaptation, which includes sanatorium-resort treatment at an altitude of 550 m a.s.l. with subsequent rehabilitation at an altitude of 2100 m a.s.l. with short-term ascent to an altitude of up to 4200 m a.s.l., thermobarotherapy, phytotherapy and diet therapy, balneotherapy, etc.

7. In the peripheral blood of persons who lived and worked in the zones of radiation contamination, with a normal content of leukocytes, pronounced neutrophilopenia and lymphocytosis were registered, and with large number (up to 50%) of altered neutrophils (nuclear fragmentation), plasmatized lymphocytes with fringed cytoplasm; a decrease in the activity (in neutrophils) of NADPH was determined; in the lymphocytogram, with a normal value of large granulomatous lymphocytes, significant decrease in the level of small lymphocytes, which were the main effectors in realization of immune response, were registered.

8. As a result of experimental studies, it was shown that in irradiated animals were registered: activation of peroxidic and catabolic processes, glycolysis and proteolytic enzymes; antioxidant reserve, ATP synthesis, number of erythrocytes, blood serum relaxation time, erythrocyte resistance, total oxygen consumption decreased; and were increased: the level of spontaneous chemoluminescence, glucose consumption by erythrocytes, deficiency of buffer bases, malonaldehyde content, lactic acid concentration. Also in in irradiated animals were registered that pH shifts towards acidification; the content of serotonin and histamine increases, which increases the narrowing of arterioles, smooth muscles and bronchi; the membranes of erythrocytes were changed, which is expressed in the increase of their star-shaped forms; destructive processes occur in the capillary walls and glial phagocytes cluster around them; pericapillary couplings and the basal layer expand due to the detection of hydration centers in the basal membrane, as well as due to an increase in the number of its petals and the number of pericytes, which leads to sharp deterioration in the permeability of the blood-brain barrier; mitochondria were destroyed, the number of lysosomes were increased, respiration and phosphorylation were uncoupled. All this leaded to the emergence of a hypoxic state of the combined type (circulatory hypoxia, deoxidation), hypoergy, decrease in the organism's resistance, premature aging. All these effects can explain the development of the clinical picture described above, and they were registered by EMBS researchers in people that were exposed to the consequences of the Chernobyl accident "chernobyltsy".

9. The developed mathematical and software tools can be successfully used to estimate the functional state of oxygen transport systems in radiation pathology, in particular, to calculate indicators of the speed and intensity of oxygen utilization, voltage cascades and the speed of oxygen transport along the entire path (from lungs to tissues), cost-effectiveness systems of external breathing, hemodynamics, etc.

10. A high degree of correlation was determined between the parameters of organism's oxygen condition, adaptability, work capacity, general non-specific resistance, and the morphofunctional state of blood cells.

Thus, in present article the last results of investigation of various characteristics of ionizing radiation in atmosphere at different altitudes above sea level were given. The results of the measurements of ionizing

radiation levels during the satellite exploring of the Earth atmosphere at different altitudes were analyzed and presented in schemes on Figs. 1–4 [1–62, 76–85]. The mechanisms of damaging radiation effects in organisms at nano- level were described; among them there are water radiolysis, "oxygen effect" as radiosensitizer, formation of various types of free radicals, peroxides, hydroperoxides with future consequences for organic compounds, cells, tissues, organs, and organisms. The results of medical treatment and rehabilitation at the EMBS of the persons irradiated by the low doses of radiation were presented, observed and discussed. With great respect we reminded some of the results of research and practical treatment and rehabilitation of victims of the accident at the Chernobyl nuclear power plant in 1986. This huge and humane work was done by the great commands of our predecessors in science at EMBS. The results they had obtained formed a scientific and practical background, based on which we can today develop new methods of research and rehabilitation of irradiated people, solve numerous emerging contemporary tasks. We described and discussed some obtained at EMBS results of medical treatment and rehabilitation of patients who obtained low doses of radiation during long time. On the base of these studies practical recommendations for such patients' medical treatment and rehabilitation were done.

The substances and preparations for some pathological states pharmacological corrections we had suggested already on the base of the works with antioxidants [83–113], including ones done by our colleague Dr O. Gonchar and her colleagues [114–123]. For pharmacological correction of disorders caused by radiation, following substances and preparations as fullerene C_{60}, yackton, sufan, splenozide, others can be used; they primarily were studied as antioxidants and suggested for hypoxia disorders corrections [1, 85, 116–118, 124–127]. Obtained results were important, too, for continuation of the works in biotechnology [124].

So, in present article some results of the works, which can be valuable in the treatment and rehabilitation of people of various contingents exposed to low doses of radiation of various natures for a long time, were observed. Final recommendations can be offered to persons of various radiation risk contingents for the purposes of their medical treatment and rehabilitation. Developed methods can be used also in the practice of health care and health recovery after the influence of ionizing radiation.

We would like to emphasize that it is necessary to implement as widely and actively as possible into the practice of health care the most effective methods of rehabilitation and treatment of persons from radiation risk contingents developed at EMBS in the conditions of adaptation to the natural conditions of the mountains (Carpathians, other mountains), as well as in the conditions of hypoxia simulation (methods of hypoxytherapy – hypobaric, normobaric, hypercapnic, interval, pulsed, periodic hypoxia, as well as hypoxia created in the conditions of barochamber, hypoxicator, hypoxic mixtures, and etc.) [64, 65].

At the same time, the research group headed by Prof. Beloshitsky P. V. had demonstrated that the methods of normobaric or hypobaric interval hypoxia were promising for the replacing of the stepwise mountain adaptation. It was demonstrated that the most effective was rehabilitation complex, which, together with "mountain-therapy", combined diet and phytotherapy, special physical and breathing exercises, thermobarotherapy, intake of bromine-iodine and silicon water, sulfate and dolomite natural waters like "Narzan", "Naftusia", etc. [64, 65].

Indeed, a trip to the Caucasus for the purpose of treatment in the mountain conditions is currently unrealistic for the population of Ukraine now. Therefore, alternative ways of treatment and rehabilitation were also studied and recommended by the authors. For Ukrainian radiation risk contingents, treatment in the conditions of the Carpathians is available and effective, where the authors have also accumulated experience in adaptation and rehabilitation [64, 65] in combination with other methods.

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this direction was done at EMBS – Elbrus medical and biological station of the National Academy of Sciences of Ukraine. Commands of Ukrainian scientists and doctors led by Prof. Beloshitsky P.V. from the first days after the accident fulfilled these tasks for many years (Baraboy V.A., Krasyuk A.N., Korkach V.I., Torbin V.F, and many others). Many sick people were saved thanks to the works at EMBS. Starting from these results, we — contemporary scientists of Ukraine — can continue to develop new methods for saving and rehabilitation of irradiated people, solve numerous emerging contemporary tasks.

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ФЕНОМЕН РАДІАЦІЇ: ДЕЯКІ ПРИРОДНІ ДЖЕРЕЛА, МЕХАНІЗМИ ЕФЕКТІВ, СПОСОБИ ЗАХИСТУ БІОЛОГІЧНИХ ОРГАНІЗМІВ ТА ЇХ РЕАБІЛІТАЦІЯ

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Радіація є важливим і небезпечним фактором в сучасній дійсності в деяких регіонах індустріальних країн, наслідком техногенних аварій на ядерних об'єктах, хімічних підприємствах тощо. Це також реальний наслідок деяких сучасних військових дій і збройних конфліктів. Радіаційні ураження організмів можуть виникати і внаслідок дії природних причин — авіаційних чи космічних польотів на великій висоті або навіть тривалого перебування на гірських висотах. Природні причини таких ефектів на сьогодні вивчені ще недостатньо.

Мета. Коротко викласти деякі результати досліджень характеристик іонізуючого випромінювання на різних висотах над Землею. Описати коротко вплив факторів радіації на біологічні організми та основні механізми цих впливів. Описати ефекти, що викликають патологічні зміни в організмі людей, які зазнали тривалого впливу низьких доз радіації, та методи реабілітації постраждалих від променевої радіації в умовах високогір'я.

Методи. Космічні супутникові дослідження атмосфери Землі на різних висотах над рівнем моря з вимірюванням різних характеристик сонячного і галактичного випромінювання (переважно рентгенівського, гамма-випромінювання, а також інших видів іонізуючого випромінювання в деяких інших діапазонах). Порівняльний аналіз результатів довготривалих спостережень за пацієнтами в стаціонарних умовах із застосуванням багатьох стандартних лабораторних методів обстеження їх стану. Проведені наукові дослідження складалися з комплексу методичних прийомів і підходів: клініко-фізіологічні дослідження дихальної та серцево-судинної систем, гематологічного та імунологічного стану, функціонального стану вищої нервової діяльності, психічного та невротичного стану; введення антигіпоксантів, гістохімічні, біофізичні та інші методи оцінювали оксибіотичні процеси. Застосовували математичну обробку результатів, а також методи математичного моделювання.

Результати. Результати вимірювань рівнів іонізуючого випромінювання під час супутникового дослідження атмосфери Землі на різних висотах проаналізовано та представлено у схемах. Описано механізми шкідливої дії радіації на організми на нанорівні: радіоліз води, «ефект кисню» як радіосенсибілізатора, утворення різних типів вільних радикалів і пероксидів з подальшими наслідками для органічних сполук, клітин, тканин, органів і організмів. Представлено, розглянуто та обговорено результати лікування та реабілітації на ЕМБС осіб, опромінених малими дозами радіації. Багато з представлених результатів були отримані завдяки колективній роботі великих команд наших попередників у науці, які досліджували можливості лікування та реабілітації пацієнтів, які протягом тривалого часу отримували малі дози опромінення; розроблено практичні рекомендації.

Висновки. Представлено деякі результати виконаних робіт, які можуть бути цінними при лікуванні та реабілітації людей різних контингентів, які протягом тривалого часу зазнали впливу малих доз радіації різної природи. Окреслені рекомендації можуть бути запропоновані особам різних контингентів радіаційного ризику з метою їх реабілітації, у практиці охорони здоров'я тощо.

Ключові слова: радіаційне ураження організмів; висота; адаптація; радіопротектори; корекція.

EXPERIMENTAL ARTICLES

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In vitro DIRECT SHOOT REGENERATION FROM *Rhodiola rosea* L. LEAF EXPLANTS

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Wild plant species are of great interest as a source of pharmacologically valuable compounds but a great number of them are endemic and/or endangered ones. Modern plant biotechnology can provide reliable methods for their utilization without disturbing natural populations. *In vitro* culture methods for *Rhodiola* species are being intensively developed to include them into various biotechnological programmes.

Aim. Development of a protocol for direct *Rhodiola rosea* L. plant regeneration from leaf explants.

Methods. The leaves of *R. rosea* aseptically growing plants were used as the explants. Several variants of Murashige and Skoog (1962) agar-solidified culture medium supplemented with different combinations of auxins (1-naphthaleneacetic acid (NAA) and 2,4-dichlorophenoxyacetic acid (2,4-D)) and cytokinins (kinetin and 6-benzylaminopurine (BAP)) were estimated as potential regeneration-inducing media. Regeneration frequency was calculated as the percentage of leaves that produced shoots.

Results. The use of MS medium supplemented with 2.5 mg/l BAP and 1.0 mg/l 2,4-D allowed inducing shoot formation with 100% frequency. An increase in the 2,4-D content up to 2.5 mg/l and decrease in BAP content to 1.0 mg/l resulted in decreasing of the regeneration frequency to 62.5%. Regeneration frequency was 25% and 62%, respectively, on the media containing 1.0 mg/l kinetin + 2.5 mg/l 2,4-D and 2.5 mg/l kinetin + 1.0 mg/l 2,4-D.

Conclusions. *R. rosea* leaf explants have demonstrated high regeneration capacity with the use of the studied combinations of plant growth regulators. MS medium supplemented with 2.5 mg/l BAP and 1.0 mg/l 2,4-D allowed inducing shoot regeneration in leaf explants with the frequency of 100%. The frequency of regeneration was lower in the case of substitution of BAP for kinetin. The other types of morphogenesis (formation of adventitious roots and/or callus) were also observed.

Key words: *Rhodiola rosea* L.; leaf explants; shoot regeneration; growth regulators.

According to different estimations the genus *Rhodiola* comprises from 130 to near 190 species of these almost 70 are accepted species names, and the others are synonyms or have not yet been clarified. *Rhodiola rosea* L. (synonyms *Sedum roseum* L. (Scop.); *Sedum rhodiola* DC.) also known as "golden root" or "roseroot" is dioecious, perennial plant of the family *Crassulaceae* distributed in mountainous regions of the Northern Hemisphere. It is included in the Red Lists of protected plant species in many countries [1–5].

For centuries, extracts of *R. rosea* roots were used in the traditional medicine as an adaptogen with various health-promoting effects to increase physical endurance, work productivity, resistance to high altitude sickness, and to treat fatigue, depression, anemia, infections, gastrointestinal ailments, and nervous system disorders. Modern phytotherapy considers it a vegetal source with an antioxidant and antistress-adaptogene action [1, 2, 5–8]. Biochemical studies of *R. rosea* rhizomes and roots have revealed the presence of six groups of compounds: phenylpropanoids (rosavin, rosin, rosarin), phenylethanol derivatives (salidroside, tyrosol), flavonoids, monoterpernes,

triterpenes, and phenolic acids. The pharmacological activity is based mainly on rosavin, rosin, and rosarin which are present in *R. rosea*, *R. sachalinensis*, *R. himalensis*, and *R. serrata* and salidroside found in the majority of *Rhodiola* species [4, 6, 7, 9]. It was shown that the content of these substances depends on the morphological part of the plant, its age and sex; the place and time of harvesting [4].

As *R. rosea* is an endangered medicinal plant its use from the natural habitats is restricted, and some new sustainable approaches are needed to avoid depletion of the natural sources. Field cultivation is challenging, costly and depending on climate and weather conditions; sufficient yields of roots/rhizomes could be obtained within 5–7 years. Chemical synthesis is another possible approach, and it has been already performed for rosavin and salidroside but not for the other biologically active compounds responsible for *Rhodiola* pharmacological properties [4, 9].

In vitro techniques provide controlled growing conditions, independency on the environmental factors, possibilities to optimize culture media, acceleration of biomass production, and ensuring of continuous production cycle [3, 5, 9]. To date, the *in vitro* cultures have been elaborated mainly for *R. rosea* and some Asian *Rhodiola* species, such as *R. crenulata*, *R. kirilowii*, *R. quadrifida* and *R. sachalinensis* [4, 5, 7]. Elaborated microclonal propagation methods can provide *in vitro* regenerated plants for repopulating native habitats of *Rhodiola* species as well as a raw material for secondary metabolites production [5, 7, 10, 11]. Shoots and roots cultured *in vitro* as well as callus and suspension cultures were studied as a source of biologically active compounds (salidroside, rosavin, triandrin, caffeic acid), and the impact of some stress factors, light and growth regulators on their production was estimated [1, 7, 12]. It was also shown that elicitation and biotransformation in *Rhodiola* cell cultures can be a feasible approach to sustainably enhance the content of active substances in *in vitro* cultures [1, 7, 12–15]. A promising way to enhance the secondary metabolite production by *R. rosea in vitro* cultures is the application of genetic engineering methods to regulate their biosynthetic pathways [5]. *Agrobacterium rhizogenes* was used to produce hairy roots of *R. rosea* [4, 9] and *R. kirilowii* [13] as a possible source of rosavinoids and salidroside.

The conditions of *R. rosea* shoot formation as a way for rapid multiplication were studied earlier [10, 11]. Plant growth regulators in different combinations were used. Leaves of aseptic plants are a fairly affordable type of explants to initiate growth of the new shoots. However, there is still lack of knowledge regarding the possibility of *R. rosea* direct shoot regeneration in leaf explants of aseptically growing plants.

The aim of the present research was elaboration of an efficient protocol for *R. rosea* regeneration in *in vitro* cultured leaf explants as a basis for including this species into further biotechnological studies.

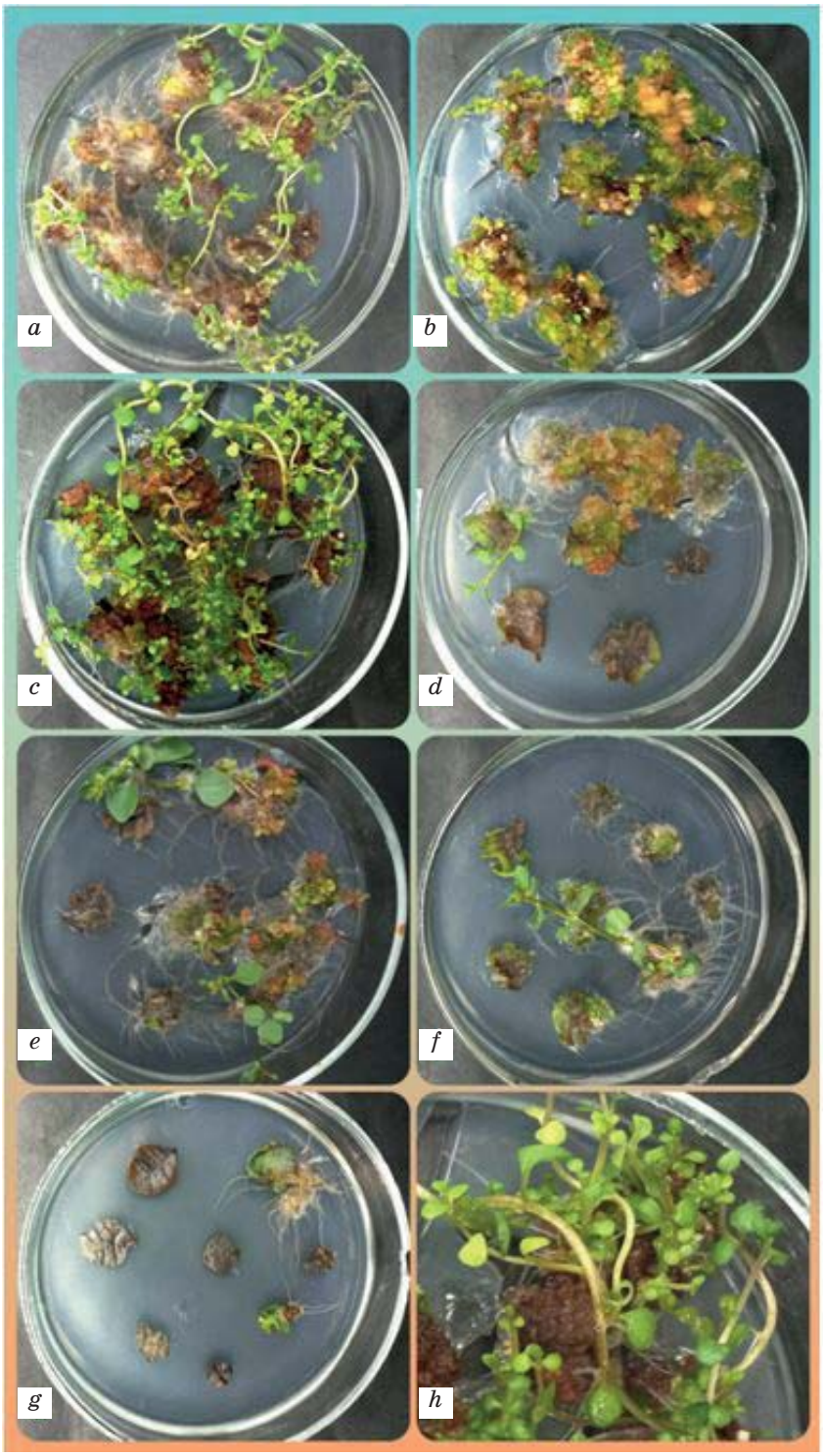
Rhodiola rosea L. plants from the collection of the Institute of Cell Biology and Genetic Engineering of NAS of Ukraine were cultured *in vitro* on the solidified Murashige and Skoog medium (MS, Duchefa, the Netherlands). Leaves of aseptically growing plants were used as explants to study their regeneration capacities. The leaves with 3–5 incisions on them were cultured under 25 °C, 16-h/8-h photoperiod and the illumination of 3000 lux in Petri dishes on MS medium supplemented with 30 g/l sucrose and different combinations of the following plant growth regulators: 6-benzylaminopurine (BAP), kinetin, 1-naphthaleneacetic acid (NAA), and 2,4-dichlorophenoxyacetic acid (2,4-D) (the Table). Combinations of plant growth regulators chosen for the experiments are the basic ones used for a number of plant species

***R. rosea* shoot regeneration on the different variants of culture media**

Medium, No	Growth regulators content, mg/l				Shoot regeneration frequency, %
	BAP	Kinetin	2,4-D	NAA	
1	1.0	–	–	0.5	87.5
2	1.0	–	2.5	–	62.5
3	2.5	–	1.0	–	100.0
4	–	1.0	2.5	–	25.0
5	–	2.5	1.0	–	62.0
6	–	1.0	–	0.5	37.5
7	–	–	–	–	0

held in *in vitro* collection of the Institute of Cell Biology and Genetic Engineering. *In vitro* morphogenesis (formation of shoots, roots and callus) was evaluated in the course of two months cultivation. The regeneration frequency was determined as the percentage of explants which formed shoots.

General results are shown in the Table. Hormone-free MS medium (No 7, the control variant) was not efficient for shoot production. The majority of explants did not form shoots, became necrotic and died, but sometimes root formation at their cut sites was observed (Fig., *g*).



Effect of growth regulators on shoot formation in *Rhodiola rosea* L. leaf explants:
media 1–7 (*a*–*g*); *h* — shoots formed on the MS medium with 2.5 mg/l BAP and 1,0 mg/l 2.4-D

On the medium supplemented with both 1.0 mg/l BAP and 0.5 mg/l NAA (medium No 1) direct regeneration of shoots was observes with 87.5% frequency during two weeks of cultivation. Besides shoot induction the mass root formation was detected with the frequency of 100% (Fig., *a*). Substitution of BAP for kinetin in the nutrient medium containing NAA (medium No 6, Fig., *f*) has led to the reduction of regeneration rate down to 37.5 %. Thus, with the same content of the auxin (NAA) in the media 1 and 6, replacing of BAP for kinetin reduced the ability of explants to form shoots.

Shoots were also induced on the medium No 2 but their growth started later, in three weeks, and the regeneration frequency was 625% (Fig., *b*). An increase in BAP concentration from 1.0 to 2.5 mg/l and decrease of 2,4-D content from 2.5 to 1.0 mg/l (medium No 3) significantly stimulated direct regeneration of shoots. Their formation was observed as early as in 10 days of cultivation with the regeneration frequency of 100% (Fig., *c, h*).

Substitution of BAP in the medium for another cytokinin, kinetin, in the presence of 2,4-D did not stimulate mass shoot regeneration in the leaf explants. Although shoot growth occurred under such conditions, the regeneration frequency was significantly lower than on the media supplemented with BAP. Thus, the regeneration frequency on the medium No 4 was no more than 25 %, and on the medium No 5 — 62 % (Fig., *d, e*). The attention must be payed to the almost identical regeneration frequency when media No 2 and No 5 with different growth regulators combination were used. Medium No 5 contained kinetin, and media No 2 — BAP. The latter regulator, as it turned out, sometimes has a more powerful effect as a phytohormone for regeneration. However, medium No 5 contained less effective kinetin, but in a much higher concentration.

A characteristic feature of morphogenesis on the medium No 4 is development of friable yellowish callus with interspersed pink areas. This may be an indirect indication of the synthesis of some secondary compounds inherent to *Rhodiola* plants. Root formation occurred in all media variants, but the most intense roots were formed on the culture media No 1 and No 5.

Root formation on the regenerated shoots was initiated after the shoots were transferred on hormone-free MS medium.

Cytokinins are usually used to induce multiple shoot formation so it is quite natural that the increase in cytokinin content contributed to the increase in regeneration frequency. At the same time, the fact is of interest that the regeneration of *R. rosea* shoots from leaf explants is also possible on the medium with auxin content of 2.5 times higher than that of the cytokinin.

Different combinations of plant growth regulators were used earlier for induction of *R. rosea* shoot formation and *in vitro* multiplication. However, in the majority of studies, the parts of plants with previously formed buds served as starting material. For example, shoot proliferation was demonstrated in pre-existing axillary buds on MS medium supplemented with thidiazuron or zeatin during 8 weeks cultivation [10]. Tasheva and Kosturkova [11] have studied apical buds, leaf nodes with leaves, stem segments, rhizome buds and segments to induce multiple shoots and have shown the differences in their regeneration rates. In our experiments, high regeneration capacity of the leaf explants of *R. rosea* aseptically cultured plants has been demonstrated, and the possibility of direct organogenesis has been proved. The elaborated regeneration protocols can be used both for *R. rosea* microclonal propagation and as a methodological basis for including this species into further biotechnological studies.

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ПРЯМА РЕГЕНЕРАЦІЯ ПАГОНІВ *in vitro* З ЛИСТКОВИХ ЕКСПЛАНТІВ *Rhodiola rosea* L.

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Дикорослі види рослин становлять великий інтерес як джерело фармакологічно цінних сполук, але багато з них є ендемічними та/або зникаючими. Сучасна біотехнологія може забезпечити надійні методи їх використання без порушення природних популяцій. Зокрема, інтенсивно розробляються методи культивування *in vitro* лікарських рослин роду *Rhodiola* для подальшого включення їх до різноманітних біотехнологічних програм.

Мета: розроблення протоколу прямої регенерації рослин *Rhodiola rosea* L. з листкових експлантів.

Методи. Використовували асептичні рослини з колекції Інституту клітинної біології та генетичної інженерії НАН України. Листки відокремлювали, робили на них надрізи та культивували на агаризованому живильному середовищі Мурасіге та Скуга (1962) з додаванням різних комбінацій таких регуляторів росту: 6-бензиламінопурин (БАП), кінетин, α-нафтилоцтова кислота (НОК) та 2,4-дихлорфеноксіоцтова кислота (2,4-Д). Оцінювали частоту регенерації як відсоток листків, на яких формувалися пагони.

Результати. Використання середовища з БАП (2,5 мг/л) та 2,4-Д (1,0 мг/л) дозволило індукувати пагони з найвищою частотою — 100%. Збільшення концентрації 2,4-Д до 2.5 мг/л та зменшення концентрації БАП до 1,0 мг/л привело до зниження показника частоти регенерації до 62,5%. На середовищах, що містили 1,0 мг/л кінетину + 2,5 мг/л 2.4-Д або 2,5 мг/л кінетину + 1,0 мг/л 2,4-Д частота регенерації становила відповідно 25% та 62%.

Висновки. Листкові експланти *R. rosea* показали високу регенераційну здатність на середовищах з різними комбінаціями регуляторів росту. Оптимальним середовищем для отримання регенерованих пагонів з листових експлантів з частотою 100% є середовище Мурасіге та Скуга з додаванням БАП та 2,4-Д у концентраціях 2,5 та 1,0 мг/л відповідно. Частота регенерації була нижчою при заміні БАП на кінетин. Спостерігали також інші варіанти морфогенезу (формування адвентивних коренів та/або калюсу).

Ключові слова: *Rhodiola rosea* L.; листкові експланти; регенерація пагонів; регулятори росту.

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L-DOPA BIOSYNTHESIS WITH *Agaricus bisporus* TYROSINASES ASSISTANCE

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L-DOPA (3,4-dihydroxyphenyl-L-alanine) is the drug of choice in treatment of Parkinson's disease, however, the chemical method of its synthesis has a number of significant drawbacks, so biotechnological approaches are being explored as an alternative.

The aim is to develop a new affordable and effective method for L-DOPA biosynthesis by mushroom tyrosinase immobilized on a cost-effective carrier, which ensures the stability and multiple use of the enzyme.

Methods. Tyrosinase isolated from *Agaricus bisporus* was used in the work. The biosynthesis of L-DOPA was carried out in aqueous and organic media. The resulting product was analyzed by mass spectrometry, specific rotation and melting point. Tyrosinase was immobilized in poly-N-vinylpyrrolidone (PVP), the interaction with the carrier, pH-optimum, and frequency of application were determined.

Results. A partially purified tyrosinase was isolated from *Agaricus bisporus*. In an aqueous solution in the presence of the enzyme, only 5.1 % L-DOPA was obtained due to the further formation of complex polycyclic compounds. L-DOPA derivative biosynthesis in methylene chloride containing buffer solution made it possible to obtain the product in 55 % yield. Tyrosinase immobilized in PVP exhibited 30% higher activity than free tyrosinase in CH_2Cl_2 and carried out biocatalysis for 7 cycles.

Conclusions. A method has been developed for the synthesis of L-DOPA using an effective cost-effective biocatalyst based on immobilized tyrosinase, which in an aqueous-organic medium made it possible to obtain L-DOPA within 7 cycles of use.

Key words: L-DOPA synthesis; biocatalyst; tyrosinase4 immobilization; poly-N-vinylpyrrolidone.

Parkinson's disease (PD) is a slowly progressive, chronic neurological disease characterized by neurons death in the brain basal ganglia and causes a range of effects, including rigidity, akinesia, bradykinesia, and resting tremor. Worldwide, about 5 million people suffer from this disease [1].

3,4-dihydroxyphenyl-L-alanine (L-DOPA, levodopa) is the drug of choice in the treatment of CP, synthetic levorotatory dihydroxyphenylalanine is used as a drug, which is much more active than the dextrorotatory isomer [2]. Currently, the main way to obtain L-DOPA is the chemical method of asymmetric synthesis, which is characterized by a low degree of conversion, low enantioselectivity and requires an expensive metal catalyst. Therefore, as an alternative, various biotechnological approaches using enzymes, microorganisms and drug isolation biological objects are being investigated. In comparison with chemical synthesis, microbial transformation usually exhibits a sufficiently high enantioselectivity, but requires long-term cultivation and complex methods of isolation and purification of the target product from the culture medium [3].

Enzymatic synthesis of L-DOPA, catalyzed by tyrosinase, is characterized by high enantioselectivity, proceeds under mild conditions of pH and temperature. However, its use on an industrial scale is restrained by denaturation and the one-time use of the biocatalyst. To eliminate the

described shortcomings, immobilization of the enzyme is used, which facilitates the separation of tyrosinase from the reaction medium, increases stability, preventing its denaturation, and makes multiple use possible. Thus, for the development of biocatalysts for the enzymatic synthesis of L-DOPA, various carriers (multilayer coating of epoxy resin with electrolytes, poly(ethylene oxide)/polypyrrole, polyhydroxyalkanoate nanogranules, agar-agar granules crosslinked with glutaraldehyde, etc.) and immobilization methods (inclusion in a gel) were used , cross-linking, covalent bonding). However, in many cases, after immobilization, the activity of tyrosinase decreased significantly, the frequency of use was small, and the price of the carrier was high [4–8]. Therefore, the purpose of this study is to develop a new, affordable and effective method of biosynthesis of L-DOPA using partially purified tyrosinase from *Agaricus bisporus* mushrooms, immobilized with an economical carrier using, which ensures stability and multiple uses of the enzyme.

However, in some cases, after immobilization, the activity of tyrosinase significantly decreased, as shown by Yildiz S. et al., who developed two methods of immobilizing tyrosinase on polypyrrole derivatives [5]. In addition, the frequency of biocatalysts use was not high in some places, for example, Botta G. and co-authors used tyrosinase immobilized in Eupergit®C250L resin with additional coating by layer method. Enzyme activity retention after immobilization was 16–38%, the multiple use was only 5 cycles [6]. Works using tyrosinase immobilized on polyhydroxyalkanoate nanoparticles are promising; the obtained drug had high activity during 8 cycles of use, however, the isolation of the enzyme and the method of its immobilization are quite complex and expensive [7, 8].

Therefore, the purpose of this study was to develop a new, affordable and effective method of biosynthesis of L-DOPA in an aqueous-organic medium using partially purified tyrosinase isolated from *Agaricus bisporus* mushrooms, immobilized using an economical non-toxic carrier, which ensures the stability and reusability of the enzyme.

Materials and Methods

In the work, a tyrosinase preparation from *Agaricus bisporus* mushrooms was used, and a partially purified preparation was isolated according to [8]. In the selected tyrosinase preparation, the protein content was determined according to the Lowry method in Hartree's modification [9], the content of copper ions [10], the activity according to L-DOPA [11] and L-tyrosine according to [12]. The L-DOPA content was determined as modified by the 4-aminoantipyrine method [13].

L-DOPA synthesis using tyrosinase in an aqueous solution was carried out as follows. 0.5–2.5 mol/dm^3 L-tyrosine in a buffer solution (0.05 mmol/dm^3 Na-phosphate buffer solution pH 6.5) was added to 100 cm^3 1–5 mol/dm^3 of ascorbic acid and 5 cm^3 of tyrosinase solution (activity of 750 units/mg of protein per minute). After 60 minutes, 10 cm^3 of 10% benzoic acid solution (pH 7.0) was added. To obtain N-acetyl derivative of L-tyrosine ethyl ester, 10.5 g of free base of L-tyrosine ethyl ester, 5 cm^3 of acetic acid and 8 cm^3 of acetic anhydride were added to a three-necked flask with a capacity of 100 cm^3 with stirring, brought to a boil, kept for 1 min and allowed to cool to room temperature. The cooled solution was evaporated, treated twice with ethyl acetate. The obtained oil was dissolved in 50 cm^3 of methyl alcohol, water was added until cloudy and placed in the refrigerator. The precipitate was filtered and dried.

L-DOPA biosynthesis in an organic solvent was carried out by adding to 10.8 mg of N-acetyl derivative of L-tyrosine ethyl ester in 5 cm^3 of methylene chloride, 0.4 cm^3 of 0.05 mol/dm^3 Na-phosphate buffer solution (pH 6.5) and 400 units/cm^3 of tyrosinase preparation. The synthesis was carried out with intensive stirring for 1 h at room temperature. The reaction was monitored by thin layer chromatography (TLC). After the substrate disappearance, the biocatalyst was removed and the organic layer was treated with an equal volume of sodium dithionite solution (1%) to reduce DOPA-quinone with protected functional groups to the corresponding catechin. The mixture was stirred for 5 min and the phases were separated. The aqueous phase was acidified with HCl (1.0 M) and extracted twice with ethyl acetate. The organic extracts were treated with saturated NaCl solution and dried over anhydrous Na_2SO_4, then filtered and concentrated to give a colored crude product. The residue was purified by flash chromatography [7]. The functional groups of the L-DOPA derivative were deprotected by boiling in 2 M hydrochloric acid solution, the reaction was monitored by TLC. The obtained product was analyzed by mass spectrometry,

TLC, specific rotation and melting point (yield 55%; M = 197 g/mol, melting point — 276–277 °C; FAB mass spectrum, m/z (I, %):198 (100%)$[M + H]^+$; specific rotation: — 13.0° (c = 5.12 in 1 N HCl), which corresponds to the literature data [14].

Immobilization of the isolated tyrosinase in poly-N-vinylpyrrolidone (PVP, M.m. 20000) was carried out as follows: 2000 units of the tyrosinase preparation were added to 3.5 cm^3 of 7.7% PVP solution and the solution was poured into a glass container. The form was kept open until drying. The enzyme activity in the organic medium was determined by increase in optical density of the solution at 389 nm. The enzyme amount that caused an increase in optical density by 0.001 per minute at 25 °C was taken as a unit of activity [7].

The pH-Optimum of free and immobilized enzyme preparations was determined by adding a solution of the substrate in methylene chloride and 0.4 cm^3 of the appropriate buffer solution with different pH values (4.0–8.0) to samples of equal activity. The effect of tyrosinase on the viscometric characteristics of PVP solutions was determined by measuring the viscosity of their aqueous solutions and the viscosity of polymer solutions when the appropriate amount of enzyme was added using an Ostwald viscometer (capillary diameter 0.73 mm). Characteristic viscosity was calculated according to [15].

The immobilized tyrosinase use multiplicity was determined by transforming the N-acetyl derivative of ethyl ester L-tyrosine according to the method described above. Multiple application of the biocatalyst was studied up to 50% preservation of enzyme activity. Statistical processing of the results was carried out in the Statistica program using the Student's t-test, the results were considered reliable at ($P < 0.05$).

Results and Discussion

Since commercial drugs have an extremely high cost, we isolated a partially purified preparation of tyrosinase with a protein yield of 0.82 mg/g of mushrooms, a copper ion content of 0.19%, and a specific activity of 750 and 4300 units/mg of protein per minute for L-tyrosine and L-DOPA, respectively, was isolated from *Agaricus bisporus* mushrooms.

Under the developed conditions (0.05 mol/dm^3 Na-phosphate buffer solution (pH 6.5), temperature 25 °C), L-DOPA biosynthesis from L-tyrosine was carried out. Tyrosinase catalyzes the tyrosine o-hydroxylation to L-DOPA, but the enzyme also catalyzes the subsequent oxidation reaction of L-DOPA to DOPA-quinone, which turns into DOPA-chromium, which further forms complex polycyclic compounds. At the same time, L-tyrosine oxidation is a rate-limiting stage in tyrosinase catalysis, i.e. the production of L-DOPA-quinone proceeds much faster, as a result of which it is impossible to isolate the L-DOPA intermediate product. Therefore, to obtain L-DOPA in the process of biocatalysis, ascorbic acid was added to the reaction mixture, which reduced DOPA-quinone to L-DOPA (Fig. 1).

However, most reducing agents, including ascorbic acid, are tyrosinase inhibitors [16]. Therefore, it was necessary to select such a concentration of ascorbic acid, which would restore the DOPA-quinone that was formed, and at the same time, to a small extent, reduce the activity of the enzyme (Table 1).

It was shown that the best concentration of ascorbic acid was 2.5 mmol/dm^3, a decrease in the concentration of the reducing agent led to almost complete conversion of L-DOPA into DOPA-quinone, and an increase in the concentration of ascorbic acid contributed to an even stronger decrease in product yield due to a significant inhibition of tyrosinase activity.

Table 1

Effect of ascorbic acid concentration on the formation of L-DOPA catalyzed by tyrosinase

Ascorbic acid concentration, mmol/dm3	L-DOPA formation *	
	mmol/dm^3	Yield, %
1	0.040±0.001	1.6±0.1
2	0.093±0.003	3.7±0.1
2.5	0.128±0.006	5.1±0.2
3	0.088±0.003	3.5±0.1
5	0.031±0.001	1.2±0.1

* $P < 0,05$; $n = 5$.

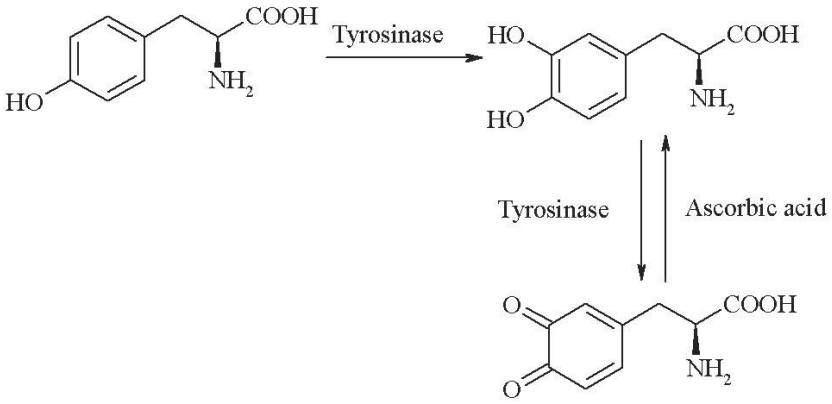

Fig. 1. **L-DOPA synthesis catalyzed by *Agaricus bisporus* tyrosinase**

The effect of tyrosine concentration on the L-DOPA formation in the presence of tyrosinase was investigated. It was found that the product concentration increases non-linearly, which is probably due to the unsaturation of the enzyme with the substrate at low concentrations of tyrosine (Fig. 2).

The highest L-DOPA formation is observed at a substrate concentration of 2.5 mmol/dm^3, further increase in concentration is impossible due to poor solubility of tyrosine in water. Thus, with the help of isolated tyrosinase in an aqueous solution, the L-DOPA formation with a yield of only 5.1% was shown, due to the further oxidation of the product by an enzyme, with the production of reactive *o*-quinone, which is transformed into DOPA-chromium with subsequent formation of complex polycyclic compounds.

Therefore, in order to prevent the intramolecular cyclization of DOPA-quinone, it was necessary to protect the functional groups of L-tyrosine. For this, the ethyl ester of L-tyrosine was subjected to the action of acetic acid and acetic anhydride (5:8 v/v) (Fig. 3).

The resulting N-acetyl derivative of the ethyl ester of L-tyrosine is almost insoluble in water. Therefore, its oxidation to the L-DOPA derivative with protected carboxyl and amino groups, catalyzed by tyrosinase (Fig. 4), was carried out in an organic solvent (methylene chloride). The resulting L-DOPA derivative is also oxidized by tyrosinase to the *o*-quinone derivative, but due to the protection of the functional groups, intramolecular cyclization does not occur.

In the course of biosynthesis, a low degree of substrate conversion is shown. It is known that adding small amounts of water to enzymes that catalyze reactions in organic solvents significantly increases speed of the process. It was established that adding 0.4 cm^3 of 0.05 mol/dm^3 Na-phosphate buffer solution pH 6.5 to the studied reaction mixture led to complete bioconversion of the tyrosine derivative. Yield of the L-DOPA derivative with protected functional groups was 83%, which agrees with the literature data [7]. After deprotection from the carboxyl and amino groups of L-DOPA, the synthesis

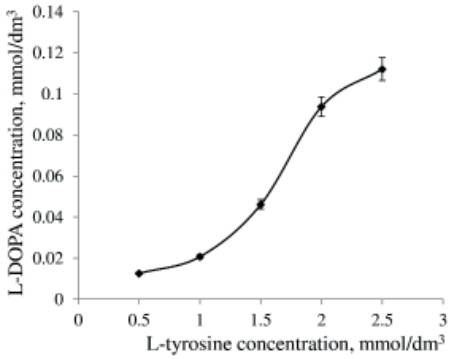

Fig. 2. **The dependence of L-DOPA concentration formed with the help of tyrosinase on the tyrosine concentration ($P < 0{,}05$; $n = 5$)**

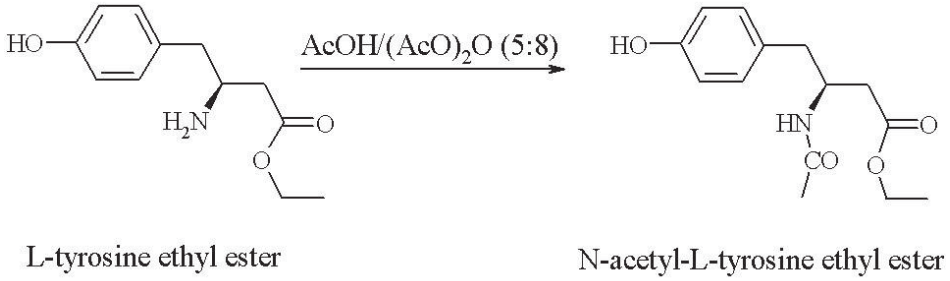

Fig. 3. **Obtaining of N-acetyl derivative of L-tyrosine ethyl ester**

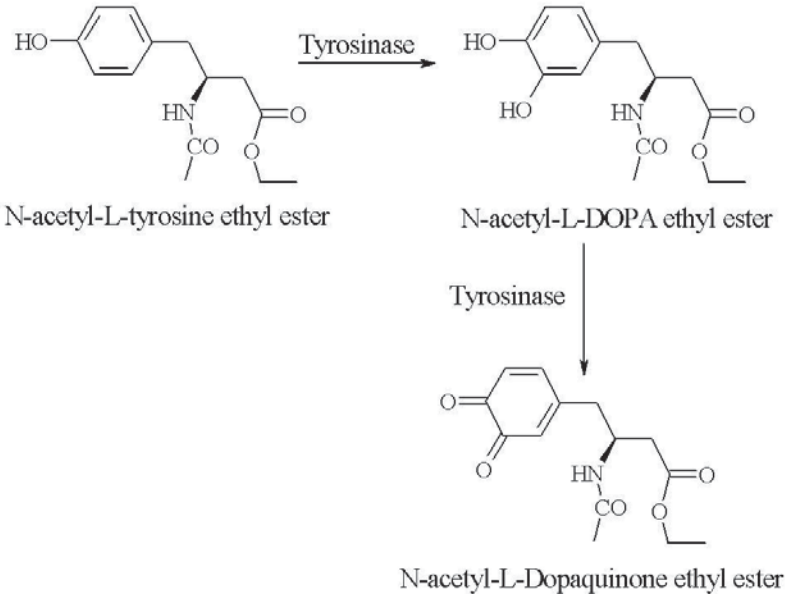

Fig. 4. **Synthesis of L-DOPA derivative with protected functional groups catalyzed by *Agaricus bisporus* tyrosinase**

product yield was 55%. The obtained product was analyzed using mass spectrometry, TLC,measurement of melting point and specific rotation. To create an effective, economical, stable, reusable biocatalyst, the selected tyrosinase preparation was immobilized in an available synthetic polymer — poly-N-vinylpyrrolidone.

Important properties of PVP, which are of great practical importance, are its high adsorption capacity and tendency to complex formation. PVP is a non-toxic promising carrier for inclusion of cells of microorganisms and enzymes, BAR. Thus, immobilized forms of enzymes with a prolonged effect were obtained on the basis of high molecular weight PVP [17, 18].

As a result of immobilization of tyrosinase in PVP, polymer films were obtained with 80% preservation of the enzyme original activity for tyrosine, when used in a buffer solution, and the characteristics presented in the Table. 2.

The interaction of tyrosinase with the carrier was studied by the viscometry method, measuring the viscosity of PVP solutions of various concentrations and the viscosity of these solutions when the appropriate amount of tyrosinase was added (Fig. 5).

When comparing the obtained viscosity values, it is shown that the addition of the enzyme to PVP solutions increases their indicated viscosity (Fig. 5).

By extrapolating the graph of the dependence of the indicated viscosity of polymer solutions on their concentration to the intersection with the ordinate axis, the values of the characteristic viscosity were obtained ($2.31 \cdot 10^2$ and $2.53 \cdot 10^2$ cm^3/g for PVP and PVP with tyrosinase, respectively), which can testify to the presence of interaction between enzyme and polymer. That is, immobilization of tyrosinase is probably carried out due to physical inclusion in the PVP structure and non-valent interactions of enzyme with the matrix.

Table 2

Characteristics of the tyrosinase preparation immobilized in PVP

Characteristics	Results *
Tyrosinase activity by tyrosine, U/g immobilized enzyme	2000 ± 22
Enzyme content, mg/g of film	3.3 ± 0.7
Organoleptic characteristics	Homogeneous transparent films
Diameter, cm	1.5 ± 0.1
Area of a film, cm^2	5.55 ± 0.3
Thickness, mm	0.20 ± 0.01
Mass, g	0.3 ± 0.07

*$P < 0,05$; $n = 5$.

Table 3

Influence of the pH of the buffer solution added to the incubation medium on the activity of free and immobilized tyrosinase

pH	Tyrosinase activity, % *	
	Free enzyme	Immobilized enzyme
4	28.3 ± 0.8	47.1 ± 1.4
5	53.3 ± 1.6	58.8 ± 1.8
6	95.8 ± 3.0	96.1 ± 2.8
6,5	100.0 ±3.1	100 ± 2.8
7	97.2 ± 2.8	97.5 ± 2.9
8	43,7 ± 1.4	50.5 ± 1.4

*$P < 0,05$; $n = 5$.

In the work, the possibility of using the obtained biocatalyst in the environment of methylene chloride (with the addition of 7.5% buffer solution) was investigated. It was established that the immobilized drug showed 30% higher activity than free tyrosinase, which is explained by stabilization of the enzyme activity — immobilized tyrosinase, under adverse conditions, which allows using a smaller amount of biocatalyst.

In the process of studying the properties of the obtained drug, the pH effect of the buffer solution added to the methylene chloride medium during the synthesis process on the activity of free and immobilized tyrosinase was determined.

It was established that as a result of immobilization, the pH-optimum of tyrosinase activity (6.5) does not change. An expansion of the tyrosinase activity pH profile in the area of acidic values was revealed (Table 3).

The inclusion of an enzyme in PVP allows the obtained biocatalyst to be used multiple times. The study of the possibility of repeated use of immobilized tyrosinase in the environment of methylene chloride with the addition of a buffer solution (7.5% v/v) showed that the obtained preparation retains high phenoloxidase activity during 6 cycles of use (Fig. 6). While G. Botta and co-authors [7] found that the biocatalyst developed by them on the basis of tyrosinase immobilized using Eupergit C250L epoxy resin covered with electrolytes, under similar conditions, retained activity only for 3 cycles of use.

Thus, the L-DOPA biosynthesis in an aqueous-organic environment, catalyzed by isolated tyrosinase from *Agaricus bisporus* mushrooms, with 55% product yield was developed. A highly efficient, stable, affordable, reusable, non-toxic biocatalyst based on tyrosinase immobilized in poly-N-vinylpyrrolidone was created to obtain L-DOPA during six cycles of use in a batch reactor.

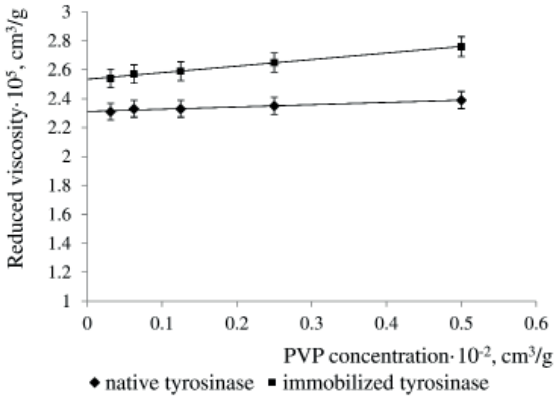

Fig. 5. **Dependence of viscosity on the concentration of free and immobilized tyrosinase ($P < 0,05$; $n = 5$)**

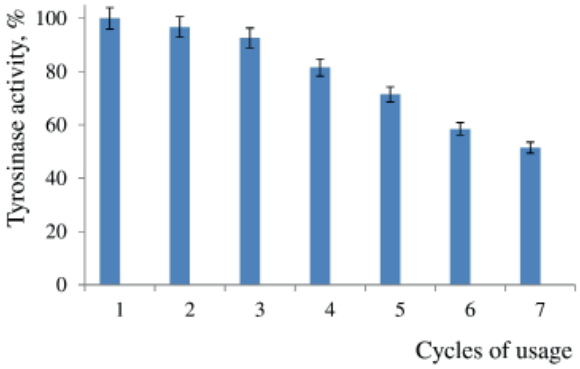

Fig. 6. **Dependence of tyrosinase activity on the frequency of use of the biocatalyst ($P < 0,05$; $n = 5$)**

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БІОСИНТЕЗ L-ДОФА ЗА ДОПОМОГОЮ ТИРОЗИНАЗИ *Agaricus bisporus*

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L-ДОФА (3,4-дигідроксифеніл-L-аланін) є препаратом вибору при лікуванні хвороби Паркінсона, однак хімічний метод його синтезу має ряд недоліків, тому як альтернатива досліджуються біотехнологічні підходи.

Мета — розробити новий доступний і ефективний спосіб біосинтезу L-ДОФА за допомогою тирозинази грибів, іммобілізованої з використанням економічного носія, що забезпечує стабільність і багаторазовість використання ензиму.

Методи. У роботі використовували виділену тирозиназу *Agaricus bisporus*. Біосинтез L-ДОФА проводили у водному і органічному середовищі. Отриманий продукт аналізували за допомогою масспектрометрії, питомого обертання і температури плавлення. Іммобілізацію ензиму проводили в полі-N-вінілпіролідон (ПВП), визначали взаємодію з носієм, pH-оптимум і кратність застосування.

Результати. З *Agaricus bisporus* виділений частково очищений препарат тирозинази. У водному розчині в присутності ензиму було показано отримання лише 5,1 % L-ДОФА, через подальше утворення складних поліциклічних сполук. Біосинтез похідного L-ДОФА у хлористому метилені з додаванням буферного розчину дозволив отримати продукт з виходом 55 %. Тирозиназа, іммобілізована в ПВП, проявляла активність на 30 % вище за вільну у середовищі CH_2Cl_2 і здійснювала біокаталіз протягом 7 циклів.

Висновки. Розроблено спосіб синтезу L-ДОФА за допомогою доступного біокаталізатора на основі іммобілізованої тирозинази, що у середовищі хлористого метилену дозволила отримувати L-ДОФА протягом 7 циклів використання.

Ключові слова: синтез L-ДОФА; біокаталізатор; тирозиназа; іммобілізація; полі-N-вінілпіролідон.

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DECOMPOSITION OF PHOSPHORUS-CONTAINING COMPOUNDS IN AQUEOUS AND POLYSACCHARIDE SOLUTIONS OF ORGANIC ACIDS

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The purpose was to study the patterns of dissolution (solubilization) of phosphorus-containing minerals in aqueous and polysaccharide solutions of organic acids in order to model the mechanism of mineral destruction by soil bacteria synthesizing organic acids and exopolysaccharides.

Methods. Model, laboratory-analytical, microbiological, statistical.

Results. The destructive effect of organic acids on minerals is manifested both in aqueous and polysaccharide solutions. The introduction of bacterial polysaccharide into an aqueous solution of acids increases the decomposition of phosphorus-containing minerals by 1.34–4.96 times. The influence of the chemical structure of acid molecules on the intensity of mineral decomposition is mainly manifested in the presence of bacterial polysaccharide, while in an aqueous solution the effectiveness of acid action depends on the nature of the mineral being destroyed. To the maximum degree, polysaccharide increases the destruction of minerals in a solution of citric acid: molten magnesium phosphate —2.55 times, thermophosphate — 2.11 times, phosphate flour 4.96 times. Decomposition of phosphorus compounds in solutions of ascorbic and oxalic acids enhances bacterial polysaccharide to a lesser extent than in citric acid solution.

Modeling the destruction of phosphorus-containing minerals under non-sterile conditions (soil conditions) made it possible to establish that organic acids under non-sterile conditions are subject to consumption by soil microbiota, especially ascorbic and citric acids, and to a lesser extent — succinic. Aqueous solutions of organic acids after 18 hours of incubation in non-sterile conditions lose their leaching activity by 1.06–12.1 times. The introduction of a polysaccharide into aqueous solutions of acids makes it possible to avoid their rapid consumption by microorganisms, because of which the efficiency of mineral leaching under non-sterile conditions decreases by only 5–20% compared to sterile ones.

Conclusions. The introduction of a bacterial polysaccharide into a solution of organic acids enables the latter to be transferred to a sorbed state, as a result of which their susceptibility to consumption by microorganisms is sharply reduced. Thus, polysaccharide-forming bacteria not only destroy minerals more intensively than microorganisms synthesizing only low-molecular-weight metabolites, but also synthesize a more stable and long-term functioning leaching complex in the soil.

Key words: phosphorus; bacteria; organic acid; polysaccharide; modeling; dissolution; mechanism.

Phosphorus (P) is one of the five essential plant nutrients. Phosphate fertilizers have played an important role in agriculture since the beginning of the 20th. By 2051, global demand for phosphate fertilizers is expected to increase by 86% on arable land, especially in developing regions. However, phosphate rocks are a limited resource, and high quality phosphate rocks can be depleted in 300–400 years [1]. Most of the soluble phosphate applied to the soil as a chemical fertilizer is immobilized shortly after application and becomes unavailable to plants, resulting in an excess of fixed phosphorus in some arable soils. Microorganisms play a significant role in the natural P-cycle [2–4]. Insoluble forms of P and phosphorus in the composition of natural minerals can be converted into soluble P by soil phosphate mobilizing bacteria [2, 5, 6]. Greenhouse and field experiments have shown that application of phosphate mobilizing microorganism preparations promote plant growth in phosphorus deficient soils, indicating their

potential as new biofertilizers to address food security issues, especially given the limited use of chemical fertilizers. However, it was shown that the effectiveness of the effect of phosphate mobilizing bacteria on plant growth varies under field conditions. Several biotic and abiotic factors can influence the activity and functions of phosphate mobilizing bacteria, as well as their effect on plant growth [2, 5]. Understanding the mechanisms of destruction (solubilization) of phosphate-containing minerals by microorganisms contributes to the development of effective strategies for using their potential to increase plant productivity, which was the goal of our research. Soil bacteria solubilize mineral phosphates mainly through the secretion of various organic acids, such as citric, gluconic, lactic, malic, oxalic, propionic, and succinic acids, which differ in the number of carboxy groups, hydroxy groups, and carbon double bonds [2, 5]. However, the role of organic acids in the processes of phosphorus mobilization is still denied by some authors. The authors base their conclusions as on the fact that there is a whole series of acid-forming bacteria that are incapable of dissolving natural phosphates, and on the basis of the fact that between the methods no acidification and decomposition of iron and aluminum phosphates no matter how significant the correlation [7].

At the same time, it seems to us that this issue can be resolved in modal experiments with the use of solutions of organic acids, which give the possibility to evaluate the ability of individual organs Czech acids to the dissolution of phosphorus containing minerals. The introduction of polysaccharide into aqueous solutions of organic acids in a concentration of 0.5–1 0 g/l will allow to model the cultural liquid of those soil microorganism, which are synthesized at the same time organic acids and polysaccharides.

Materials and Methods

In the experiment, 0.1 M aqueous and polysaccharide solutions of ascorbic, oxalic, citric and succinic acids were used. Sterilization of acid solutions was carried out by filtration using cellulose acetate membrane filters MCA020013H with a pore diameter of 0.2 μm. Bacillus mucilaginosus C-3 polysaccharide was used as a bacterial polysaccharide. It is an anionic heteropolysaccharide, containing in its composition glucose — 10.6%, galactose — 4.0%, mannose — 11.9%, fukose — 0.5%, unidentified sugar — 0.5% (of dry weight).

We used the bacterial strain *B. mucilaginosus C-3*, obtained from the collection of the Institute of Mineral Resources of Mingeo of Ukraine. Bacteria were stored at room temperature on agar mineral medium with glucose, g/l: glucose — 15.4, $MgSO_4$ $7H_2O$ — 0.7, Na_2HPO_4 2 H_2O — 2.0, KNO_3 — 1.0, $FeCl_3$ $6H_2O$ — 0.05. The cultivation of *B.mucilaginosus* was carried out in a periodic process, which was carried out in 750 ml bah by volume with 100 ml of medium on a shaker at 240 rpm. The temperature of the cultivation was 30 °C, cultivation time was 18–24h. The sowing material was introduced in the amount of 5% of the medium volume. The polysaccharide preparation was obtained as follows: the culture liquid (18–24 h) was diluted 2–3 times with distilled water and centrifuged to separate the cells at 40000g for 40 minutes. The supernatant was drained and polysaccharide was precipitated from it with three volumes of ethanol. Precipitated EPS was separated from the supernatant by centrifugation at 6000 rpm for 10 min. The ethanol precipitate was dissolved in distilled water and dialyzed against tap water for one day, against distilled water for two days. The resulting polysaccharide was freeze-dried.

The lyophilized polysaccharide preparation was added to acid solutions at a concentration of 0.5 g/L. To carry out the process of polysaccharide macromolecules swelling and sorption of organic acid molecules on them, the samples were placed for 3 hours in a refrigerator at + 5 °C. For sterile variants of the experiment, the samples of minerals were sterilized at 1.5 atm for 1 hour by autoclaving and aseptically added to acid solutions. For non-sterile variants of the experiment, the samples of non-sterile mineral and 100 μl of water extract from meadow chernozem in a dilution of 10^5 were introduced into test tubes after 3 hours. The test tubes were incubated at 28 °C on a shaker with 160 rpm. The incubation time was 18 hours. The amount of polysaccharide in the solutions at the end of the experiment was controlled by viscosity (with the help of a VZh-1 viscometer) and by the weight method with preliminary precipitation of 3 times the ethanol volume.

The concentration of phosphorus in solutions was determined by the Murphy-Reilly method, after preliminarily precipitating the polysaccharide with three volumes of ethanol [8]. Measurement of the optical density of

the solutions of the probes on FEK 56-2 and KFK-2 (light filter No. 8, λ = 590 nm, cuvette No. 30). The statistical significance of the obtained differences between the experimental variants was assessed by the value of the error of the mean result, calculated using the Statistica 10 program.

Results and Discussion

In aqueous solutions, oxalic acid has the greatest dissolving effect on the studied phosphorus compounds, followed by citric and ascorbic acids (Tables 1–3). Succinic acid realizes its dissolving effect depending on the properties of the minerals subjected to destruction: when dissolving fused magnesium phosphate and phosphate rock — at the level of citric acid, when dissolving thermophosphate — to a lesser extent than ascorbic acid.

In the presence of *B.mucilaginosus* polysaccharide, the action effectiveness of the studied acids is identical in relation to all three connections: oxalic > citric > ascorbic > amber. and does not depend on the structural features of decomposed phosphorus fertilizers. Consequently, the minerals decomposition intensity in polysaccharide solutions of organic acids are defined, first of all, by the chemical structure of the acid molecule lots and does not depend on the structural features of the destructurable phosphorus fertilizers. As can be seen from the data presented in Table 3, the most effective bactericidal polysaccharide strengthens the decomposition of phosphate flour: the phosphorus removal in the presence of a polysaccharide exceeds the destruction of phosphate flour in an aqueous solution of ascorbic acid by 4.3 times, citric acid by 4.96, oxalic acid by 4.1, and succinic acid by 1.94 times.

In the maximum degree, polysakharid increases the destruction of minerals in the created citric acid: fused magnesium phosphate — 2.55 times, thermal phosphate — 2.11 times for, phosphate flour —

Table 1

Removal (solibilization) of phosphorus from fused magnesium phosphate in 0.01 M solutions of organic acids in the presence of *B. mucilaginosus* polysaccharide (2) and without it (1)

Organic acid Sterile conditions		Phosphorus removal (solibilization), P_2O_5 mg/l	
		Non-sterile conditions	
Ascorbic acid	1	100.0 + 8.14	30.3 + 2.88
	2	211.3 + 17.5	175.5 + 18.2
Lemon acid	1	124.2 + 10.6	39.4 + 5.11
	2	316.7 + 21.1	301.7 + 21.9
Oxalic acid	1	142.5 + 13.8	50.5 + 5.17
	2	213.1 + 15.9	218.4 + 20.5
Succinic acid	1	128.2 + 11.8	75.8 + 6.88
	2	190.4 + 18.0	192.2 + 20.1

Table 2

Removal (solibilization) of phosphorus from thermophosphate in 0.01 M solutions of organic acids in the presence of *B. mucilaginosus* polysaccharide (2) and without it (1)

Organic acid Sterile conditions		Phosphorus removal (solibilization), P_2O_5 mg/l	
		Non-sterile conditions	
Ascorbic acid	1	130.0 + 8.14	10.7 + 2.88
	2	175.2 + 17.5	165.5 + 18.2
Lemon acid	1	147.2 + 10.6	19.4 + 5.11
	2	310.7 + 21.1	223.8 + 21.9
Oxalic acid	1	188.5 + 13.8	34.5 + 5.17
	2	367.5 + 15.9	332.9 + 20.5
Succinic acid	1	99.4 + 11.8	85.6 + 6.88

Table 3

Removal (solibilization) of phosphorus from phosphate rock in 0.01M solutions organic acids in the presence of *B. mucilaginosus* polysaccharide (2) and without it (1)

Organic acid Sterile conditions		Phosphorus removal (solibilization), P_2O_5 mg/l	
		Non-sterile conditions	
Ascorbic acid	1	26.8 + 1.14	15.1 + 0.65
	2	115.4 + 17.5	120.5 + 8.24
Lemon acid	1	37.5 + 2.62	17.6 + 1.15
	2	185.9 + 12.5	185.6 + 11.4
Oxalic acid	1	55.2 + 1.84	50.5 + 5.07
	2	226.5 + 14.9	224.3 + 20.5
Succinic acid	1	42.6 + 2.18	40.3 + 2.88

4.96 times. Bacterial polysaccharide enhances decomposition of phosphorus compounds in solutions of ascorbic and oxalic acids to a lesser extent than in a solution of citric: fused magnesium phosphate by 1.50 times, phosphate rock — 4.31 and 4.1 times, respectively. However, during temophosphate decomposition, the opposite pattern is observed — in oxalic acid solution, the polysaccharide enhances the mineral decomposition by 1.95 times, and in ascorbic acid solution — by 1.34 times. To a minimal extent, the polysaccharide enhances the decomposition of the studied phosphorus-containing compounds in succinic acid solution.

Conducting an experiment on the minerals destruction in organic acids solutions in sterile conditions enables to find out in what way the structure of an acid molecule affects the degree of phosphate compounds decomposition. However, in soils, secondary microorganisms' metabolites experience the effects of the microbiota sides existing in soil, in particular, they can be consumed as sources of carbon and energy. Therefore, we delivered an experiment on the dissolution of minerals in non-sterile conditions I. As a result, it was found that the efficiency of minerals dissolution in aqueous solutions of organic acids under non-sterile conditions decreased compared to sterile conditions (Table 1–3). In particular, in the ascorbic acid and citric acids, the removal of phosphorus became minimal, especially when dissolving thermal phosphate and phosphate flour. This is probably related to the fact that citric and ascorbic acids to a greater extent are consumed by micro-organisms in comparison with oxalic and succinic acid [9]. In the smallest degree, succinic acid was consumed, therefore, the removal of phosphorus in its aqueous solution remained practically at the level of the experiment carried out under sterile conditions (Tables 1–3.). The consumption of organic acids by natural waters microbiota, soils is described in the literature [10,11], while it is noted that acids (acetic, alkaline, succinic) are consumed by soil micro flora with different intensities, and that the zinc content (leachable element) in percolate is closely correlated with the acids content. H.V. Hue [12] also notes that when conducting vegetation experiments, protocatechuic acid turned out to be the most effective in increasing the availability of phosphorus to plants as the most resistant to microbial destruction from the studied acids (acetic, malic acid, etc.).

The effectiveness of organic acids in aqueous solutions under non-sterile conditions decreased significantly after 18 hours of incubation. The solubilizing ability of acids decreased to the maximum when dissolving thermophosphate: ascorbic acid — 12.1 times, citric acid — 7.59, oxalic — 5.46, succinic — 1.16 times (Table 2). The dissolution of phosphate rock decreased minimally under non-sterile conditions: in ascorbic acid — 1.77 times, citric acid — 2.13, oxalic — 1.09, succinic — 1.06 times (Table 3). The removal of phosphorus from fused magnesium phosphate (Table 1) decreased under non-sterile conditions in a solution of ascorbic acid — 3.3 times, citric — 3.15, oxalic — 2.82, succinic — 1.69 times.

The effectiveness of the organic acids action in the presence of bakterial polysaccharide in non-sterile conditions turned out to be identical to that in sterile conditions (Table 1–3). The removal of phosphorus in polysaccharide solutions under non-sterile conditions did not decrease such significantly as in water. The reduction amount in phosphorus removal compared with sterile conditions was 5–20%. These data indicate that organic acids in the sorbed state on polysaccharides not only destroy minerals more effectively, but are also less accessible for consumption by soil microflora. The control of polysaccharide amount in solutions after the end of the experiment made it possible to draw a conclusion about its preservation for 18 hours by 95%. This is also confirmed by the data of E.I. Andreyuk et al.[13] about the fact that during the first 36 hours of cultivation bacteria do not consume in the same way as bacteria.

For exopolysaccharides, the function of extracellular enzymes action prolonging and antibiotics is known. [14]. Extracellular polysaccharides of some microorganisms are able to protect the producers exoenzymes from proteolytic degradation and other external influences. There is reason to believe that such an ability has a universal character. For example, the exopolysaccharide Arthrobacter citreus stimulated the activity of exoproteases Nocardia sp. [15, 16], and the extracellular glycan Mycobacterium lacticolum 121 doubled the thermal stability of the alkaline protein basics, derived from *Actinomyces* sp. [17]. The mechanism of the exglycans protektor action is apparently in the formation of a stable glycoprotein complex.

Preservation of exoenzymes activity is probably a special case of the universal protective function of bacterial polysaccharides. The second manifestation of it, as we believe, is protection from the consumption of low-molecular metabolites and

ions absorbed by the polysaccharide during vegetative cell growth. Thanks to the synthesis of exopolysaccharide, microorganisms, as it were, create their own microenvironment around cells, ensuring more complete manifestation of trophic and functional capabilities.

Obviously, the ecological role of microorganisms synthesizing organic acids in the dissolution of hard-to-reach phosphates is limited by the susceptibility of these acids to their consumption by soil microorganisms at a fairly high rate. These are microorganisms with sufficiently high speed. Microorganisms synthesizing exopolysaccharides simultaneously with organic acids represent not more active (2–5 times) higher complex, but less protective — molecular secondary metabodies from the rapidly consumed soil microbiota consequently the absorbed state is less accessible.

Conclusions

The effectiveness of the organic acids action in aqueous solutions depends on the chemical properties of their molecules and the structural features of the dissolved compounds. The greatest dissolving effect on the studied phosphorus compounds has an oxalic acid, then — citric and ascorbic acids

Bacterial polysaccharide accelerates the minerals decomposition compared to acids aqueous solutions, for example, phosphate flour: in a solution of ascorbic acid by 4.3 times, citric acid — 4.96, oxalic acid — 4.1, succinic acid — 1.94 times.

Under nonsterile conditions, the effectiveness of aqueous solutions of organic acids is significantly reduced. For 18 hours of incubation, the solubilizing ability of acids decreases to maximum when dissolving thermal phosphate: ascorbic by 12.1 times, citric — 7.59, oxalic — 5.46, succinic — 1.16 times.

The solubilizing ability of polysaccharide solutions of organic acids in non-sterile conditions decreases slightly — by 5–20%.

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ДЕСТРУКЦІЯ ФОСФОРОВМІСНИХ СПОЛУК У ВОДНИХ І ПОЛІСАХАРИДНИХ РОЗЧИНАХ ОРГАНІЧНИХ КИСЛОТ

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Мета: дослідження закономірностей розчинення (солюбилизації) фосфоровмісних мінералів у водних і полісахаридних розчинах органічних кислот з метою моделювання механізму деструкції мінералів ґрунтовими бактеріями, які синтезують органічні кислоти і екзополісахариди.

Методи: модельний, лабораторно-аналітичний, мікробіологічний, статистичний.

Результати: Розчинювальна дія органічних кислот на мінерали проявляється як у водному, так і полісахаридному розчині. Внесення бактеріального полісахариду у водний розчин кислот посилює розкладання фосфоровмісних мінералів у 1,34–4,96 раза. Вплив хімічної будови молекул кислот на інтенсивність розчинення мінералів проявляється, в основному, в присутності бактеріального полісахариду. У водному ж розчині ефективність дії кислот залежить від природи мінералу, що деструктується. В максимальному ступені полісахарид інтенсифікує розкладання мінералів у розчині лимонної кислоти: плавлений магнієвий фосфат – у 2,55 раза, термофосфат — в 2,11 раза, фосфоритне борошно — в 4,96 раза. Розкладання фосфорних сполук в розчинах аскорбінової та щавлевої кислот бактеріальний полісахарид інтенсифікує у меншому ступені, ніж у розчині лимонної кислоти.

Моделювання розкладання фосфоровмісних мінералів у нестерильних умовах (умови ґрунту) дозволило встановити, що органічні кислоти у нестерильних умовах споживаються ґрунтовою мікробіотою, особливо аскорбінова і лимонна, у меншому ступені — бурштинна кислота. Водні розчини органічних кислот через 18 годин інкубування у нестерильних умовах втрачають деструктувальну активність у 1,06–12,1 раза. Внесення полісахариду *Bacillus mucilaginosus* у водні розчини кислот дозволяє запобігти швидкому споживанню їх мікроорганізмами, в результаті чого ефективність розкладання мінералів у нестерильних умовах зменшується порівняно зі стерильними лише на 5–20%.

Висновки. Внесення бактеріального полісахариду до розчину органічних кислот дозволяє перевести їх у сорбований стан, у результаті чого схильність кислот до споживання мікроорганізмами різко зменшується. Таким чином, бактерії, що синтезують екзополісахариди, не тільки більш інтенсивно деструктують мінерали, ніж мікроорганізми, що синтезують тільки низькомолекулярні вторинні метаболіти, але й синтезують більш стійкий і довго функціонувальний у ґрунті комплекс метаболітів, що розкладають мінерали.

Ключові слова: фосфор; бактерії; органічна кислота; полісахарид; моделювання; розкладання, механізм.

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LOSS OF AN ABUNDANT QUANTITY OF RIBONUCLEIC ACID DURING MINI COLUMN ISOLATION METHOD

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Aim. The Isolation of nucleic acid is an important step for conducting different molecular assays in many laboratories around the world. It is also a common practice that user is isolating the ribonucleic acid (RNA) from the samples with mini column once and throwing away the supernatant. This makes isolated RNA as limiting factor in many studies as this issue has not been addressed in literature. Therefore, we decided to conduct whether it is a loss of ribonucleic acid during the mini column isolation method.

Method. In this research, we have made analysis whether there is a loss of RNA during the isolation process. The mini column isolations were done with different samples of human tissues from placenta and umbilical cords. These were assessed with spectrometric instrument and real time PCR machine.

Results. It was found that there is loss of abundant quantity of RNA during the subsequent isolations. The amount measured with UV spectrometer indicates that some times 2nd and 3rd isolation have more RNA than the first isolation. Realtime PCR for house keeping gene beta actin shows that presence of RNA can be seen up to 6 isolation cycles from supernatants.

Conclusion. There is loss of RNA in subsequent isolations with mini column method, therefore it is possible to isolate more RNA from the next supernatant isolations. User should do the multiple isolations to get higher yield of RNA.

Key words: mini column isolation, RNA; nucleic acid; ribonucleic acid; polymerase chain reaction; real time PCR; viruses; biomarkers; vaccines.

Isolation of nucleic acid is an essential step for conducting the different nucleic acid analysis methods like PCR and hybridization for various viral, bacterial, biomarkers and genetic targets [1–4]. Not only this, the nucleic acid can be used as therapeutic as well as vaccine options in many clinical settings, therefore there is an urgent need to isolate the sufficient amount of nucleic acid in highly pure form [5–8]. Ribonucleic acid (RNA) can be isolated from different sources e.g., blood, tissue, buccal swabs, nasal samples, plasma, serum, urine and cell cultures. There are different methods available to isolate the RNA, which are magnetic beads, solutions-based and mini column methods for examples. The mini column isolation method is widely used in the laboratories througaround the world. Usually most of the laboratories isolate the RNA from the sample once and the supernatant of this method is discarded. Many times, there is limited volume of isolated RNA e.g., 50 or 100 µl available, which is not sufficient to conduct the whole studies, where user has to conduct a set of experiments on the same isolated RNA (homogenous material). In case of development of vaccines, one needs sufficient quantity of RNA, which may not be available from a single isolation source [4, 9, 10].

In this study, the experiments are conducted to find whether RNA is still in supernatant and this surplus RNA can be isolated to conduct the further analysis.

A protocol of local ethics committee approval of Genekam Biotechnology AG (Number: GEN01-23) about conducting the experiments of this research work is available. In this experiment, 4 samples from different tissues like umbilical cord, placenta tissue, placenta membrane etc. are used to isolate the RNA with mini column RNA isolation kit

(Genekam, Germany). 100 µl of the samples were added to lysis buffer with proteinase K. These samples were kept at 56 °C for 10 min. After that guanidine solution was added, while keeping at 70 °C for 5 min. 400 ul of molecular ethanol (Applichem, Germany) was added to each well.

Mini column step. These mixtures were added to mini columns, which were centrifuged at the speed of 11000 rpm for 1 minutes. The supernatants from each sample were collected in separate tubes. Mini columns were washed with 500 µl B and C tubes each while centrifuging at the speed of 11 000 rpm for one minute. At the end mini columns were centrifuged in order to make them dry at the speed of 13 000 rpm for 1 minute. At last, 70 °C warm elution buffer was added to each mini column, while keeping this for 2 minutes at room temperature. In order to get the elution buffer containing RNA, the mini columns were centrifuged. The tubes were labelled as first isolation and stored at 4 °C for further analysis.

Collected supernatants were processed as mentioned in mini column step till there was use of elution buffer at the end to elute RNA. During this step, the supernatants were collected and processed again as mentioned above. There were two groups. In one group, there were 2 isolations (one original isolation and 1 isolation from supernatants, these are labelled as Isolation 1 and 2) and in 2nd group, there are 7 isolations (one original isolation and 6 subsequent isolations from supernatants, which were labelled as Isolation 1 to 7). In both groups, isolation 1 is the original and first isolation and rest isolations are from subsequent supernatants. (Tables 1 and 2).

Total Volume of isolated RNA was 50 µl per isolation. The full RNA isolation protocol can be also obtained from manufacturer.

Measurement of RNA content with UV spectrometer, Nanodrop (Thermofischer, USA): The instrument was calibrated with elution buffer. After that, 2 ul of each isolated was measured and the values are recorded at µg/2 µl.

Real time PCR for human internal control. All isolated samples were tested for the presence of RNA with housekeeping gene (β-Actin) through real time kit FR799 (Genekam, Germany) in 96 well plate in a real time machine ABI 7500 (Thermofischer, USA). 2 ul of isolated RNA was used during the real time PCR reaction in 18 ul RNA mastermix containing cDNA synthesizing enzymes. The temperatures and cycling conditions were one cycle 42 °C for 60 minutes and 70 °C for 10 min as well as 45 cycles of 90 °C for 15 s and 60 seconds for 60 °C. The results were analyzed with software in linear and log modes along with recording of Ct values. The full protocol for conducting real time PCR for internal control can be obtained from the manufacturer.

Group 1, where the 2 isolations were done. The values of these versions of different samples measured with spectrometer is shown in the Table 1. The values indicate that there is loss of RNA during 2 isolations. Therefore, it is decided to conduct second experiment, where the isolation was repeated up to 7 isolations. The results are shown in the Table 2. The results indicate that there is a loss of RNA during all steps and this loss decreases with the increase in the number of isolations. In the sample 2, it varies better 0.170 µg/2 µl at the beginning to 0.006 µg / 2 µl. It was very surprising to see that yield of isolated RNA was more in the first supernatant passage (second isolation) than the first isolation.

Real time PCR of isolated RNA in different isolated versions show that real time for beta actin housekeeping gene is in position to detect RNA in all isolations regardless whatever quantity was detected in spectrometer. Results are shown as curves in the Figure. It is hard to find any correlation between the amount of RNA measured in spectrometer and Ct values achieved in the real time machine.

Since the availability of possibility to isolate the nucleic acid with mini columns, it has become a common practice in all laboratories working with molecular methods, to conduct the RNA isolation from different samples to analyze them for different targets as these may be pathogenic agents and biomarkers of different cells e.g., stem cells, tumor cells, immune cells etc. It is also daily

Table 1

Measurement of UV spectrometer for the concentrations of RNA in different isolations (Group 1)

Sample *	Isolation 1	Isolation 2
Sample 1	0.006	0.009
Sample 2	0.053	0.042
Sample 3	0.005	0.022
Sample 4	0.005	0.09

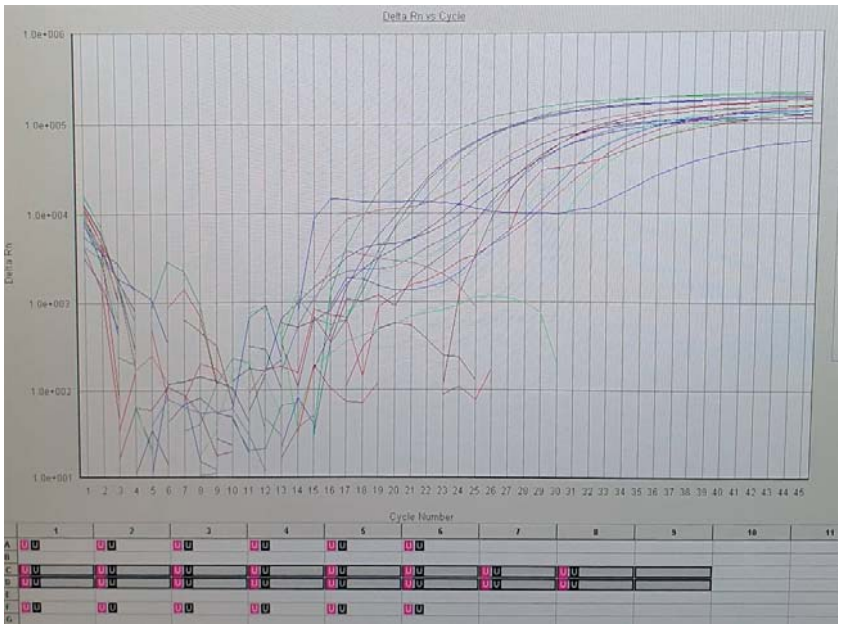
* µg / 2µl.

Table 2

Measurement of UV spectrometer for the concentrations of RNA in different isolations (Group 2)

Sample *	Isolation 1	Isolation 2	Isolation 3	Isolation 4	Isolation 5	Isolation 6	Isolation 7
Sample 1	0.005	0.003	0.002	0.002	0.002	0.008	0.001
Sample 2	0.018	0.007	0.004	0.001	0.001	0.008	0.006
Sample 3	0.077	0.163	0.073	0.001	0.001	0.001	0.003
Sample 4	0.01	0.4	0.136	0.014	0.002	0.002	0.005

* µg / 2µl.



The results of real time PCR for internal control for the presence of human RNA in isolated samples

practice in the laboratories to measure the presence of viral RNA in different samples. [1, 2, 11–13] Most of the laboratories are doing only once the isolation and throw away the samples as supernatant. Amount of isolated RNA with only one-time isolation is very limited e.g., 100 µl. If user is conducting experiments for a number of different targets, this small amount can be a limiting factor because of its small size.

In the literature, there is hardly to find any report that some groups have tried to look into this important problem in spite of the fact that each group needs sufficient amount of nucleic acid to conduct the assays from the same source. This study is indicating that it is possible to isolate the more RNA from the supernatants of the same sample. In this way, many laboratories have sufficient amount of isolated RNA to conduct bigger studies and compare them also [14].

There are research groups, which are working on development of vaccines and therapies, they may face many times a challenge to isolate the sufficient RNA to conduct their experimental studies. This publication provides them the way to isolate more amount of valuable RNA rather throwing of the rest of supernatant.

These studies shown here can help many institutes and laboratories to save money in terms of chemicals and time used to isolate the samples. In two samples shown Table 2, the amounts of isolated RNA from sample number 3 and 4 were less than that of RNA isolated from two following supernatants from the first isolation. It should be investigated further as it indicates that there may be some time a lot of loss of RNA in the first isolation or binding capacity of membrane may be playing a role. User can have more isolated RNA in 2nd and 3rd subsequent isolations.

There are many publications, where research groups are using 5 or 10 µl during the PCR studies, hence a lot of quantity of isolated RNA can be obtained from supernatant, which is usually thrown away as waste product. [15, 16] This publication is showing a way to get larger volume of isolated RNA to bigger studies.

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ВТРАТА ЗНАЧНОЇ КІЛЬКОСТІ РИБОНУКЛЕЇНОВОЇ КИСЛОТИ ЗА УМОВ ЇЇ ВИДІЛЕННЯ З ВИКОРИСТАННЯМ МІНІ-КОЛОНКИ

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Мета. Виділення нуклеїнової кислоти є важливим кроком для проведення різних молекулярних аналізів у багатьох лабораторіях по всьому світу. Також поширеною практикою є те, що користувач один раз виділяє рибонуклеїнову кислоту (РНК) із зразків за допомогою міні-колонки та викидає супернатант. Це робить ізольовану РНК обмежувальним фактором у багатьох дослідженнях, оскільки це питання не розглядалося в літературі. Тому ми вирішили визначити, чи є це втратою рибонуклеїнової кислоти під час методу ізоляції на мініколонці.

Метод. Проводили виділення в міні-колонці з різними зразками людських тканин із плаценти та пуповини та подальші виділення супернатантів. Виходи та успішні виділення РНК оцінювали за допомогою спектрометричного приладу та машини ПЛР у реальному часі.

Результати. Було виявлено, що при наступних виділеннях відбувається втрата великої кількості РНК. Кількість, виміряна за допомогою УФ-спектрометра, вказує на те, що іноді 2-й і 3-й виділення містять більше РНК, ніж перше виділення. ПЛР у реальному часі для бета-актину гена домашнього обслуговування показує, що присутність РНК можна побачити до 6 циклів ізоляції з супернатантів.

Висновок. При наступних виділеннях за допомогою методу міні-колонки відбувається втрата РНК, тому можна виділити більше РНК із наступних виділень супернатанту. Користувач повинен виконати багаторазові виділення, щоб отримати більший вихід РНК.

Ключові слова: виділення мініколонки, РНК; нуклеїнова кислота; полімеразна ланцюгова реакція; ПЛР в реальному часі; віруси; біомаркери; вакцини.