



NIKOLAY YANKOV SOLAKOV

Development of technology for bioproducts based on
organic sources

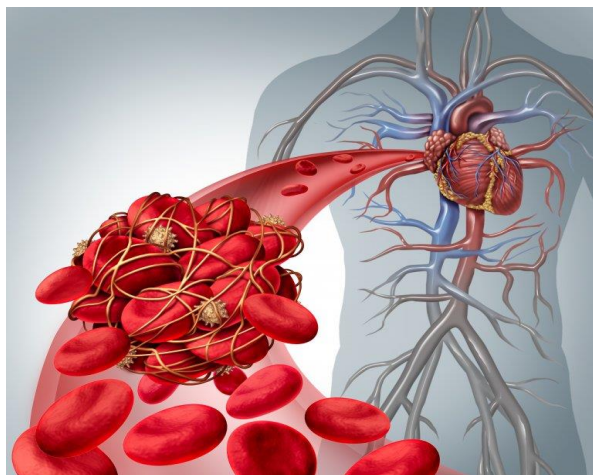
ABSTRACT

of a dissertation for awarding the educational and scientific degree
"DOCTOR"

Scientific field 5. Technical sciences

Professional direction 5.12. food technology,

Scientific specialty "Technology of biologically active substances
(including enzymes, hormones, proteins).



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The numbering of the sections, figures and tables in the abstract corresponds to those in the dissertation.



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DEVELOPMENT OF TECHNOLOGY FOR BIOPRODUCTS
BASED ON ORGANIC SOURCES

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Doctoral program "Technology of biologically active
substances (incl. enzymes, hormones, proteins).

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I. INTRODUCTION

The dissertation work is aimed at developing a technology for obtaining biologically active complexes from wild-growing higher mushrooms and microbial cultures and their inclusion in a functional product with a targeted health effect.

In nature, thousands of representatives of higher wild-growing mushrooms are known, of which about 600 species have been tested for their impact on human health. All of them have been found to exhibit immunomodulatory properties. The mushroom matrix contains several main groups of biologically active substances, such as terpenes, sterols, functional polysaccharides, powerful antioxidants and inhibitors. Depending on the composition of the biological matrix, additional health effects are also established, which determines the scientific interest in their use as a substrate for obtaining bioproducts with diverse functional activity.

The object of development are the species *Ganoderma lucidum* (Reishi) and *Cordyceps sinensis*, which are the best studied in terms of their biologically active composition. They have a powerful adaptogenic effect (impact on more than 300 biochemical processes and functions).

Experiments were conducted to obtain biologically active extracts by fermentation hydrolysis of the fungal substrate with the producer strain *Bacillus subtilis*. This microorganism is one of the few resistant to different conditions and a well-studied producer for industrial use. Enzymes produced by different strains of *Bacillus subtilis* are widely used in various fields, including the food and pharmaceutical industries.

In the conventional method for obtaining proteolytic and fibrinolytic enzymes, in the cultivation of *Bacillus subtilis*, legumes with a high content of protein components are included in the composition of the nutrient media. Such a nutrient environment provides conditions for the production of high molecular weight proteolytic enzymes that cannot overcome a large part of biological barriers in the body. Adding a fungal substrate to the nutrient medium of the microbial strain will ensure the production of enzymes with different molecular masses and specific biological action. The emphasis in the development of this dissertation work is aimed at achieving the following innovative elements:

- Optimizing the cultivation technology of wild medicinal mushrooms by varying the composition of the nutrient medium and cultivation conditions.
- Combined use of fungal and microbial extracts in order to improve the biologically active effect of the final product.
- Creation of a new technology in which the nutrient medium for cultivating the microbial strain is part of the composition of the final product and is a source of proteolytic, fibrinolytic, cellulase, glucanase, chitinase enzymes and other biologically active components.

The application of the combined biotechnological approaches for the use of biologically active components from natural organic sources, in which a final product with high biological activity is expected, determines the relevance of the current dissertation work and the applicability of the obtained results in practice.

II. CONCLUSIONS FROM THE LITERATURE REVIEW

- The obtained data prove the enormous potential of medical mushrooms as a source of biologically active substances and possibilities for their application.
- It has been established that these organisms can extract maximum amounts of nutrients from air, water, soil, as well as by parasitizing plant hosts and some arthropods, representatives of insects. This ability provides them with the concentration of a large amount of biologically active substances available in the mycelium, fungal body and spores.
- The ability of fungi to spread by spores or by a network of filamentous structures called hyphae allows them to unimpededly occupy vast territories with favorable conditions for living. Developing in different habitats, fungi of the same species use diverse food substrates and produce different biologically active substances.
- Controlled cultivation of medicinal mushrooms, both outdoors and in laboratory conditions, provides various opportunities to obtain a certain composition of active components in medicinal mushroom extracts.
- Cultivation outdoors, but in a controlled environment, ensures the maximum accumulation of biologically active substances from all groups, and the obtained extract has a composition as close as possible to that obtained from wild-growing representatives.
- By changing the cultivation method in a controlled environment, it is possible to achieve a large amount of the desired biologically active substance.
- From the overview, it was found that the representatives of the genera ***Ganoderma*** and ***Cordyceps*** have the highest potential, in order to achieve the highest yield of biologically active substances.
- From the genus ***Ganoderma***, the species ***Ganoderma lucidum*** and ***Ganoderma neo-japonicum*** were selected, and from the genus ***Cordyceps*** respectively ***Cordyceps sinensis*** and ***Cordyceps militaris***.
- ***Cordyceps sinensis*** provides a variety of biologically active substances that affect blood circulation and support collagen production. ***Cordyceps militaris*** has a proven high content of adenosine precursors having a beneficial effect on cell energy, which is the basis for improving blood circulation.
- ***Ganoderma lucidum*** is one of the most well-studied medicinal mushrooms with a proven rich biologically active extract. ***Ganoderma neo-japonicum*** is one of the least researched laboratory-grown species of the ***Ganoderma*** genus, which allows for experimentation: development of extraction methods and creation of technology for the production of a functional product with a variety of composition-rich final dosage forms.
- It has been established that the producer strain ***Bacillus subtilis*** is suitable for providing a rich enzyme complex with proteolytic action, including peptides of

different molecular weight, necessary for the composition of the developed product.

- ***Bacillus subtilis*** is a good producer of chitinase, cellulase and betaglucanase enzymes, which makes it suitable for hydrolyzing cellular structures in mushroom raw materials. It can be successfully applied in biotechnological processes to obtain extracts from fungal and plant sources.

III. GOAL AND OBJECTIVES

PURPOSE

- The aim of the dissertation work is to develop a technology for obtaining bioproducts based on organic sources

TASKS

- Cultivation of the higher medicinal mushrooms ***Ganoderma lucidum* (Reishi)**, ***Ganoderma neo-japonicum***, ***Cordyceps sinensis***, and ***Cordyceps militaris*** in experimental technological regimes.
- Cultivation of ***Bacillus subtilis*** microbial culture in different nutrient media with a view to obtain high total proteolytic and chitinase enzyme activity.
- Testing of different biotechnological methods for obtaining a biologically active extract by using enzymes or living cells from the producer strain ***Bacillus subtilis***.
- Concentration of the biologically active substances in the obtained extracts by sublimation drying.
- Development of a semi-industrial method for cultivating the medicinal mushroom *Cordyceps militaris* in order to achieve a higher content of the biologically active substance Cordycepin.
- Experimental proof of the presence of the biologically active components: derivatives of adenosine (cordycepin), peptides with different molecular mass, phenolic profile and antioxidant activity in the obtained extracts.
- Creation of formulation composition and technology for obtaining final solid dosage form of functional bioproduct "***FibrinoZIME***".
- Preparation of technological documentation of a functional bioproduct "***FibrinoZIME***", according to the requirements of HACCP and good manufacturing practice.

IV. MATERIALS AND METHODS

4.1. Materials

4.1.1. Cell cultures of higher medicinal fungi

The studies were carried out with starter cell cultures of the following higher medicinal mushrooms:

- ***Ganoderma lucidum*** – Zhejiang Kangzhou Biotechnology Co., Ltd – Jinhua city, China;

- *Ganoderma neo-japonicum* OGGNJ100 - Out-Grow USA;
- *Cordyceps sinensis* OGCS100- Out-Grow USA;
- *Cordyceps Militaris* OG1000CM- Out-Grow USA
- *Cordyceps Militaris* – producer strain Shanghai- (used in the semi-industrial method) - Zhejiang Kangzhou Biotechnology Co.,Ltd – Jinhua city, China

4.1.2. Producer strain

- *Bacillus subtilis* NBIMCC 2353. The producer strain *Bacillus subtilis* NBIMCC 2353 purchased from NBIMCC was used

4.1.3. Components used in the preparation of the functional product:

- Extract of fresh mycelium of medical higher mushrooms *Cordyceps sinensis*, *Cordyceps militaris*, *Ganoderma lucidum*, *Ganoderma neo-japonicum*, cultivated in IKHT; *Cordyceps militaris* fruiting body extract and industrially grown *Ganoderma lucidum* mycelium extract, *Natto* extract, *Carica Papaya* plant dried extract, purchased from Zhejiang Kangzhou Biotechnology Co.,Ltd – Jinhua city, China; dried *Bombyx mori* L. cocoon extract providing peptides with enzymatic action produced by *Serratia* sp. E-15 purchased from Dr. T&T Health UK Ltd; fructooligosaccharides; mineral supplements: magnesium bis-glycinate and zinc citrate; polyvinylpyrrolidone (E1201); microcrystalline cellulose; anti-caking agents: magnesium stearate (E 572) and silicon dioxide (E551), purchased from the company "Oreon Mateev" EOOD - Karlovo.

4.2. Technological methods

- Cultivation of wild mushrooms includes the following stages:
- Propagation of cell culture to obtain inoculum;
- Formation of a fresh mycelial mass in a liquid medium (phase I);
- Formation of a mycelial mass in a solid medium (II phase);
- Formation of conglomerates (nodules) in the mycelial mass;Нарастване на възлите;
- Formation of the embryo of the fruiting body;
- Growth of the fetal body;
- Spore formation.

Within the framework of the conducted experiment, the biologically active substances sought are in the mycelial mass and the fruiting body. In order to obtain substrates with a high content of biologically active substances, a different approach is required at each individual stage of mushroom culture development.

4.2.1. Propagation of cell culture of the medicinal mushrooms *Ganoderma neo-japonicum*, *Ganoderma lucidum*, *Cordyceps sinensis* and *Cordyceps militaris* in liquid nutrient medium to obtain starting inoculum.

Starter cultures were propagated in a standardized medium purchased from Out-Grow USA, provided in powder form. A liquid medium was prepared from it, dissolving 6 g in 500 ml of distilled water heated to boiling with slow homogenization for 2-3 minutes. The solution was filtered through 40 micron filter paper and the filtrate was sterilized at 1.02 atmospheres pressure and 125°C temperature for 15 minutes. The liquid media thus prepared were poured into sterilized and cooled to 20°C glass jars equipped with a 220 nm pore size filter to ensure air aeration during cultivation. After cooling, 1 ml of sterile liquid cell culture from *Ganoderma neo-japonicum*, *Cordyceps sinensis* and *Cordyceps Militaris* was injected into the obtained medium. To inoculate the obtained liquid medium with *Ganoderma lucidum* cells, 5 g of grown mycelium on a solid medium was used. It is left at a temperature of 28°C for 7 days without light and 7 days with light. Culture growth was monitored visually from day 4 to 14.

To obtain a high number of viable cells, the resulting inoculum was cultured in liquid nutrient medium **M0** (table 3), after which cultures were made from it in liquid and solid nutrient medium.

Table 3. Composition of the liquid nutrient media g/l.

Substance	Standard startup medium / g	Standard startup medium / g	Standard startup medium / g	Standard startup medium / g	M0/ g	M0/ g	M0/g	M0/ g
startup medium	6	6	6	6	-	-	-	-
Dextrose	-	-	-	-	20	20	20	20
Potato starch	-	-	-	-	5	5	5	5
Yeast autolysate	-	-	-	-	5	5	5	5
FOS	-	-	-	-	5	5	5	5
<i>Ganoderma neo-japonicum</i> cell culture [ml]	1	-	-	-	1	-	-	-
<i>Ganoderma lucidum</i> cell culture [g]	-	5	-	-	-	5	-	-
<i>Cordyceps sinensis</i> cell culture [ml]	-	-	1	-	-	-	1	-
<i>Cordyceps militaris</i> cell culture [ml]	-	-	-	1	-	-	-	1
Distilled water [ml]	to : 1000	to : 1000	to : 1000	to : 1000	to : 1000	to : 1000	to : 1000	to : 1000

4.2.2. Cultivation of fresh mycelium of medicinal mushrooms *Ganoderma neo-japonicum*, *Ganoderma lucidum*, *Cordyceps sinensis* and *Cordyceps militaris* in liquid nutrient medium (phase I).

A series of experiments were conducted to optimize the composition of the nutrient medium during cultivation of the fresh mycelium from higher fungi, aiming to increase the yield of mycelial mass.

On the basis of nutrient medium M0 (control), four variants of enriched liquid nutrient mediums (M1, M2, M3 and M4) were developed with a composition presented in Table 4.

Table 4. Composition of liquid nutrient media for cultivation of fresh mycelial culture g/l.

Substance	M0 control	M1	M2	M3	M4
Potato starch [g]	20	20	20	20	20
Peptone [g]	-	10	10	10	10
Yeast extract [g]	6	6	6	6	6
Glucose [g]	-	25	25	-	-
Dextrose [g]	25	-	-	25	25
FOS [g]	5	5	5	5	5
MgSO ₄ x 7 H ₂ O [g]	-	0,2	0,2	0,2	0,2
Vitamin B ₁ [g]	-	-	0,03	0,03	0,03
KH ₂ PO ₄ [g]	-	-	0,1	0,1	0,1
K ₂ HPO ₄ [g]	-	-	0,2	0,2	0,2
FeSO ₄ [g]	-	-	-	-	0,1
Distilled water [ml]	to: 1000	to: 1000	to: 1000	to: 1000	to: 1000

The media were autoclaved at a pressure of 1.02 atmospheres and a temperature of 125°C for 15 minutes, then cooled to room temperature. After sterilization, the media were poured into 500 ml Ellermail flasks to a volume of 300 ml and inoculated with 5 ml each of pre-expanded cell culture. The cell culture of *Ganoderma lucidum*, *Ganoderma neo-japonicum*, *Cordyceps sinensis* and *Cordyceps militaris* was cultivated in the nutrient media (**M0**, **M1**, **M2**, **M3** and **M4**) for 72 hours in a shaker

at 28°C, shaking amplitude 100 rpm and absence of light, followed by a photoperiod of 16 h light (300 lm) to 8 h no light for a period of 12 days.

4.2.3. Quantification of mycelial growth in liquid media.

Based on a method for indirect qualitative determination of the growth of mycelial culture (Kuyumdzhieva, A. et al., 2007) an analytical method for quantitative determination of the growth of mycelial culture in a liquid nutrient medium was developed. At startup mycelium growth (72 hours after inoculation) every 24 hours the mycelium formed separated by filtration from the liquid culture medium after tempering for 30 minutes under static conditions at room temperature. The volume of the separated nutrient medium is measured. Изчисления:

- The resulting volume (V_i) from the measurement taken is subtracted from the volume (V_0) of the nutrient medium before inoculation.
- The volume (V_m) of the mycelial structure represents the difference between the separated culture medium during the measurement and the initial volume of the culture medium before inoculation.

$$V_m = V_0 - V_i$$

Determination of mycelial growth rate:

- Determine ΔV_m for every 24 h after the end of the dark period
- From the obtained results, the % growth of the mycelium relative to V_{m1} is recalculated for each subsequent interval of 24 hours until the end of cultivation.

4.2.3. Cultivation of mycelium of medicinal mushrooms *Ganoderma neo-japonicum*, *Ganoderma lucidum*, *Cordyceps sinensis* and *Cordyceps militaris* on solid nutrient medium (phase II).

M0 medium was used as the basis, and in its composition the FOS content was replaced by the addition of agar-agar to form the main solid medium (S1). From it, by adding additional organic sources - whole-grain rice (S2) and old aged oak shavings (S3), enriched nutrient media were obtained (table 5).

Table 5. Composition of solid nutrient media for the cultivation of higher medicinal mushrooms.

Substance/Mediums	Option S1	Option S2	Option S3
Dextrose [g]	20	20	20
Картофено нисестре [g]	5	5	5
Yeast autolysate [g]	5	5	5
Agar-Agar [g]	15	15	15
Rice [g]	-	200	-
Oak shavings [g]	-	-	200
Inoculum [ml]	5	5	5
Distilled water [ml]	to :1000	to :1000	to :1000

The dry ingredients are dissolved in 1 liter of distilled water. They are heated in a water bath to 60°C until a gel-like mass is obtained. The finished solution is dosed in glass jars of 270 ml in a dose of 100 g. The jars are equipped with an inoculating hole and a bactericidal filter with a pore size of 220 nm. The culture medium was autoclaved at a pressure of 1.02 atmospheres and a temperature of 125°C for 15 minutes and cooled to room temperature. The prepared medium is inoculated with 5 ml of the liquid inoculum from the cell cultures (*Ganoderma lucidum* KBGL001, *Ganoderma neo-japonicum* GGNJ100, *Cordyceps Sinensis* OGCS100 and *Cordyceps Militaris* OG1000CM, from which 25 samples are loaded for each individual culture.

The inoculated media are arranged in a controlled culture incubator at 28°C and access to natural light.

4.2.4. A semi-industrial method for cultivating *Cordyceps militaris* cell culture to obtain a fruiting body and a high *Cordycepin* content.

The semi-industrial method covers all stages of development of the fungal organism from cell culture to obtaining a mushroom body. Cultivation was carried out in a closed room, equipped with additional ice lighting, emitting light with a wavelength of $\lambda=450$ nm., with the possibility of adjusting the photo-period.

To prepare the media for the cultivation of *Cordyceps militaris*, all raw materials were micronized by mechanical grinding and homogenized. The dry substances were mixed in an industrial blender and dissolved in deionized water.

2 nutrient media (**IM-1** and **IM-2**) were prepared, which differ in the composition of the starting raw materials (**Table 6**).

Table 6. Composition of nutrient media for semi-industrial cultivation of *Cordyceps Militaris* – Shanghai

Substance	IM 1, g	IM 2, g
Grated potato skins	30	30
Crushed sugarcane husks	30	30
Soy peptone	10	10
Yeast extract	6	6
Glucose	20	20
Whole grain rice	150	150
Powdered shrimp shells		5
Monosodium glutamate	-	10
L-Glycine	-	5
(NH ₄) ₂ SO ₄	-	20
Azomite	5	5
MgSO ₄ x 7 H ₂ O	0,2	0,2
Vitamin B1	0,03	0,03
KH ₂ PO ₄	-	0,1
K ₂ HPO ₄	-	0,2
FeSO ₄ · 7H ₂ O	-	0,8
Distilled water [ml]	to :1000	to :1000

The media were distributed in glass jars with a capacity of 750 ml and isolated from the environment by a polymer film. The jars were autoclaved at a pressure of 1.02 atmospheres and a temperature of 125°C for 15 minutes, then cooled to room temperature. After sterilization, the media were inoculated with 5 ml of cell inoculum of *Cordyceps militaris* - producer strain Shanghai and placed on separate racks for subsequent observation. During the first 7 days, cultivation was carried out in the absence of light and with partial manual shaking at 8-h intervals to initiate mycelial growth. The cultivation process continues for another 60 days until harvest. The resulting crop was collected dried and subjected to extraction by fermentation with *Bacillus subtilis*.

The finished liquid extract was filtered and lyophilized.

4.2.5. Propagation of the producer strain *Bacillus subtilis*

To restore and multiply the cells of the original lyophilized producer strain *Bacillus subtilis* NBIMCC 2353, a standard nutrient medium with the following composition was used: Meat extract - 10.0; Peptone – 10.0; NaCl – 5.0; Distilled water - up to :1000. The medium was sterilized in an autoclave for 20 minutes at 121°C and 1 atmosphere pressure.

In 500 ml Erlenmeyer flasks, place 100 ml of the pre-sterilized standard culture medium, inoculate with the producer strain and place in a circular shaker

BS/4. The process of formation and growth of cell mass is carried out at a temperature of 30°C and a shaking amplitude of 100 revolutions/minute, for a period of 24 hours.

The resulting cell culture was used for subsequent inoculation of the nutrient media.

4.2.5.1. Optimizing the composition of the nutrient medium for the cultivation of *Bacillus subtilis* NBIMCC 2353 in order to increase the number of viable cells.

A change was made to the standard nutrient medium by adding prebiotics and other substances related to stimulating the proliferation of cells from the inoculum. Autolyzed yeast extract from *Saccharomyces cerevisiae* and *Cichorium intybus* root extract (FOS) with a high content of oligosaccharides, which have strong prebiotic properties, K₂HPO₄ and MgSO₄ were used. The resulting medium is the control (K).

The composition of the media used in the experiment is presented in **Table 7**.

Table 7. Composition of nutrient media for the cultivation of *Bacillus subtilis* strain (g)

Standard medium	Quantity	Control medium (K)	Quantity
Meat extract	10 g	Meat extract	10 g
Peptone	10 g	Peptone	10 g
NaCl	5 g	Yeast extract	1,5 g
Distilled water [ml]	до :1000	FOS	1 g
		NaCl	5 g
		K ₂ HPO ₄	1,3 g
		MgSO ₄	0,3 g
		Distilled water [ml]	до :1000

From the control (K), by adding dry extracts of cultivated mycelium of *Ganoderma lucidum* and *Cordyceps sinensis*, 4 variants of optimized nutrient media were obtained (C1-C4) **Table 8**

Table 8. Composition of optimized nutrient media (g)

Substance	Control (K) g	C 1, g	C 2, g	C 3, g	C 4, g
Meat extract	10	10	10	10	10
Peptone	10	10	10	10	10
Yeast extract	1,5	1,5	1,5	1,5	1,5
FOS	1	1	1	1	1
NaCl	5	5	5	5	5
K ₂ HPO ₄	1,3	1,3	1,3	1,3	1,3
MgSO ₄	0,3	0,3	0,3	0,3	0,3
<i>Cordyceps sinensis</i> ext.	-	1	2	-	-
<i>Ganoderma lucidum</i> ext.	-	-	-	1	2
Distilled water [ml]	до :1000	до :1000	до :1000	до :1000	до :1000

1 ml of the inoculum of the producer strain is placed in 100 ml of the sterilized optimized nutrient media (1-4) and placed in a circular shaker BS/4. The process of the formation and growth of the cell mass is carried out at a temperature of 30°C and a shaking amplitude of 100 rpm. The duration of cultivation is 24 hours for the first and 72 hours for the second stage. The amount of viable cells was determined by the **McFarland** method immediately before the start of the second EPAP, followed by consecutive measurements at an interval of 24 hours.

4.2.5.2. Optimizing the composition of the nutrient medium for the cultivation of *Bacillus subtilis* NBIMCC 2353 in order to increase the specific enzyme activity:

- Increase of total proteolytic activity by adding native bovine collagen protein and mycelium from *Ganoderma neo-japonicum*, *Ganoderma lucidum*, *Cordyceps sinensis* and *Cordyceps militaris*

Different amounts of native bovine collagen were added to the control medium (K) at pH=8 (**Table 9**).

Table 9. Composition of nutrient media P1-P3 (g)

Substance	1-stage medium (control), g	P1, g	P2, g	P3, g	P4 g	P5, g
Meat extract	10	10	10	10	10	10
Peptone	10	10	10	10	10	10
Yeast extract	1,5	1,5	1,5	1,5	1,5	1,5
FOS	1	1	1	1	1	1
NaCl	5	5	5	5	5	5
K ₂ HPO ₄	1,3	1,3	1,3	1,3	1,3	1,3
MgSO ₄	0,3	0,3	0,3	0,3	0,3	0,3
Native bovine collagen	-	40	-	-	-	-
GNJM	-	-	40	-	-	-
GLM	-	-	-	40	-	-
CSM	-	-	-	-	40	-
CMM	-	-	-	-	-	40
Distilled water [ml]	to :1000	to :1000	to :1000	to :1000	to :1000	to :1000

FOS – Fructooligosaccharides, GNJM – *Ganoderma Neo-Japonicum Mycelium*, GLM – *Ganoderma Lucidum Mycelium*, CSM – *Cordyceps Sinensis Mycelium*, CMM – *Cordyceps Militaris Mycelium*

1 ml of *Bacillus Subtilis* NBIMCC 2353 cell culture was inoculated into 100 ml of nutrient medium. The process of cell mass formation and growth was carried out at a temperature of 30°C and a shaking amplitude of 100 rpm. Aliquots were taken from each culture medium to determine the proteolytic activity immediately before the start of the process, at 24, 48 and 72 hours.

- **Enhancement of chitinase activity by addition of non-hydrolyzed live mycelium fragments of four species of medicinal mushrooms *Ganoderma lucidum*, *Ganoderma neo-japonicum*, *Cordyceps sinensis*, *Cordyceps militaris* and shrimp shell chitin**

5 types of different sources of native chitin were added to the control medium (K): non-hydrolyzed fragments of living mycelium of four species of higher fungi *Ganoderma lucidum*, *Ganoderma neo-japonicum*, *Cordyceps sinensis*, *Cordyceps militaris* and chitin from shrimp shells at pH=8 (Table 10).

Table 10. Composition of the environments CN1-CN5 (g)

Substance	Control medium (K) g	CH1 g	CH2 g	CH3 g	CH4 g	CH5 g
Meat extract	10	10	10	10	10	10
Peptone	10	10	10	10	10	10
Yeast extract	1,5	1,5	1,5	1,5	1,5	1,5
FOS	1	1	1	1	1	1
NaCl	5	5	5	5	5	5
K ₂ HPO ₄	1,3	1,3	1,3	1,3	1,3	1,3
MgSO ₄	0,3	0,3	0,3	0,3	0,3	0,3
Chitin from shrimp shells	-	8	-	-	-	-
<i>Ganoderma neo-japonicum</i> (fresh mycelium)	-	-	8	-	-	-
<i>Ganoderma lucidum</i> (fresh mycelium)	-	-	-	8	-	-
<i>Cordyceps sinensis</i> (fresh mycelium)	-	-	-	-	8	-
<i>Cordyceps militaris</i> (fresh mycelium)	-	-	-	-	-	8
Distilled water [ml]	to :1000	to:1000	to:1000	to:1000	to:1000	to:1000

1 ml of cells of *Bacillus Subtilis* NBIMCC 2353 were inoculated into 100 ml of the nutrient media thus obtained. Incubation was carried out at a temperature of 30°C and a shaking amplitude of 100 revolutions/minute on a BS/4 shaker. Aliquots were separated from each culture medium for determination of chitinase activity immediately before starting the experiment, at 24, 48 and 72 hours from the start of the process.

4.2.6. Preparation of mycelium extract by enzymatic digestion of the cell wall

Mycelium from *Ganoderma Lucidum*, *Ganoderma neo-japonicum*, *Cordyceps sinensis*, *Cordyceps militaris*, cultivated on a solid nutrient medium (II phase), for a period of six months in controlled conditions at IKHT-Sofia, was used as the starting material.

The separated 5 g fungal mycelium was mechanically chopped, placed in 1% citric acid solution in H₂O and micronized at 10,000 rpm for 2 min. 0.5 g were added to the resulting solution. of the enzymes DeniLight (*Laccase*), Lipopan (*Lipase*), Fungamyl 4000 SG (*Alpha Amylase*), Celluclast 1.5 L (*Cellulase*) and homogenized for 24 hours at 600 rpm at a temperature of 20° C. The solution was shock frozen to temperature -40° C, then thawed and upon reaching a temperature of 20° C the Chitinase enzyme was added in an amount of 0.5 g. and homogenized with a magnetic stirrer for 48 h at 600 rpm. The clear part of the solution was decanted and

lyophilized. The cloudy part of the solution was separated into a heavy and a light fraction by centrifugation at 10000 rpm and a temperature of 7° C for 10 minutes. A sample was separated from the resulting supernatant for chromatographic analysis. The light and heavy fractions from the obtained complex extract were lyophilized.

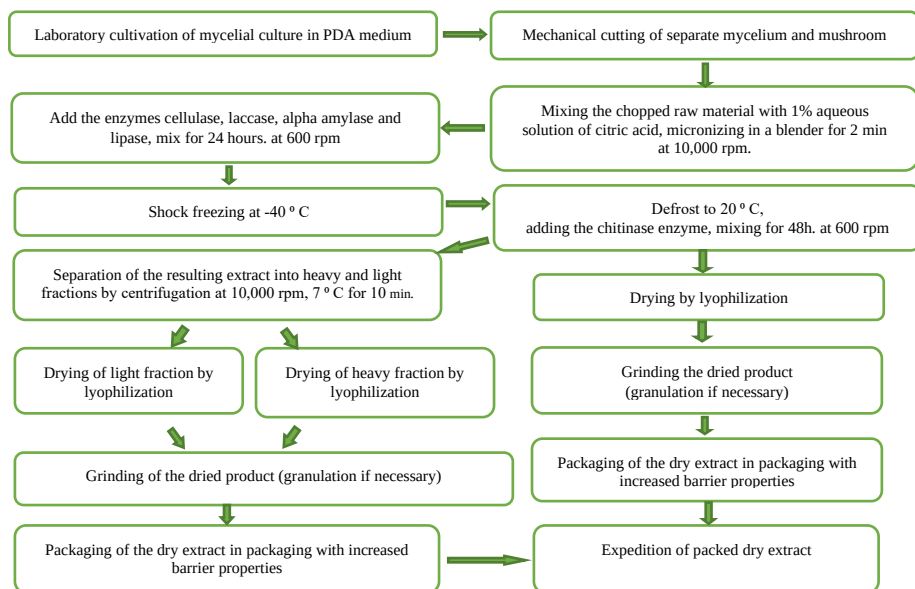


Figure 11. Block diagram of processing steps to obtain a dry enzyme-treated extract

4.2.7. Preparation of mycelium extract by fermentation with living cells.

A mycelium of higher medicinal mushrooms *Ganoderma lucidum*, *Ganoderma neo-japonicum*, *Cordyceps sinensis* and *Cordyceps militaris* was used (phase I) cultivated for 21 days in liquid nutrient medium M2.

The technological order of implementation is presented in **Figure 12**.

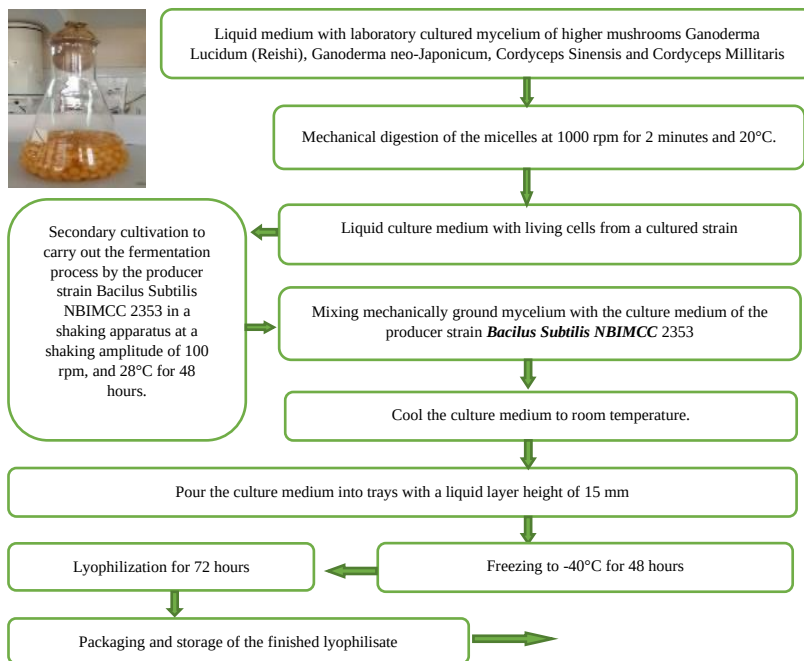


Figure 12. Block diagram of the technological processes for obtaining an extract from a laboratory-cultivated mycelium obtained by fermentation with living cells.

The cultured mycelium in **M2** medium together with the liquid fraction was subjected to grinding in a blender at 1000 rpm for 2 minutes at room temperature. The finely ground mycelial medium was mixed with the pre-cultivated producer strain *Bacillus subtilis* NBIMCC 2353 in **CH1** at a ratio (mycelial medium : bacterial medium) of 6:1.

The new culture fluid thus obtained was distributed in 500 ml Erlenmeyer flasks until a volume of 300 ml was occupied. The photoperiod is performed with natural lighting at 12 h light/12 h dark intervals. Cultivation was carried out for 48 hours, at 28°C and a shaking amplitude of 100 rpm, after which the flasks were cooled to room temperature. The resulting fermented liquid product is poured into stainless steel trays with a height of the liquid layer 15mm, frozen to -40°C for 48 hours and lyophilized for 72 hours.

The resulting dry lyophilizate is packed in three-layer aluminum foil packages with inserted desiccants containing silica gel (SiO₂) to protect the product from lost moisture.

4.2.8. A semi-industrial method for obtaining fruiting body extract by fermentation.

The experiment was conducted in a mushroom extract factory located in Jinhua City, China. Raw material cultivated for 67 days and representing the fruiting body of *Cordyceps millitaris* was used.

The technological order of implementation is presented in **Figure 13**.

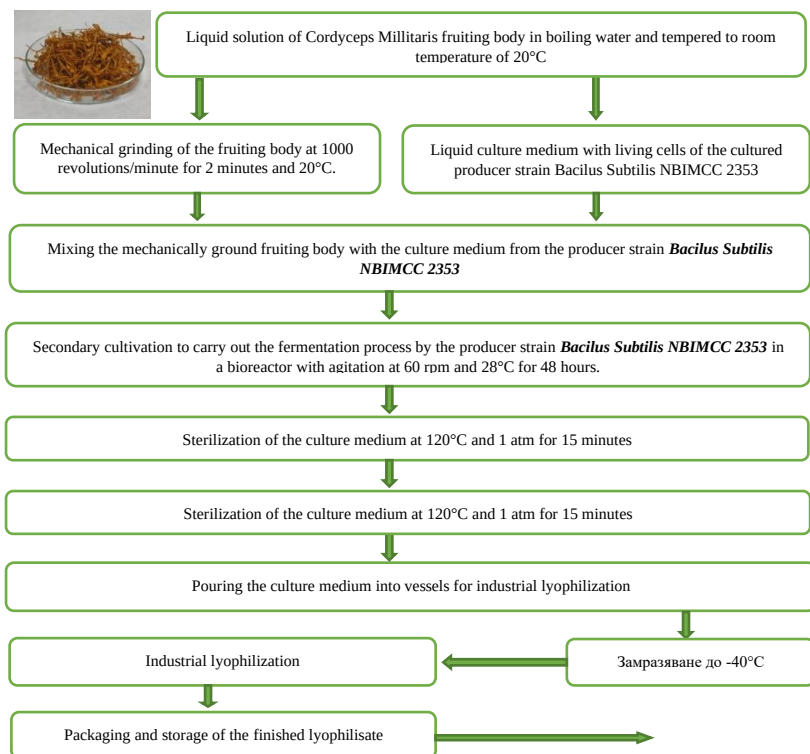


Figure 13. Block diagram of technological processes for obtaining fruiting body extract from industrially cultivated mushroom culture *Cordyceps millitaris* obtained by fermentation with living cells.

The obtained fruiting bodies from the semi-industrial cultivation in the two nutrient media **IM-1** and **IM-2** were distributed in pre-sterilized containers and poured with boiling water in a ratio (water: fruiting body) 1:1. After tempering to reach 20°C, they were subjected to grinding in a blender at 1000 rpm for 2 minutes. The finely ground raw material was mixed with a pre-cultivated producer strain *Bacillus subtilis* **NBIMCC 2353** in **CH1** in a ratio (mycelial medium : bacterial medium) of 6:1.

The culture fluid was placed in a 1000 L semi-industrial bioreactor filled to a

volume of 350 L. Aeration of the 650 L air bed (residual capacity of the reactor volume) was performed by agitating the fluidized bed using a built-in "Anchor" stirrer at a stirring rate 60 rpm. The necessary light for the cultivation is provided by a controlled LED element mounted on the lid of the reactor providing a light flux intensity of 1000 lm. Cultivation was carried out under a photo period of 12 hours light / 12 hours no light for 48 hours, at 28°C. After completion of the cultivation, the culture medium was subjected to sterilization by heating to 120°C for 15 minutes and a pressure of 1 atm, in the reactor itself before draining it.

The fermented raw material thus obtained is tempered to room temperature.

The culture medium was frozen to a temperature of -40°C for a period of 48 hours, after which it was lyophilized.

A comparative evaluation of the obtained raw materials cultured in nutrient media IM-1 and IM-2 was prepared in terms of increasing the availability of the biologically active substance *Cordycepin* by HPLC analysis.

4.2.9. Freeze drying of obtained extracts of cultivated mycelium and fruiting body of medicinal mushrooms

- The lyophilization process includes the following steps:
- Freezing – Freezing is carried out in refrigerating chambers with forced air convection at a temperature of -25°C to -40°C, for a minimum of 24 hours.
- Sublimation drying was carried out in a sublimation installation of the company "Hochvakuum-TG-16.50" - Germany, with contact heating of the plates. Before starting the process, the plates of the installation are cooled to temperatures from -20°C to -40°C, and the desublimator to a temperature from -50°C to -60°C. The drying temperature of the examined samples is in the range of +25°C to +32°C, and the final products have a minimal residual moisture content (2.0 – 5.0%). The total duration of sublimation drying of the samples is 12-24 hours. Lyophilized samples are stored in multi-layer aluminum foil packages, hermetically sealed under vacuum.

4.3. Analytical methods

4.3.1. Determination of the number of viable cells in the culture fluid.

The total number of viable cells was counted according to a standard McFarland method (Souza F.A. et al., 2017, Sapiun Z. et al., 2020). Using a UV-Vis spectrophotometer, model Biochrom Libra S20, the absorbance was measured, indicating the degree of turbidity resulting from cell accumulation. Standard solutions were developed by mixing a 1% solution of BaCl₂ with a 1% solution of H₂SO₄, demonstrating varying degrees of turbidity corresponding to a corresponding number of cells in 1 ml of an equivalent solution (**Table 11**).

Таблица 11. McFarland стандарти.

№	McFarland standart	1% BaCl ₂ (mL)	1% H ₂ SO ₄ (mL)	CFU/ml
TM50	0,5	0,05	9,95	1.5 x 10 ⁸
TM51	1,0	0,1	9,9	3.0 x 10 ⁸
TM52	2,0	0,2	9,8	6.0 x 10 ⁸
TM53	3,0	0,3	9,7	9.0 x 10 ⁸
TM54	4,0	0,4	9,6	1.2 x 10 ⁹
TM55	5,0	0,5	9,5	1.5 x 10 ⁹
TM56	6,0	0,6	9,4	1.8 x 10 ⁹
TM57	7,0	0,7	9,3	2.1 x 10 ⁹
TM58	8,0	0,8	9,2	2.4 x 10 ⁹
TM59	9,0	0,9	9,1	2.7 x 10 ⁹
TM60	10,0	1,0	9,0	3.0 x 10 ⁹

To calibrate the method, a standard TM50 of McFarland, detected at a wavelength of 625 nm, placed in a cuvette with a 10 mm length of the transmitted light flux, was used. Under these conditions, a **McFarland TM50** standard was detected with an absorbance value in the range of 0.08 to 0.1 corresponding to 1.5 x 10⁸ CFU/ml.

4.3.2. Determination of total phenolic number in extracts of mushroom mycelium and fruiting body

The content of total phenolic substances in mushroom mycelium and fruiting body extracts was determined by a standard method with Folin-Ciocalteu reagent (Carmona-Hernandez J.C. et al., 2021; Marcelino C.P. et al., 2020). A UV-Vis spectrophotometer, model Biochrom Libra S20, and a calibration curve obtained by measuring standard solutions of gallic acid (GA) at concentrations of 10, 25, 50, 75 and 100 µg/ml dissolved in MeOH(50%) were used.

The sample was prepared as a solution of the dry extract in MeOH(50%) with a concentration of 100 mg/ml for the first stage of the preparation. The resulting solution was homogenized in an ultrasonic bath until the extract was completely dissolved. The homogenate was diluted with MeOH (50%) to a concentration of 1 mg/ml, then filtered with a 220 nm pore size filter.

3 ml of distilled water and 0.25 ml of Folin-Ciocalteu reagent are added to 0.5 ml of the sample solution. After 2 min, 0.75 ml of 20% sodium carbonate solution and 0.5 ml of distilled water were added. The reaction mixture was homogenized and allowed to stand at room temperature in the dark for 120 min. Absorbance was measured at 765 nm against a blank in which the extract solution was replaced by 0.5 ml MeOH(50%).

4.3.3. Determination of antioxidant activity by DPPH radical versus Trolox in extracts of mushroom mycelium and fruiting body

0.5 ml of methanol and 0.5 ml of a solution with a concentration of 1 mg/ml of the studied raw materials are added to 0.3 ml of a solution of DPPH in methanol (0.2 mmol). The mixed solution was homogenized and allowed to stand in the dark at room temperature for 120 minutes. Absorbance was measured at 517 nm against methanol. Trolox solutions with a concentration of 0.50 to 3.0 µg/ml are used for the standard plate (Pankaj S. and Singh R.P., 2013.).

4.3.4. Determination of proteolytic activity by the Anson method

The following reagents were used for the analysis: 0.1 M universal buffer pH 8 - three solutions A, B, C, mixed in equal amounts (solution A - 0.1 mol/l glacial acetic acid, solution B - 0.1 mol/l orthophosphoric acid and solution C - 0.1mol/l orthoboric acid); 1N NaOH; 0.3M trichloroacetic acid (THO); 0.5M Na₂CO₃; working solution of Folin-Ciocalteu reagent.

The method is based on the hydrolysis of sodium caseinate protein by the enzyme preparation found in the tested solution, with subsequent inactivation of the enzyme and precipitation of the non-hydrolyzed protein with (THO). A unit of proteolytic activity is the amount of enzyme which in 1 min at 30°C releases from sodium caseinate an amount corresponding to 1 µmol of Tyrosine. A 2% solution of sodium caseinate was used as a substrate.

4.3.5. Qualitative determination of fungal cell wall degradation by microscopic observation.

Microscopic observation of fungal cell wall degradation was done with an Accuscope 3012 microscope with a Micrometrics 5MP imaging camera mounted on it.

4.3.6. Qualitative determination of glucosamines as products of enzymatic degradation of mycelial cell wall by HPLC.

HPLC analysis was performed on a sample separated from the resulting supernatant after separation of the extract. The sample was diluted 1:5 with MeOH and sonicated until complete dissolution. The resulting solution was purified by multi-stage filtration using filter paper with a pore size of 100 µm, a glass filter with a pore size of 40 µm, a glass filter with a pore size of 1 µm, and a syringe cartridge with a pore size of 0.45. µm and a syringe cartridge with a pore size of 0.2 µm. Chromatographic analysis was performed with an HP Agilent 1260 Infinity Quaternary LC apparatus, DAD [λ =270 nm], RP-C18 [250 mm], isocratic mode and liquid phase with composition: (A) 1% Acetic acid (H₂O) : ACN [70:30], (B) MeOH, A:B (90:10). As a standard, a solution containing Caffeine (10mg/ml) Glucosamine HCL (1 mg/ml), N-Acetyl-D-Glucosamin (2 mg./ml) dissolved in

mobile phase (A) and filtered with a cartridge the size of pores 0.2 μm . A 1:10 dilution with MeOH was made to perform the analysis.

4.3.7. Molecular mass determination of peptides from fermented fresh mycelium by electrophoresis

Electrophoresis was performed in a polyacrylamide gel - SDS-PAGE (according to Laemmli, 1971) to determine protein fractions. A collecting gel – 6.0% and a separating gel – 12% were prepared. Lyophilized samples of mycelium from *Ganoderma lucidum*, *Ganoderma neo-japonicum*, *Cordyceps sinensis* and *Cordyceps militaris* were rehydrated in deionized water with a concentration of the resulting solution of 200 mg/ml and centrifuged for 10 min at 4°C and a speed of 8000 rpm on a Beckman centrifuge. For final sample preparation, 1 part of the supernatant was mixed with 1 part of sample solvent containing 0.2M Tris HCl buffer (pH 6.8), SDS, glycerin, DTT and Bromophenol Blue.

A Protein Electrophoresis System was used - Pharmacia LCB MultiDrive XL, equipped with an OmniPAGE WAVE Electrophoresis System for the following markers: Sigma marker, Mix 5, and Mix 6, STD (BSA, Lc, Lg, La) and BSA. Electrophoresis was carried out at a constant current strength of 30 mA and an initial voltage of 105 V with a duration of the process of 3.20 hours. The gel was stained with Coomassie brilliant blue G – 250 solution for 30 minutes, then washed and destained. Gel reading and molecular mass calculations were performed using GelAnalyzer-19 software.

4.3.8. Quantification of Adenosine and Cordycepin content in raw mushroom mycelium and fruiting body by HPLC.

HPLC analysis for the quantification of *Adenosine* and *Cordycepin* was performed using an HP Agilent 1260 Infinity Quaternary LC instrument, DAD [$\lambda=260$ nm], Agilent Poroshell 120 HPH-C18, [4.6 x 100 mm, 2.7 μm], elution mode isocratic mode with eluent rate 0.7 ml/min, Run [15 min] and liquid phase with composition: (A) H₂O: MeOH [92:8], (B) H₂O: MeOH [85:15], A: B (98:2). Separate aliquots of the extracts were diluted with MeOH (50%) to a concentration of 100 mg/ml. The samples were treated with an ultrasonic bath until they were completely dissolved and before injection they were filtered with a cartridge filter with a pore size of 220 nm.

Adenosin and *Cordycepin* reference standards purchased from Sigma Aldrich were used. A standard solution containing a mixture of the two standards at a concentration of 100 $\mu\text{g/ml}$ was prepared and used to make a calibration curve. The calibration solutions were diluted with MeOH(50%) in the linear range, with concentrations of 25 $\mu\text{g/ml}$, 50 $\mu\text{g/ml}$ and 100 $\mu\text{g/ml}$, respectively.

4.3.9. Quantification of the protein content in extracts of cultivated medicinal mushrooms - by the *Kjeldahl* method by determining total nitrogen.

The method is based on the quantification of the nitrogen obtained from the separated ammonia obtained as a product of the sulfuric acid reaction of mineralization during the combustion of the protein sample.

Principle of the method. When heated to 400⁰ C, with concentrated sulfuric acid, organic compounds are oxidized to carbon dioxide (CO₂) and water (H₂O), and nitrogen (N₂) without diazo (-N=N-) is released in the form of ammonia (NH₃) and combines with sulfuric acid (H₂SO₄), becoming sulfate [(NH₄)₂SO₄] by the reaction:



Catalysts such as copper and mercury salts, selenium mixture, hydrogen peroxide, etc. are used to speed up the process.

To release the formed ammonia from the sulfate, a 35% sodium hydroxide solution (NaOH) is used according to the reaction:



The solution required for titration is obtained by mixing the distillate with a previously prepared 4% solution of boric acid (H₃BO₃).

The amount of ammonia separated is determined by titration with a 0.1N solution of sulfuric acid (H₂SO₄) and an indicator mixture representing an alcoholic solution of methyl rot and methylene blue, leading to a change in the color of the reaction mixture from green (in an alkaline medium) to violet (in an acidic medium) medium).

4.3.10. Residual moisture content of the extracts after lyophilization (%) - Moisture scale with infrared heating "Sartorius".

To carry out the density measurement, the heat-drying method is used, for which a 1g measured amount (accurate to the 4th decimal place) of the sample is deposited in a thin layer on the Sartorius apparatus. The drying procedure is started, after which the remaining amount of dry residue is measured.

The result is presented in %, calculated by the formula:

$$\%, (\text{sample moisture}) = \frac{\mathbf{M}(\text{source sample}) - \mathbf{M}(\text{solids})}{\mathbf{M}(\text{source sample})} \times 100$$

Accordingly, the following formula is used to calculate % (solids):

$$\%, (\text{solids}) = \frac{\mathbf{M}(\text{solids})}{\mathbf{M}(\text{source sample})} \times 100$$

Where:

- %, (sample moisture) – percentage moisture content of the starting sample
- % (solids) – percentage content of the total amount of dry residue in the starting sample
- **M** (source sample) – mass of the starting sample, g (accuracy ±0.0001g)

- **M** (solids) – mass of the dry residue after drying the sample, g (accuracy $\pm 0.0001\text{g}$)

4.3.11. Microbiological studies of the lyophilized extracts: (BDS 1670-82).

- Total number of mesophilic aerobic and facultatively anaerobic microorganisms - CFU/g (BDS EN ISO 4833-2013);
- *Escherichia coli* in 0.1 g of the product (ISO 16649 - 1,2,3);
- Pathogenic microorganisms, incl. *Salmonella* (БДЦ EN ISO 6579:2003) in 25.0 g of the product;
- Coagulase-positive staphylococci in 1.0 g of the product (BDS EN ISO 6888-1-2005);
- Sulfite-reducing clostridia in 0.1 g of the product (ISO 15213-2003);
- Spores of microscopic molds and yeasts, CFU/g (BDS ISO 6611-2006);

V. RESULTS AND DISCUSSION

A technological model for creating a biologically active product based on organic sources has been developed. Extracts obtained as a result of cultivation of the higher medicinal mushrooms *Ganoderma lucidum*, *Ganoderma neo-japonicum*, *Cordyceps sinensis*, *Cordyceps Militaris* and the producer bacterial strain *Bacillus subtilis* were used as raw material for the production of the functional product.

5.1. Cell culture cultivation of higher medicinal mushrooms *Ganoderma neo-japonicum*, *Ganoderma lucidum*, *Cordyceps sinensis* and *Cordyceps millitaris* in liquid medium.

5.1.1. Propagation of starter cultures *Ganoderma lucidum*, *Ganoderma neo-japonicum*, *Cordyceps sinensis*, *Cordyceps Militaris* to obtain starting inoculum.

The first stage of cultivation involves propagating the starter cultures in a standard medium recommended by the manufacturer. Propagation of the mushrooms *Ganoderma neo-japonicum*, *Cordyceps sinensis* and *Cordyceps Militaris* was carried out by inoculating the culture medium with a starter cell culture (Fig. 14), while for *Ganoderma lucidum* industrially grown live mycelium was used. (Fig. 15).



Figure 14. Inoculated liquid medium *Ganoderma neo-japonicum* *Cordyceps sinensis* and *Cordyceps Militaris*



Figure 15. Inoculated liq *Ganoderma lucidum* mycelium medium

The proliferation of the cell cultures was monitored in the starter medium suggested by the manufacturer and the **M0** liquid medium. Visually, it was found that in all four cell cultures there was a more intensive growth process in **M0** medium (**Figure 16**). Thus propagated cell cultures were used for subsequent inoculation.

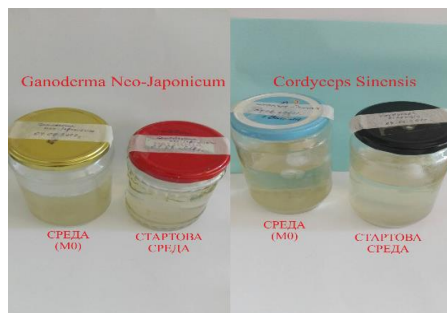


Figure 16. Development of *Ganoderma neo-japonicum*, *Cordyceps sinensis* cell cultures in standard and **M0** media

5.1.2. Cultivation of fresh mycelium of medicinal mushrooms *Ganoderma neo-japonicum*, *Ganoderma lucidum*, *Cordyceps sinensis* and *Cordyceps militaris* in liquid nutrient medium (phase I).

Fresh mycelium is considered developed when spherical forms are formed from the mycelial culture. Observation of the development of the four medicinal mushrooms *Ganoderma neo-japonicum*, *Ganoderma lucidum*, *Cordyceps sinensis* and *Cordyceps militaris* shows the formation of spheres of different sizes (**Figure 17**)



Figure 17. Grown fresh mycelial culture in liquid nutrient medium (a) *Ganoderma neo-japonicum*, (b) *Ganoderma lucidum*, (c) *Cordyceps sinensis* (d) *Cordyceps militaris*

In the development of the mycelium, through the multiplication of the fungal cell culture, connected cell structures are formed in the form of hyphae, representing channels for the transfer of nutrients and water for nutrition. In this way, part of the solution from the nutrient medium passes into the hyphae. By measuring the volume

of the undigested nutrient medium, the degree of growth of the mycelium is determined (**Figura 18-21**)

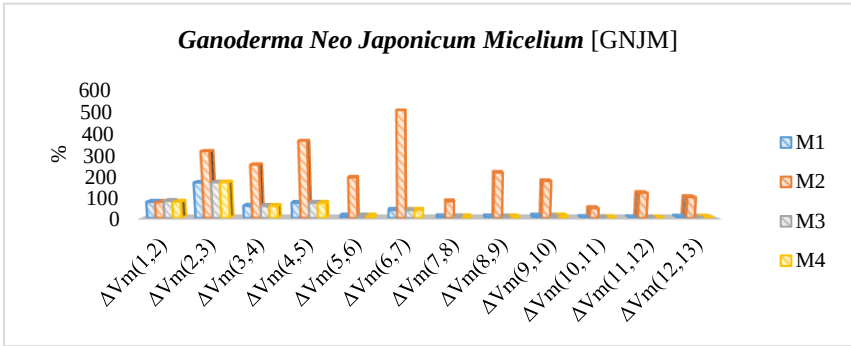


Figure 18. Mycelial growth of *Ganoderma neo-japonicum* (%)

Δ Vm – rate of micelle growth

medium M1 - potato starch, peptone, yeast extract, glucose, FOS, $\text{MgSO}_4 \times 7 \text{H}_2\text{O}$

medium M2 - potato starch, peptone, yeast extract, glucose, FOS, $\text{MgSO}_4 \times 7 \text{H}_2\text{O}$, Vitamin B1, KH_2PO_4 , K_2HPO_4 , FeSO_4 .

medium M3 - potato starch, peptone, yeast extract, dextrose, FOS, $\text{MgSO}_4 \times 7 \text{H}_2\text{O}$, Vitamin B1, KH_2PO_4 , K_2HPO_4 , FeSO_4 .

medium M4 - potato starch, peptone, yeast extract, dextrose, FOS, $\text{MgSO}_4 \times 7 \text{H}_2\text{O}$, Vitamin B1, KH_2PO_4 , K_2HPO_4 , FeSO_4 .

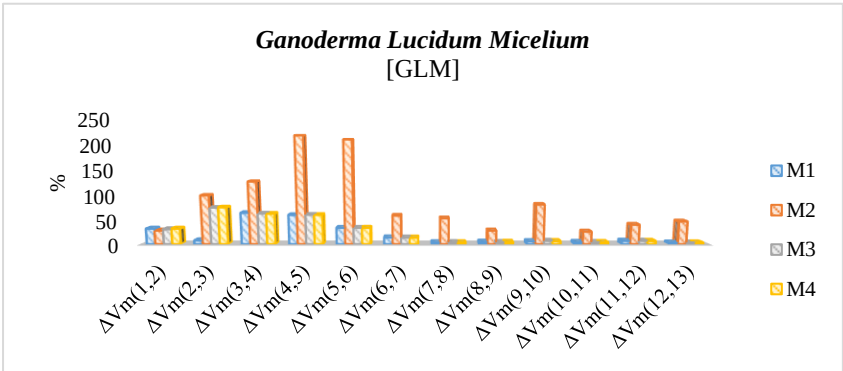


Figure 19. *Ganoderma lucidum* mycelial growth (%)

Δ Vm – rate of micelle growth

medium M1 - potato starch, peptone, yeast extract, glucose, FOS, $\text{MgSO}_4 \times 7 \text{H}_2\text{O}$

medium M2 - potato starch, peptone, yeast extract, glucose, FOS, $\text{MgSO}_4 \times 7 \text{H}_2\text{O}$, Vitamin B1, KH_2PO_4 , K_2HPO_4 , FeSO_4 .

medium M3 - potato starch, peptone, yeast extract, dextrose, FOS, $\text{MgSO}_4 \times 7 \text{H}_2\text{O}$, Vitamin B1, KH_2PO_4 , K_2HPO_4 , FeSO_4 .

medium M4 - potato starch, peptone, yeast extract, dextrose, FOS, $\text{MgSO}_4 \times 7 \text{H}_2\text{O}$, Vitamin B1, KH_2PO_4 , K_2HPO_4 , FeSO_4 .

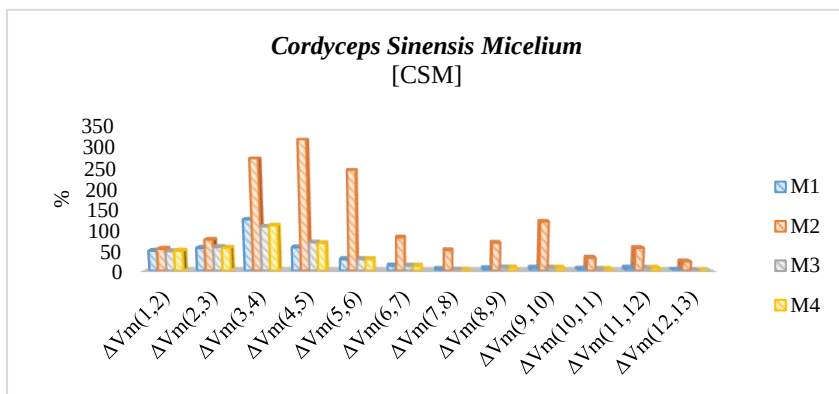


Figure 20. *Cordyceps millitaris* mycelial growth (%) Δ Vm – rate of micelle growth
 medium M1 - potato starch, peptone, yeast extract, glucose, FOS, $\text{MgSO}_4 \times 7 \text{H}_2\text{O}$
 medium M2 - potato starch, peptone, yeast extract, glucose, FOS, $\text{MgSO}_4 \times 7 \text{H}_2\text{O}$, Vitamin B1, KH_2PO_4 , K_2HPO_4 , FeSO_4 .
 medium M3 - potato starch, peptone, yeast extract, dextrose, FOS, $\text{MgSO}_4 \times 7 \text{H}_2\text{O}$, Vitamin B1, KH_2PO_4 , K_2HPO_4 , FeSO_4 .
 medium M4 - potato starch, peptone, yeast extract, dextrose, FOS, $\text{MgSO}_4 \times 7 \text{H}_2\text{O}$, Vitamin B1, KH_2PO_4 , K_2HPO_4 , FeSO_4 .

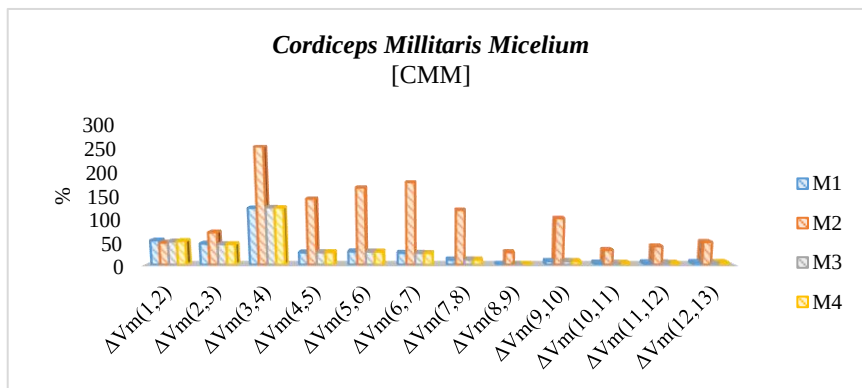


Figure 21. *Cordyceps sinensis* mycelium growth (%) Δ Vm – rate of micelle growth
 medium M1 - potato starch, peptone, yeast extract, glucose, FOS, $\text{MgSO}_4 \times 7 \text{H}_2\text{O}$
 medium M2 - potato starch, peptone, yeast extract, glucose, FOS, $\text{MgSO}_4 \times 7 \text{H}_2\text{O}$, Vitamin B1, KH_2PO_4 , K_2HPO_4 , FeSO_4 .
 medium M3 - potato starch, peptone, yeast extract, dextrose, FOS, $\text{MgSO}_4 \times 7 \text{H}_2\text{O}$, Vitamin B1, KH_2PO_4 , K_2HPO_4 , FeSO_4 .
 medium M4 - potato starch, peptone, yeast extract, dextrose, FOS, $\text{MgSO}_4 \times 7 \text{H}_2\text{O}$, Vitamin B1, KH_2PO_4 , K_2HPO_4 , FeSO_4 .

Experimentally, it was found that the best mycelial growth was in **M2** medium, followed by **M4** and **M3**, **M1** medium, and the lowest growth was observed in the **M0** control medium.

From the evaluation, **M2** medium was selected for subsequent mycelium cultivation of the selected higher fungi. The resulting mycelial culture will be subjected to fermentation with a live *Bacillus subtilis* cell culture to obtain a biologically active extract and produce a functional product.

5.1.3. The mycelium cultivation of medicinal mushrooms *Ganoderma neo-japonicum*, *Ganoderma lucidum*, *Cordyceps sinensis* and *Cordyceps militaris* on solid nutrient medium (stage II).

Laboratory models for the cultivation of the higher mushrooms *Ganoderma lucidum*, *Ganoderma neo-japonicum*, *Cordyceps sinensis*, *Cordyceps militaris* in solid food media were studied.

The procedure for processing the solid culture media is presented in **Figure 22**.



Figure 22. Processing of solid food media.

After starting the cultivation, the samples from the thermo cabinet were left without a light source for 168 hours, after which a photoperiod of 12 hours with natural light and 12 hours without light at 28°C was observed. On growth monitoring, surface development of the mycelial culture was found between days 7 and 20 (Figure 23).

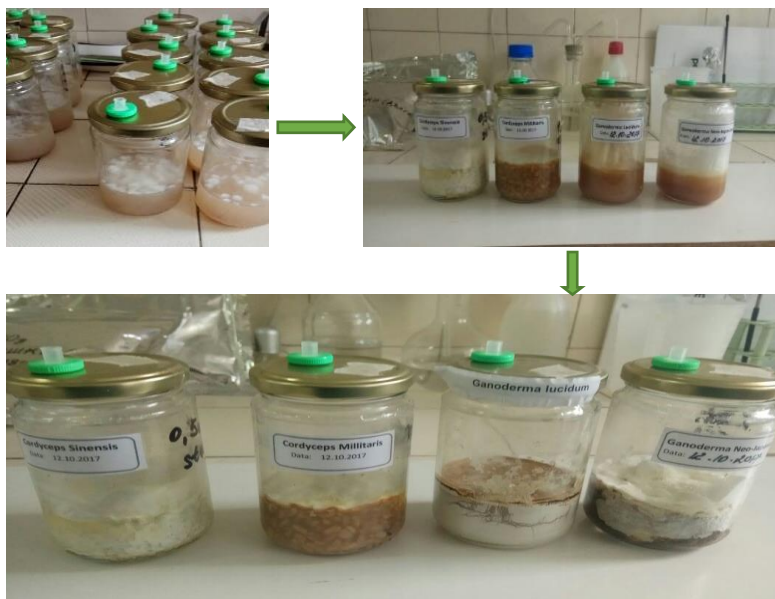


Figure 23. Tracking mycelial growth of *Ganoderma lucidum*, *Ganoderma neo-japonicum*, *Cordyceps sinensis*, *Cordyceps militaris*.

A visual evaluation of the mycelial growth of the higher fungi in the deep culture was carried out, observing at intervals of 15 days.

Profound development of the mycelial culture was observed between the 30th and 90th day, with the best result for all higher fungi being reported in medium S2 (with added carbon source rice).

5.2. A semi-industrial method for cultivating *Cordyceps militaris* cell culture to obtain a fruiting body and a high *Cordycepin* content.

The study was conducted in a medicinal mushroom farm with an extract production plant located in Jinhua City, China.

A total of 5000 L of working medium were prepared, respectively 2500 L of medium IM1 and 2500 L of medium IM2. The working media thus obtained were poured into 12,500 jars of 750 ml, filled to a volume of 200 ml for medium IM1 and 12,500 jars for medium IM2. After sterilization, the culture media were inoculated with a liquid cell culture of *Cordyceps militaris* - producer strain Shanghai.

Cultivation was carried out for 67 days, starting in the absence of light for the first 7 days, then continued under the planned photoperiod of 16 h light to 8 h dark for another 60 days.

Figure 24 shows the cultivation of a fruiting body from *Cordyceps militaris* – Shanghai.



Figure 24. *Cordyceps militaris* fruiting body cultivation – Shanghai.

During the conducted experiment, a total of 4 types of starting raw materials were obtained for extracts for use in final dosage forms (**Table-12**).

Table 12. Received raw materials from semi-industrial cultivation of *Cordyceps sinensis* – Shanghai

Food environment	output quantity, L	Inoculated jars, pcs	mycelial culture, kg	Fruiting body, kg	Mycelial culture in a jar, g	Fruiting body in a jar, g
IM1	500	2500	462,5 ±33,76	175 ±12,78	185 ±13,5	70 ±5,1
IM2	500	2500	475 ±38,95	275 ±22,55	190 ±15,58	110 ±9

Nutrient medium IM1 - potato peels, sugar cane peels, soy peptone, yeast extract, glucose, whole grain rice, Azomite, $\text{MgSO}_4 \times 7 \text{H}_2\text{O}$, Vitamin B1,

Nutrient Medium IM2- Potato Peels, Sugar Cane Peels, Soy Peptone, Yeast Extract, Glucose, Whole Grain Rice, Shrimp Shell Powder, Monosodium Glutamate, L-Glycine, $(\text{NH}_4)_2\text{SO}_4$, Azomite $\text{MgSO}_4 \times 7 \text{H}_2\text{O}$ Vitamin B1, KH_2PO_4 ,, K_2HPO_4 , $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$

The analysis of the obtained data shows that in the IM1 medium, the amount of mushroom body obtained represents 35% of the starting material, the mycelial culture represents 92.5% of the starting material, and the total amount of raw material for the production of extracts represents 127.5% of the starting material.

In **IM2 medium**, the amount of fungal body obtained represents 55% of the starting material, the mycelial culture represents 96% of the starting material, and the total amount of raw material for the production of extracts represents 151% of the starting material.

This result shows that under these cultivation conditions, the total amount of substrates in the system in **IM1 medium** increased by 27.5% and in **IM2 medium** by 51%, which is probably due to the nitrogen, carbon dioxide and water absorbed from the air.

The total amount of raw materials obtained from conducting the experiment is 937.5 kg of fresh mycelial culture and 450 kg of fresh fruiting body. The raw materials are subjected to extraction by fermentation and lyophilized.

5.3. Cell culture propagation of a *Bacillus subtilis* strain

5.3.1. Increase the number of viable cells by optimizing the composition of the nutrient medium

A rehydrated lyophilized producer strain *Bacillus subtilis* NBIMCC 2353 was used, which was initially propagated in standard culture medium, and the resulting culture solution was used for subsequent inoculation. Control medium (K) was obtained from the inoculum by adding prebiotic and other substances related to stimulation of cell proliferation. Powdered extracts of two species of higher mushrooms *Cordyceps sinensis* and *Ganoderma lucidum* were added to it in two different concentrations: 1 g/l and 2 g/l, and media **C1-C4** were obtained. In all variants, the producer strain inoculum had an initial cell concentration of 1.6×10^7 CFU/ml.

Determination of cell culture density and calculation of the number of cells of the strain in different nutrient media was done spectrophotometrically, according to the **McFARLAND** standard.

Table 13. Number of *Bacillus subtilis* NBIMCC 2353 cells (log N) in the investigated media, reported at the beginning of cultivation, at the 24th, 48th and 72nd hours after the start of cultivation (n=3)

Food environment	Number of cells (log N) counted at the start of cultivation	Number of cells (log N) counted at 24 h	Number of cells (log N) counted at 48 h	Number of cells (log N) counted at 72 h
K - control	$7,2 \pm 0,009$	$8,75 \pm 0,008$	$9,07 \pm 0,007$	$8,14 \pm 0,006$
C1	$7,2 \pm 0,006$	$9,79 \pm 0,007$	$10,28 \pm 0,008$	$9,30 \pm 0,006$
C2	$7,2 \pm 0,007$	$9,83 \pm 0,008$	$11,58 \pm 0,008$	$11 \pm 0,007$
C3	$7,2 \pm 0,005$	$9,75 \pm 0,005$	$10,26 \pm 0,003$	$9,60 \pm 0,006$
C4	$7,2 \pm 0,008$	$9,88 \pm 0,006$	$11,65 \pm 0,007$	$11,50 \pm 0,003$

Nutrient medium K – meat extract, peptone, yeast extract, FOS, NaCl, K_2HPO_4 , $MgSO_4$

Nutrient medium C1 - meat extract, peptone, yeast extract, FOS, NaCl, K_2HPO_4 , $MgSO_4$, *Cordyceps sinensis* ext.,

Nutrient medium C2 - meat extract, peptone, yeast extract, FOS, NaCl, K_2HPO_4 , $MgSO_4$, *Cordyceps sinensis* ext.

Nutrient medium C3 - meat extract, peptone, yeast extract, FOS, NaCl, K_2HPO_4 , $MgSO_4$, *Ganoderma lucidum* ext.

Nutrient medium C4 - meat extract, peptone, yeast extract, FOS, NaCl, K_2HPO_4 , $MgSO_4$, *Ganoderma lucidum* ext.

In the control medium (K), where the cell culture was propagated without added enhancers, the number of cells increased to 1.87 log units and the peak of growth was at 40 - 48 hours after inoculation. After this period, death of the cell culture begins and by the 72nd hour, a decrease in cells of almost a full log unit is observed. In media **C1** and **C3** with added 1g each of *Cordyceps sinensis* and *Ganoderma lucidum* extract, a certain similarity was observed in the reproductive time range of the culture. The peak of reproduction was also at the 48th hour from the start of the experiment and the increase was just over 3 log units (10.28 log N for medium **C1** and 10.26 log N for medium **C3**). By the 72nd hour, there was a decrease in **C1** medium by 1 log unit to 9.30 log N, and in **C3** medium supplemented with *Ganoderma lucidum* extract (1 g) the cells decreased by 0.66 to 9.60 log N. media **C2** and **C4** with added 2 g of extract from the two higher fungi showed a higher number of cells, almost 4.5 log units. The highest number was reported for medium **C4** – 11.65 log N (at the 48th hour). The death of the cells after the peak of their reproduction until the 72nd hour is also marked by a smaller number (about 0.5 log units), respectively in **C2** medium – up to 1 to :1000log N, and in **C4** – up to 11.50 log units, in which by the 72nd hour a very small number of cells had died and the number of living cells remained almost the same as that recorded at the 48th hour.

It was found that in all environments - control (K) and **C1-C4**, the peak of cell population growth was reached at the 48th hour. In the nutrient media supplemented with extracts of both medicinal mushrooms, higher maximum cell counts were reported than in the control variant. Of these, the largest number is in the variant samples with 2 g of extracts introduced, where by the 48th hour the number of cells increases by almost 4.5 log units, and by the 72nd hour a small amount of cells die. This is particularly clear for the medium supplemented with 2 g of *Ganoderma lucidum* extract.

When the powdered extracts of *Cordyceps sinensis* and *Ganoderma lucidum* were added to the nutrient medium for the cultivation of *Bacillus subtilis strain NBIMCC 2353*, an increase in the number of cells was observed, which proves their positive impact on population growth.

5.3.2. Enhancement of Total Proteolytic Activity of *Bacillus Subtilis* Culture Medium Enriched with Native Bovine Collagen Protein and Higher Medicinal Mushroom Mycelium

The experimental data obtained are presented in **Table 14**.

Table 14. Proteolytic activity of *Bacillus Subtilis* in medium supplemented with native bovine collagen protein and mycelium of higher medicinal fungi.

Medium	PU/ml, 24 h.	PU/ml, 48 h.	PU/ml, 72 h.
K	1,38 ± 0,004	2,27 ± 0,006	1,89 ± 0,003
P1	15,74 ± 0,006	23,74 ± 0,007	19,71 ± 0,005
P2	17,37 ± 0,004	26,83 ± 0,008	22,18 ± 0,007
P3	18,34 ± 0,007	30,57 ± 0,006	25,47 ± 0,008
P4	14,91 ± 0,003	24,42 ± 0,007	20,56 ± 0,005
P5	13,67 ± 0,005	21,51 ± 0,007	17,85 ± 0,004

K-Control - meat extract, peptone, yeast extract, FOS, NaCl, K₂HPO₄, MgSO₄, **P1**- medium with added native bovine collagen, **P2**- medium with added *Ganoderma neo-japonicum* mycelium, **P3**- medium with added *Ganoderma lucidum* mycelium, **P4**- medium with added mycelium of *Cordyceps sinensis* and **P5**- medium with added mycelium of *Cordyceps militaris*

The total proteolytic enzyme activity detected was highest at 48 h after inoculation. A higher proteolytic activity was found when protein sources were added compared to the control. In the variants with added mushroom substrate, a higher proteolytic activity was found compared to the variant with added collagen, which is probably due to the richer composition of the fungal mycelium compared to collagen. This gives grounds for using this approach when developing the technology of the new product.

5.3.3. Enhancement of chitinase enzyme activity of *Bacillus Subtilis* culture medium upon addition of non-hydrolyzed live mycelium fragments of four species of medicinal mushrooms *Ganoderma lucidum*, *Ganoderma neo-japonicum*, *Cordyceps sinensis*, *Cordyceps militaris* and shrimp shell chitin

Table 15 presents the results of a study of the chitinase activity of a nutrient medium for the cultivation of *Bacillus Subtilis*. A tendency to increase enzyme activity was observed when adding components containing shrimp chitin (**CH1**) and fresh mycelium from *Ganoderma lucidum* (**CH2**), *Ganoderma Heo-Japonicum* (**CH3**), *Cordyceps sinensis* (**CH4**) and *Cordyceps militaris* (**CH5**).

Table 15. Chitinase enzyme activity of *Bacillus Subtilis*

Medium	Chitinase activity (U/mL) reported at 24 h	Chitinase activity (U/mL) reported at 48 h	Chitinase activity (U/mL) reported at 72 h
Control medium (K)	4,37 ±0,002	8,65 ±0,001	6,83 ±0,006
CH1	32,57 ±0,007	40,86 ±0,006	37,68 ±0,007
CH2	33,78 ±0,007	41,62 ±0,003	38,43 ±0,002
CH3	34,46 ±0,007	42,73 ±0,003	39,58 ±0,002
CH4	31,64 ±0,007	39,93 ±0,003	36,82 ±0,006
CH5	33,47 ±0,007	41,29 ±0,003	38,62 ±0,004

Control medium (K) meat extract, peptone, yeast extract, FOS, NaCl, K₂HPO₄, MgSO₄; **CN1-medium** with added shrimp chitin; **CN2-medium** with added fresh mycelium from *Ganoderma lucidum*; **CH3-medium** with added fresh mycelium from *Ganoderma Heo-Japonicum*, **CH4-medium** with added fresh mycelium from *Cordyceps sinensis*; **CH3 medium** with added fresh mycelium from *Cordyceps militaris*

The reported chitinase enzyme activity of the media supplemented with fresh mushroom mycelium was higher compared to the variant supplemented with shrimp chitin and the control medium. The obtained results prove that *Bacillus Subtilis* is a suitable producer strain for use in a biotechnological process for enzymatic digestion of fungal substrate from fresh mycelium and can be successfully applied in the production of the developed functional product. Given the highest reported result at 48 hours, a two-day processing cycle can be assumed in the production technology.

5.4. Preparation of biologically active extracts from mycelial cultures by fermentation with living cells of *Bacillus subtilis* and using enzymes.

To release the biologically active substances from the cell and the cell wall, without the need to use organic solvents, an enzymatic method was applied to break down the structures of the cell, which takes place in a temperature range of 18-20°C with the solvent water. The necessary acidity of the solution is achieved by citric acid and the processing conditions keep the biologically active substances unchanged, as processes occur naturally in natural systems. Solvent removal in this approach is carried out under vacuum and low temperature through a lyophilization process, which ensures the preservation of volatile and thermolabile molecules. Living mycelium from *Ganoderma lucidum*, which contains more than 400 types of biologically active substances, was used as the raw material. The enzymes DeniLight (*laccase*), Lipopan (*lipase*), Fungamyl 4000 SG (*alpha amylase*), Celluclast 1.5 L (*cellulase*), Chitinase in amounts of 0.5 g each were used to break down the cell wall. The raw material from the living mycelium of *G. Lucidum* was grown for a period of six months in laboratory conditions at the ICFT-Sofia. Microscopic and chromatographic examination were carried out to establish the degradation of the cell wall. For microscopic observation, a sample of the solution was taken before the start of the enzymatic hydrolysis process and a sample of the obtained complex extract at the end of the process. Fermentation was carried out

with living cells of the producer strain *Bacillus subtilis* and a light and heavy fraction was separated from the resulting culture liquid. Chromatographic analysis was performed on a sample separated from the obtained supernatant of the complex extract.

Figure 25 shows a microscopic image of the starting material after the initial mechanical micronization step. Cellular structures in the form of fibrous hyphae from the fungal material are clearly visible. From the microscopic observation, it is clear that the cells in the initial stage after micronization are in their entirety without destroyed cell walls.

Figure 26 presents a microscopic image of the obtained complex extract at the final stage after enzymatic hydrolysis of the cell wall.



Figure 25. Field of view of microscopic observation of a live *Ganoderma lucidum* mycelium after the mechanical micronization step.

The absence of entire structures of cells with an intact cell wall is clearly visible. This indicates that the cell walls have been degraded and the intracellular substances have passed into the solution, thus producing a biologically active extract with preserved biologically active substances.

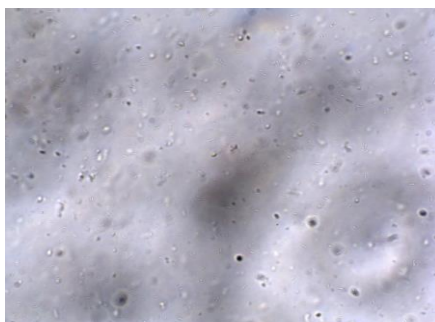


Figure 26. Field of view microscopic observation of a complex extract preparation at the final stage after enzymatic degradation of the cell wall.

Figure 27 shows the chromatograms of the samples - standard and supernatant after separation of the complex extract. On the first chromatogram, the peaks of the **Glucosamin HCL** and **N-Acetyl-D-Glucosamin** standards are reported. The chromatogram from the extract supernatant again shows peaks for glucosamine fractions in the time domain of the standards, which is further evidence of degradation of the chitin layer from the cell wall. Chromatographic research was conducted with a view to qualitative determination without quantitative calculations, which are not the subject of the present project. The purpose of this study is to prove the degradation of the chitin layer of the fungal cells

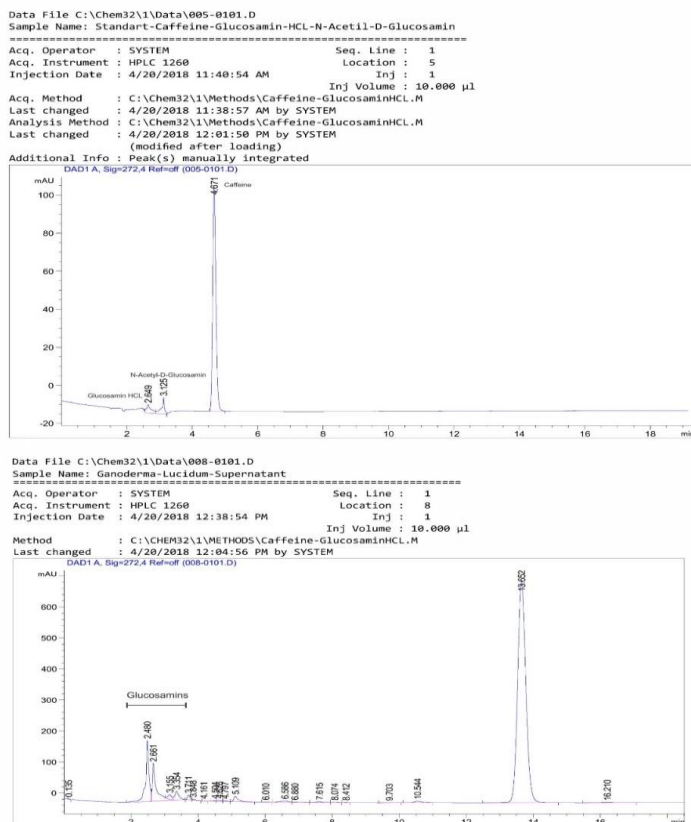


Figure 27. Chromatograms from an HPLC, Agilent 1260 study of a separated sample from the supernatant after separation of an enzyme-treated extract from mycelium and fruiting body of *Ganoderma Lucidum*

The results prove that when using this method, it is possible to obtain extracts in which all active substances are preserved, due to the processes taking place at temperatures sparing the biological activity of thermolabile compounds. The method can be adjusted depending on the need for maximum preservation of target biologically active substances by changing the stages involving the enzymatic reaction, as well as the use of different enzymes depending on the processed plant matrix of the raw material used.

5.5. Experimental evidence of the presence of biologically active components in extracts of the mycelium of *Ganoderma neo-japonicum*, *Ganoderma lucidum*, *Cordyceps sinensis* and fruiting body of *Cordyceps militaris*

5.5.1. Establishing the presence of proteins with different molecular mass.

A biochemical study was carried out on the protein spectrum of experimental samples of medicinal mushrooms and a culture sample of *Bacillus subtilis* NBIMCC 2353 according to the method of Laemmli (1970). **Figure 28** shows the result of polyacrylamide gel electrophoresis of medicinal mushrooms and a sample of *Bacillus subtilis* NBIMCC 2353.

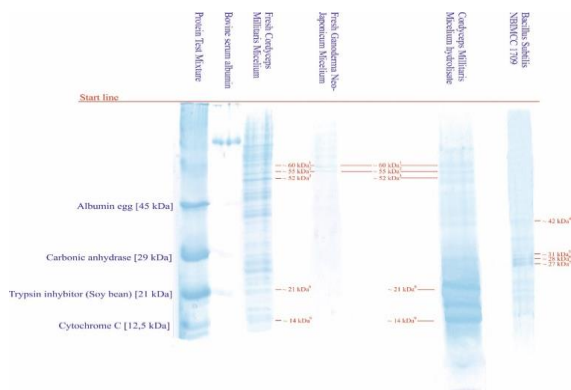


Figure 28. SDS–PAGE of medicinal mushrooms and *Bacillus subtilis* culture sample1-mycelium from *Cordyceps militaris*; 2 – culture fluid from *Cordyceps militaris*; 3 – *Ganoderma neo-japonicum* mycelium; 4 – culture fluid from *Ganoderma neo-japonicum*; 5 – old mycelium from *Ganoderma lucidum*; 6 – old mycelium from *Cordyceps sinensis*; 7 – lyophilisate from *Cordyceps militaris*; 8 – *Bacillus subtilis* NBIMCC 2353; PTM – a mixture of proteins with a known molecular mass; BSA – bovine serum albumin

By densitometric analysis, the electrophoretic mobility of the protein fractions of the studied experimental variants was determined. The results are shown in **Table 16**.

Table 16. Electrophoretic mobility of protein fractions of samples of medicinal mushrooms and culture sample of *Bacillus subtilis* NBIMCC 2353.

Sample	Stripe/ Ribbon	Rf	Raw volume	Cal. volume	MW (molecular mass)
1. Protein Test Mixture	1.	0,407	2027	-	45000
1. Protein Test Mixture	2.	0,612	2550	-	29000
1. Protein Test Mixture	3.	0,763	2697	-	21000
1. Protein Test Mixture	4.	0,883	1620	-	12500
1. Protein Test Mixture	5.	0,918	470	-	6500
2. Bovine serum albumin	1.	0,158	967	-	62582
3. Fresh mycelium CMM	1.	0,064	132	-	69265
3. Fresh mycelium CMM	2.	0,082	234	-	67941
3. Fresh mycelium CMM	3.	0,103	343	-	66491
3. Fresh mycelium CMM	4.	0,136	523	-	64095
3. Fresh mycelium CMM	5.	0,183	400	-	60754
3. Fresh mycelium CMM	6.	0,21	589	-	58863
3. Fresh mycelium CMM	7.	0,243	701	-	56530
3. Fresh mycelium CMM	8.	0,275	630	-	54198
3. Fresh mycelium CMM	9.	0,301	660	-	52370
3. Fresh mycelium CMM	10.	0,354	451	-	48587
3. Fresh mycelium CMM	11.	0,37	692	-	47453
3. Fresh mycelium CMM	12.	0,441	684	-	42410
3. Fresh mycelium CMM	13.	0,553	273	-	34467
3. Fresh mycelium CMM	14.	0,598	397	-	31252
3. Fresh mycelium CMM	15.	0,629	639	-	29045
3. Fresh mycelium CMM	16.	0,66	739	-	26839
3. Fresh mycelium CMM	17.	0,691	317	-	24632
3. Fresh mycelium CMM	18.	0,741	371	-	21039
3. Fresh mycelium CMM	19.	0,812	299	-	15996
3. Fresh mycelium CMM	20.	0,841	215	-	13979
3. Fresh mycelium CMM	21.	0,852	156	-	13159
4. Fresh mycelium GNJ	1.	0,19	94	-	60315
4. Fresh mycelium GNJ	2.	0,267	128	-	54783
5. Lyophilisate CMM	1.	0,148	490	-	63303
5. Lyophilisate CMM	2.	0,204	495	-	59302
5. Lyophilisate CMM	3.	0,226	300	-	57747
5. Lyophilisate CMM	4.	0,258	345	-	55469
5. Lyophilisate CMM	5.	0,301	340	-	52413
5. Lyophilisate CMM	6.	0,407	385	-	44857
5. Lyophilisate CMM	7.	0,534	829	-	35801
5. Lyophilisate CMM	8.	0,625	1173	-	29356
5. Lyophilisate CMM	9.	0,689	1111	-	24800
5. Lyophilisate CMM	10.	0,74	1046	-	21133
5. Lyophilisate CMM	11.	0,774	728	-	18688
5. Lyophilisate CMM	12.	0,84	354	-	14021
5. Lyophilisate CMM	13.	0,888	508	-	10632
5. Lyophilisate CMM	14.	0,985	135	-	3687
6. Bacillus subtilis NBIMCC2353	1.	0,4	399	-	45369
6. Bacillus subtilis NBIMCC2353	2.	0,446	196	-	42064
6. Bacillus subtilis NBIMCC2353	3.	0,507	315	-	37733
6. Bacillus subtilis NBIMCC2353	4.	0,537	401	-	35568
6. Bacillus subtilis NBIMCC2353	5.	0,596	132	-	31408
6. Bacillus subtilis NBIMCC2353	6.	0,643	106	-	28046
6. Bacillus subtilis NBIMCC2353	7.	0,655	219	-	27191
6. Bacillus subtilis NBIMCC2353	8.	0,73	271	-	21835

Analysis of the protein spectrum of culture fluid reports the presence of protein fractions with molecular masses ranging from 3.7 to 100 kDa. According to Kim et al., 2006; Choi & Sa, 2000; Lee et al., 2005; Lee et al., 2007 et al. authors in this range are also the molecular masses of the fibrinolytic enzymes **Nattokinase** (*Subtilisin NAT* 27.7 kDa), **Streptokinase** (47 kDa), **Urokinase** (34 kDa), **Metalloprotease from *Armilaria mellea*** (18.5 kDa). The presence of fractions with a molecular mass as close as possible to the molecular masses of the fibrinolytic enzymes indicates the potential for developing a technology for the production of a biologically active product using the studied extract.

5.5.2. Determination of the content of cordycepin and adenosine derivatives.

A comparative assessment of the content of cordycepin and adenosine derivatives in extracts obtained from laboratory-grown fresh mycelium versus industrially cultivated mycelium of the higher medicinal mushrooms ***Ganoderma lucidum***, ***Cordyceps sinensis*** and ***Cordyceps militaris*** was made. For the cultivation of ***Cordyceps militaris***, two technological methods have been applied - industrial and optimized.

HPLC analysis was performed to determine the content of cordycepin and adenosine derivatives. The results of the study are presented in **figures 29, 30, 31** and in **table 17**.

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                                           Inj Volume: 10.000 µl
Acq. Method     : C:\Chem32\1\Methods\Cordycepin
Last changed    : 1/29/2021 10:36:46 AM by SYSTEM
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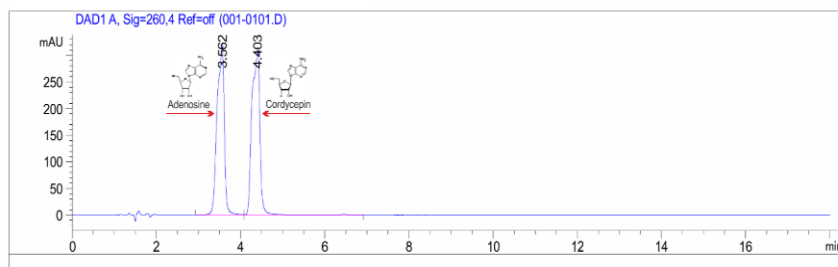


Figure 29. Chromatogram from an HPLC analysis of **Adenosin** and **Cordicepin** standards

Data File C:\Chem32\1\Data\001-0101.D

Sample Name: Cordyceps Militaris

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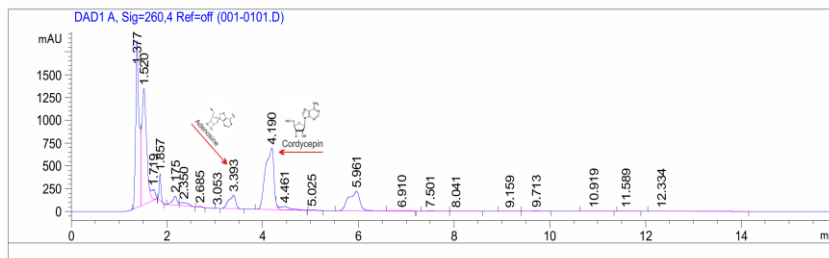


Figure 30. Chromatogram from an HPLC study of *Cordyceps militaris*, industrially cultivated according to a standard method

Data File C:\Chem32\1\Data\001-0101.D

Sample Name: Cordyceps Militaris Sample-optimisation

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Acq. Instrument : HPLC 1260                  Location  :    1
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Last changed    : 1/29/2021 11:13:57 AM by SYSTEM
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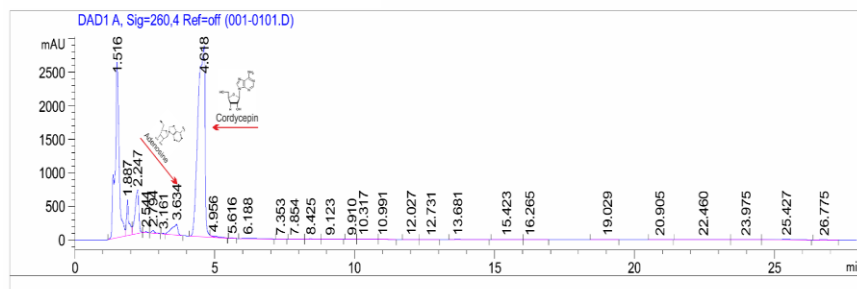


Fig. 31. Chromatogram from an HPLC study of *Cordyceps militaris* extract, industrially cultivated according to an optimized method

Table 17. Content of cordycepin and adenosine derivatives in extracts obtained from laboratory (fresh mycelium) and industrial cultivation of the higher medicinal mushrooms *Ganoderma lucidum*, *Cordyceps sinensis* and *Cordyceps militaris*.

	GLM Industrial	CSM Industrial	CMM Industrial	CMM Optimized Industrial	CM Fresh	CS Fresh	GL Fresh	GNJ Fresh
Adenosins Dry%	0,3	0,59	0,4	0,64	0,09	0,12	0,1	0,05
Adenosins Dry c[mg/g]	29,94	59,14	40,35	64,22	8,46	12,16	10,54	4,52
Cordycepsins Dry%	0,09	0,28	0,32	2,22	0,08	0,05	0,1	0,11
Cordycepsins Dry c[mg/g]	9,01	28,3	32,25	221,69	7,83	4,88	9,72	10,79

GLM (Industrial) - *Ganoderma lucidum* Mycelium industrially cultivated, CSM - *Cordyceps sinensis* Mycelium industrially cultivated, CMM (Industrial) - *Cordyceps militaris* Mycelium industrially cultivated, CMM (Optimized Industrial) - industrially cultivated by an optimized method; CMFresh - fresh mycelium from *Cordyceps militaris*, CSFresh - fresh mycelium from *Cordyceps sinensis*, GLFresh - fresh mycelium from *Ganoderma lucidum*, GNJFresh - fresh mycelium from *Ganoderma neo-japonicum*

The results of the chromatographic studies show a clear tendency to increase the content of *Cordycepin* and *Adenosin* in the sample of *Cordyceps militaris* cultivated in a nutrient medium with an optimized composition, with the presence of *Cordycepin* being 2.22% and *Adenosin* being 0.64%, while in the sample from *Cordyceps militaris* cultivated in medium with a standard composition is respectively for *Cordycepin* 0.32% and for *Adenosin* 0.4%. The realized increase is respectively for *Cordycepin* 1.9% and for *Adenosin* 0.24%.

This result shows that the culture medium with the optimized composition in terms of increasing *Cordycepin* content can be successfully applied for laboratory and industrial production in small and large volumes.

In addition, a comparison was made of the data for the content of *Cordycepin* and *Adenosin* in samples of mycelium cultivated for 9 months, compared to samples of fresh mycelium laboratory grown in a liquid medium for a period of 72 hours. The results showed a lower content of the target substances in the samples of laboratory cultivated fresh mycelium compared to the samples of industrially grown mycelium over a period of 9 months. This is an expected result, due to the fact that during the initial phase of cultivation during the first 72 hours, intense enzymatic reactions take place in the mycelial culture leading to the utilization of a greater amount of *Adenosine* and, accordingly, a weaker synthesis of its derivatives, whatever the substance is and *Cordycepin*. This result demonstrates the fact that the use of mycelial culture to produce enzymes is most productive in the interval up to 72 hours.

5.5.3. Determination of total phenolic content.

A spectrophotometric study was conducted to determine the total phenolic content of the mushroom mycelium extracts. Gallic acid was used as a reference standard.

Table 18. Total phenol content (mg/g)

Sample	GAE mg/g	STD $\pm$ mg/g	STD%
CMF (Industry standard)	15,91	0,003	2,12
CMM (Industrial)	16,20	0,012	8,44
CSM (Industrial)	5,525	0,003	7,89
GLM (Industrial)	46,37	0,042	9,43
CMFB (Industrial optimized)	113,66	0,039	3,44
CMFresh	17,01	0,007	4,72
CSFresh	20,19	0,013	7,03
GLFresh	17,22	0,012	7,89
GNJFresh	22,16	0,017	8,14

CMF (Industrial Standard) *Cordyceps militaris* fruit part industrially grown by standard method, **CMM (Industrial)** – *Cordyceps militaris* Mycelium industrially grown, **CSM (Industrial)** – *Cordyceps sinensis* Mycelium industrially grown, **GLM (Industrial)** – *Ganoderma lucidum* Mycelium industrially grown, **CMFB (Industrial optimized)** *Cordyceps militaris* fruit part industrially grown according to an optimized method, **CMFresh** - fresh mycelium from *Cordyceps militaris*, **CSFresh** - fresh mycelium from *Cordyceps sinensis*, **GLFresh** - fresh mycelium from *Ganoderma lucidum*, **GNJFresh** - fresh mycelium from *Ganoderma neo-japonicum*

The results of the study (**table 18**) show that the highest content of phenolic substances is reported in the extract of the fruit part of *Cordyceps militaris*, cultivated industrially by an optimized method 113.66 mg/g. Compared to the result obtained from the extract of the fruit part of *Cordyceps militaris*, grown industrially by a standard method 15.91 mg/g, a significant increase of the phenolic substances contained by 7.14 times was observed, which is probably due to the applied optimization in the cultivation method .

In the samples of fresh micelles separated after the fermentation process, good values for the presence of phenolic substances are reported, but this could be due to unused substances from this group found in the nutrient medium.

5.5.4. Antioxidant activity in mushroom mycelium extracts.

Table 19 presents the result of a spectrophotometric study to determine antioxidant activity in mushroom mycelium extracts. To calculate the obtained results, a calibration curve was developed using the standard substance tocopherol neutralizing the **DPPH•** radical. Results are presented as tocopherol antioxidant equivalent capacity (**TEAC**).

Table 19. Spectrophotometric determination of antioxidant capacity of medicinal mushroom extracts.

Sample	TEAC mg/g	STD $\pm$ mg/g	STD %
CMF (Industry standard)	0,71	0,042	4,71
CMM (Industrial)	0,67	0,003	0,33
CSM (Industrial)	0,50	0,008	1,19
GLM (Industrial)	1,18	0,062	4,77
CMFB (Industrial optimized)	1,85	0,019	1,02
CMFresh	0,65	0,018	3,24
CSFresh	0,10	0,021	5,70
GLFresh	0,09	0,02	5,47
GNJFresh	0,15	0,005	1,19

CMF (Industrial Standard) *Cordyceps militaris* fruit part industrially grown by standard method, **CMM (Industrial)** – *Cordyceps militaris* Mycelium industrially grown, **CSM (Industrial)** – *Cordyceps sinensis* Mycelium industrially grown, **GLM (Industrial)** – *Ganoderma lucidum* Mycelium industrially grown, **CMFB (Industrial optimized)** *Cordyceps militaris* fruit part industrially grown according to an optimized method, **CMFresh** - fresh mycelium from *Cordyceps militaris*, **CSFresh** - fresh mycelium from *Cordyceps sinensis*, **GLFresh** - fresh mycelium from *Ganoderma lucidum*, **GNJFresh** - fresh mycelium from *Ganoderma neo-japonicum*

The results obtained with the extract of *Cordyceps militaris* cultivated in industrial conditions by the optimized method demonstrate a higher antioxidant activity against the **DPPH•** radical, which correlates with the results obtained for the total phenolic content. Also, the difference between the samples of the fresh mycelium and the other samples of mushroom cultures stands out clearly. This is probably due to the fact that in the initial stage of cultivation, the accumulation of substances with antioxidant activity is weak, and it is more likely that at this stage enzymes involved in the metabolism of mushrooms are present in the mycelial cultures. This allows to select a target model for cultivation according to the need to obtain a biologically active extract. In cases where the presence of phenolic compounds with high antioxidant activity is required, the optimized cultivation method should be applied until the stage of obtaining fruiting bodies, and in cases where it is necessary to obtain a biologically active extract with high enzyme activity, the preparation method should be applied of fresh mycelial culture.

5.5.5. Determination of protein content

The protein content was determined by measuring the total nitrogen content in samples of lyophilized fresh mycelium from the medicinal mushrooms *Ganoderma neo-japonicum*, *Ganoderma lucidum*, *Cordyceps sinensis*, *Cordyceps militaris* according to the standard **Kjeldahl** method. The results of the analysis are presented in **Table 20**.

Table 20. Results for determination of protein content by the *Kjeldahl* method of samples of lyophilized fresh mycelium from medicinal mushrooms *Ganoderma neo-japonicum*, *Ganoderma lucidum*, *Cordyceps sinensis*, *Cordyceps militaris*.

Sample	Protein content, %
Ganoderma neo-japonicum	16,97
Ganoderma lucidum	16,23
Cordyceps sinensis	20,11
Cordyceps militaris	22,04

6. Creation of a technology for obtaining a functional bioproduct "FibrinoZIME".

6.1. Determination of recipe composition - qualitative and quantitative selection of the constituent components of the product

- Based on the preliminary research on different types of cultivation substrates and obtained extracts from higher medicinal mushrooms, the types and quantitative ratio between the selected biologically active substances that will be included in the composition of the new product have been determined.
- The bioformula of the new bioproduct complies with the requirements for physiological activity, harmlessness and microbial stability. It is a multi-component functional bioproduct. Its composition includes sources of building and energy elements, as well as physiologically active substances of various origins:
- Fermented fresh mycelium from 4 medicinal mushrooms: *Ganoderma neo-japonicum*, *Ganoderma lucidum*, *Cordyceps sinensis*, *Cordyceps militaris*. The raw material obtained is rich in enzymes with a proteolytic action, falling within the range of enzymes with a pronounced fibrinolytic action of low molecular mass (in the range of 7 - 40 kDa) produced during the growth of fresh mycelium, as well as of medium and high molecular mass (in the range of 40 – 100 kDa) produced during the fermentation process caused by *Bacillus Subtilis*.
- *Ganoderma lucidum* mycelium extract (industrially grown for a period of 9 months) – the raw material provides a rich complex of biologically active substances such as β -glucans, triterpenes, sterols, etc., which ensure improvement of the work of the immune defense, circulatory system, heart activity and the nervous system. β -glucans are the most commonly consumed immunomodulator due to the fact that they increase the body's resistance to bacterial and fungal pathogens and accordingly reduce the strength and frequency of colds and respiratory infections. β -glucans have great potential in the treatment of diabetes, reducing blood sugar levels, regulating high blood pressure and helping with allergies.
- Extract from the fruiting body of *Cordyceps militaris* - the raw material has a proven high content of *Cordycepin*, providing a biologically active

effect for the human body, such as improving microcirculation in the capillary network, increasing the energy of cells, improving protection against viruses, etc.

- Natto extract – is a raw material obtained from fermented soybean sprouts by *Bacillus Subtillis*, providing a high content of fibrinolytic enzymes with the common name Nattokinase with a molecular mass in the range of 20-30 kDa.
- Extract from *Bombyx mori* L (intestine) – the raw material provides the fibrinolytic enzyme Serratia E-15 protease with a molecular mass of 52 kDa to complement the general fibrinolytic complex and regulate the final enzyme activity of the received dosage form.
- Carica Papaya L. extract – the raw material provides the enzyme Papain, which has a proven physiological effect for tissue restructuring in humans, providing a restorative effect of the extracellular matrix when using the product and regulating the final enzyme activity of the received dosage form.
- Fructooligosaccharides (FOS) – these are low-calorie, non-digestible carbohydrates that are used as a food additive. Fructooligosaccharides improve the taste of food, its durability and density, and at the same time have a beneficial effect on the immune system, bone health and the balance of important bacteria in the gastrointestinal system. These compounds refer to prebiotics, as they actively stimulate the reproduction of the gastrointestinal microbiota in the large intestine - beneficial microorganisms that support the absorption of nutrients and are important for normal digestion. Included in the composition of the tablet form, they improve the absorption of biologically active substances.
- Zn-Citrate – is a zinc salt of citric acid. This biologically active form provides an efficient delivery of the element Zn, its ionic form being released when the element has already passed into the blood. The released Zn-ion acts as a cofactor for the activation of fibrinolytic enzymes.
- Mg-bis-Glycinate – is a complex salt of the amino acid Glycine ensuring complete absorption of the element Mg through the small intestine on the way of protein absorption. The released Mg-ion acts as a cofactor for the activation of all proteolytic enzymes.
- PVP (polyvinylpyrrolidone) is a polymer that is safe for human health and is not absorbed by the body. It is used in the composition as a binding agent.
- Microcrystalline cellulose – provides a cross-linked structure to the tablet form and protects it from premature destruction.
- Mg-Stearate – is a salt of stearic acid providing fluidity of the microgranulated mixture in the production process.
- SiO₂ – is an anti-caking agent improving the production process.

The selection of the component composition is consistent with the need to obtain a product with a balanced composition and high biological value.

As part of the project program, a formulation of the **FibrinoZIME** product was prepared and sample quantities of it were prepared.

The component composition in quantitative ratio is presented in **table 21**

Table21. Component and quantitative composition of a functional product **FibrinoZIME**.

Component	Quantity, g	Content, %
Fermented fresh mycelium from 4 types of medicinal mushrooms	100	10
Ganoderma lucidum mycelium extract	50	5
Cordyceps militaris fruiting body extract	50	5
Natto extract	400	40
Bombyx mori L. extract (intestine)	300	30
Milk extract of Carica Papaya L.	50	5
Fructooligosaccharides	80	8
Zn-Citrate	10	1
Mg-bis-Glycinate	10	5
PVP (polyvinylpyrrolidone)	10	1
Microcrystalline cellulose	30	3
Mg-Stearate	10	1
SiO₂	0,001	0,0001
Total:	1000,001	100

The starting raw materials for the new product are lyophilized, which guarantees high quality and long-term storage.

6.2. Lyophilization of components of the product composition.

For the successful implementation of the lyophilization process, technological factors are particularly important: freezing temperature, post-drying temperature, packaging and storage conditions. These indicators were tracked through the relevant stages of freeze drying.

Lyophilization took place in three phases:

- First phase – Freezing – extract from the mycelium cultivated in liquid and solid nutrient media from 4 types of higher fungi and the fruiting body of *Cordyceps militaris* are placed in special trays for freeze-drying, with a layer thickness of 5 to 12 mm and are frozen in a chamber with forced convection of the air environment at temperatures from -30°C to -37°C for 12-15 hours;

The freezing process takes place in three successive stages: 1) cooling to the cryoscopic temperature of the product surface; 2) freezing to the cryoscopic

temperature in the center of the product; 3) freezing the product to the set temperature in the thermal center or the set average volume temperature.

- Second phase - Sublimation - when developing the drying regime, the peculiarities of the product composition, the properties of the main components and the nature of their changes in the dehydration process must be taken into account. The temperature regime of the sublimation drying is programmed to obtain an optimal drying speed that ensures good quality of the final product. Before drying, the plates of the installation are cooled to temperatures of -40°C to -55°C. The desublimator of the installation is minimally cooled to a temperature of -55°C to -60°C, which already in the initial period ensures a sufficiently low partial pressure of the water vapor above the desublimator and creates favorable conditions for mass transfer. The process takes place with the removal of water at -22°/-25°C under deep vacuum;
- Third phase – Desorption (further drying), by heating under deep vacuum, at positive temperatures +28 to + 31°C.

The correct determination of the temperature and duration of drying ensures the production of products with a minimum residual moisture content in the range of 2.25 - 4.7%.

It is known from general theoretical statements that the amount of heat supplied to the material during drying should not be greater than the amount of heat required for the sublimation of the ice, because otherwise protein denaturation occurs due to an increase in the concentration of electrolytes.

A summary of the results of the technological tests related to the complete cycle of freeze-drying of mycelium and fruiting body from higher fungi is presented in **table 22**.

Table 22. Parameters of the freeze-drying process of mycelium and fruiting body of higher fungi

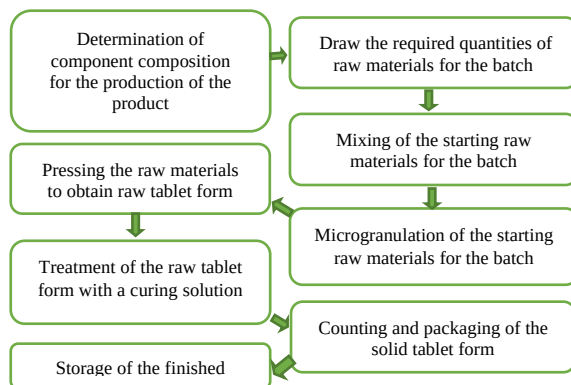
Parameter s/ raw material	Mycelium (stage I) Liquid nutrient medium				Mycelium (stage II) Solid nutrient medium				Fruiting body Cordyceps militaris	
	<i>Gano derma lucidu m</i>	<i>Gano derma neo- Japon icum</i>	<i>Cordy ceps Sinen sis</i>	<i>Cordy ceps Milita ris</i>	<i>Ganod erma lucidu m</i>	<i>Gano derma neo- Japon icum</i>	<i>Cor dyce ps Sine nsis</i>	<i>Cordyceps Militaris</i>	med ium IM 1	med ium IM 2
I. Layer thickness / mm	10-12	10-12	10-12	10-12	8-10	8-10	8-10	8-10	5	5
II. Freezing										
1. Freezing temperature / °C	-30	-32	-35	-37	-30	-33	-32	-32	-30	-32
2. Eutectic temperature / °C	-35	-38	-38	-39	-39	-40	-38	-40	-38	-37
III. Sublimation drying										
1. Load bearing surface / kg/m ²	9,67	9,02	8,98	9,40	10,02	11,01	9,12	10,2	9,81	10,0
2. Drying temperature / °C	-22 to - 25	-22 to -25	-22 to -25	-22 to -25	-22 to - 25	-22 to -25	-22 to -25	-22 to -25	-22 to -25	-22 to -25
3. Desublimato r temperature / °C	-55 to -60	-55 to -60	-55 to -60	-55 to -60	-55 to - 60	-55 to -60	-55 to -60	-55 to -60	-55 to -60	-50 to -60
4. Chamber pressure / Pa	20 to 25	20 to 25	20 to 25	20 to 25	20 to 25	20 to 25	20 to 25	20 to 25	20 to 25	20 to 25,
5. Post- drying temperature / °C	30	30	31	30	28	29	28	28	30	30
6. Duration of the process / h	21	20	19,5	20,5	14	14,5	13,5	14	20,5	21,0
7. Residual moisture content / %	3,51	4,02	4,7	3,97	2,25	2,40	3,10	2,97	3,2	3,8

6.3. Preparation of a technological scheme for obtaining a solid dosage form - a tablet of lyophilized functional bioproduct *FibrinoZIME*

The functional bioproduct *FibrinoZIME* is produced in a solid dosage form (tablet) according to the following technological algorithm (**Fig. 32**):

1. Determining the number of tablets used for a single dose, as well as the amount of the dose (single or daily) contained in one package. Calculations are made based on the activity required for a single intake of the respective ingredient, represented as a % of the total content, and based on it, the required amount of the component for the respective batch is calculated.
2. Measuring the amount of the individual components of the product - it is done on a working scale with an accuracy of 1g, after which they are placed in a clean container equipped with a lid or in a clean polymer bag.
3. The mixing and homogenization of the individual ingredients of the product is done in a blender or drum with cleaned surfaces.
4. Microgranulation is carried out in a granulator or drum using an alcoholic granulation solution.
5. Pressing is carried out on a tablet press providing a pressure of 15 kN/sm²,
6. When setting up the press, the weight of the tablet is checked by measuring 10 tablets on a laboratory scale with an accuracy of 0.01 g and the hardness of the tablet is checked by a hardness tester at the moment of breaking.
7. The curing of the tablet form is done with a curing solution that does not change the quality of the tablet.
8. Packaging and labeling - the finished product is packed in packages containing the intended doses of the product, and a desiccator containing 0.5-1 g of pearl silica gel (SiO₂) is placed in each individual package, after which it is labeled and, if necessary, packaged in a transport package.

Figure 32. Block diagram of the algorithm for preparing solid tablet dosage form of functional bioproduct "FibrinoZIME".



VI. MAIN CONCLUSIONS

1. It was established that when cultivating the higher medicinal mushrooms *Ganoderma lucidum*, *Ganoderma neo-japonicum*, *Cordyceps sinensis* and *Cordyceps Militaris* in a liquid nutrient medium, the best results in mycelial growth were achieved in the enriched **M2** media (with added carbon source glucose, vitamin B1, KH_2PO_4 and K_2HPO_4), followed by media **M4** and **M3**, **M1**, and the lowest growth was observed in the control medium **M0**.
2. It was found that the mycelial growth when cultivating the higher medicinal mushrooms *Ganoderma lucidum*, *Ganoderma neo-japonicum*, *Cordyceps sinensis* and *Cordyceps Militaris* on solid nutrient medium was observed between the 30th and 90th day as the best result and for the four mushroom species, it was reported in nutrient medium **S2** (with added carbon source rice).
3. An increase in the number of viable cells was achieved in the cultivation of *Bacillus subtilis strain NBIMCC 2353* by adding powdered extracts of *Cordyceps sinensis* and *Ganoderma lucidum* to the composition of the nutrient medium. In the variant samples with 2 g of extracts introduced, by the 48th hour, the number of cells increased by almost 4.5 log units, and the amount of dead microbial cells by the 72nd hour was small. This is especially clearly expressed in the nutrient medium with added 2 g of *Ganoderma lucidum* extract.
4. An increase in the total proteolytic activity of the medium for the cultivation of *Bacillus Subtilis NBIMCC 2353* was achieved by adding native bovine collagen protein and mycelium from *Ganoderma lucidum*, *Ganoderma neo-japonicum*, *Cordyceps sinensis*, *Cordyceps militaris*. The highest total proteolytic enzyme activity was found at 48 h after inoculation, with variants with added fungal substrate having higher proteolytic activity than the variant with added collagen.
5. An increase in the chitinase enzyme activity of a culture medium for *Bacillus Subtilis NBIMCC 2353* was achieved by adding non-hydrolyzed fragments of living mycelium of the four types of medicinal mushrooms *Ganoderma lucidum*, *Ganoderma neo-japonicum*, *Cordyceps sinensis*, *Cordyceps militaris* and chitin from shrimp shells. The highest chitinase enzyme activity was found at 48 hours, being higher in the media supplemented with fresh mushroom mycelium compared to the variant supplemented with shrimp chitin and the control medium.
6. Biologically active extracts from mycelial cultures were obtained by the action of an enzyme complex of *laccase*, *lipase*, *alpha amylase* and *cellulase* and by fermentation with living cells of *Bacillus Subtilis NBIMCC 2353*. Degradation of the cell wall was detected by microscopic observation, and chromatographic peaks were reported for *Glucosamine*

fractions, which is further evidence of degradation of the chitin layer from the cell wall of the fungal cells.

7. Protein fractions with molecular masses ranging from 3.7 to 69.2 kDa were detected in the protein spectrum of samples of the medicinal mushrooms ***Ganoderma neo-japonicum***, ***Ganoderma lucidum***, ***Cordyceps sinensis*** and fruiting body of ***Cordyceps militaris*** and culture fluid of ***Bacillus subtilis* NBIMCC 2353**, which are as close as possible to the molecular masses of fibrinolytic enzymes.
8. An increase in ***Cordycepin*** content by 1.9% and ***Adenosine*** by 0.24% was found in industrially cultivated *Cordyceps militaris* in a nutrient medium with an optimized composition compared to a nutrient medium with a standard composition.
9. The presence of phenolic substances was experimentally established in the samples of fresh micelles separated after the fermentation process. The highest phenolic content of 113.66 mg/g was reported in ***Cordyceps militaris*** fruiting body extract cultivated industrially by an optimized method, which was 7.14 times higher than the amount in ***Cordyceps militaris*** fruiting body extract, grown industrially by a standard method.
10. Antioxidant capacity of extracts from ***Ganoderma neo-japonicum***, ***Ganoderma lucidum***, ***Cordyceps sinensis*** and ***Cordyceps militaris*** was determined. The highest antioxidant activity was demonstrated for ***Cordyceps militaris*** extract - TEAC 1.85 mg/g, cultivated in industrial conditions using the optimized method. The difference between the samples of the fresh mycelium, which have lower values compared to the other samples of mushroom cultures, clearly stands out.

VII. CONTRIBUTIONS

1. Scientific contributions

1. Technological modes of cultivation of the higher medicinal mushrooms ***Ganoderma lucidum***, ***Ganoderma neo-japonicum***, ***Cordyceps sinensis*** and ***Cordyceps Militaris*** in enriched nutrient media have been established.
2. Component compositions of nutrient media for the cultivation of ***Bacillus subtilis* strain NBIMCC 2353** were formulated with the aim of increasing the number of viable cells and increasing the total proteolytic and chitinase activity.
3. The presence of biologically active components (***adenosine*** derivatives (***cordycepin***), peptides with different molecular mass, phenolic profile and antioxidant activity) in extracts from mycelium of ***Ganoderma neo-japonicum***, ***Ganoderma lucidum***, ***Cordyceps sinensis*** and fruiting body of ***Cordyceps*** has been experimentally proven ***militaris***.
4. The main parameters of the sublimation drying process of mycelium from ***Ganoderma neo-japonicum***, ***Ganoderma lucidum***, ***Cordyceps sinensis***

and fruiting body from *Cordyceps militaris*, as components of the composition of the functional bioproduct "*FibrinoZIME*", have been established.

2. Scientific and applied contributions

1. A method was developed for enzymatic digestion of raw material of mushroom and plant origin to obtain a water-based extract with a high content of biologically active substances, by using a complex of microbially produced enzymes (*laccase*, *lipase*, *amylase*, *cellulase* and *chitinase*) for hydrolyzing the biopolymers.
2. The possibility of using the producer strain *Bacillus Subtilis* as a producer of a complex of enzymes for hydrolyzing biopolymers has been proven. Optimum conditions for enzymatic digestion have been achieved in which the fermentation process can be carried out for an interval of 12 to 48 hours, and thus optimize the technology for the production of various raw materials.
3. A method was developed to cultivate the producer strain *Bacillus subtilis* strain **NBIMCC 2353** in order to increase the number of viable cells in a shorter period of time, by optimizing the composition of the nutrient medium with the addition of an industrially obtained extract of *Ganoderma lucidum* and *Cordyceps sinensis* with a high content (40 %) of β -glucans.
4. A method was developed for producing fresh mycelium from higher medicinal mushrooms (*Ganoderma lucidum*, *Ganoderma neo-japonicum*, *Cordyceps sinensis* and *Cordyceps militaris*) in a liquid nutrient medium in order to obtain a high content of biologically active peptides with enzymatic action. A fresh spherical mycelium was obtained which could be easily separated from the liquid phase of the culture medium, which improved the speed of the production cycle. By adding native (non-hydrolyzed fragments – biopolymers, including specific proteins, chitin, etc.) to the composition of the nutrient medium, the cultivation process is successfully directed towards the production of peptides with targeted enzymatic action (general proteolytic, fibrinolytic, cellulase hydrolyzing, chitinase hydrolyzing).
5. An analytical method was developed to determine the growth of the fresh mycelium, in which the ratio of the volume fractions occupied by the spheres of the fresh mycelium to the remaining part of the total volume occupied by the culture liquid is used. ($V_m = V_{p-p} - V_{liquid}$), V_m - volume of the micelle spheres, V_{p-p} - total volume of the solution together with the micelle, V_{liquid} - volume of the decanted culture medium.
6. A method was developed for the production of mycelia from higher medicinal mushrooms (*Ganoderma lucidum*, *Ganoderma neo-japonicum*, *Cordyceps sinensis* and *Cordyceps militaris*) on a solid nutrient medium

enriched with rice in order to obtain a high content of biologically active polysaccharides (**β -glucans**) . In this method, the time for deep cultivation of the mycelial culture in order to increase the polysaccharides is reduced from 270 days (in standard industrial approaches) to 90 days.

7. A semi-industrial method for the production of mycelium and fruiting body from higher medicinal mushrooms (***Cordyceps sinensis*** and ***Cordyceps militaris***) on a solid nutrient medium was experimentally applied, and the content of ***Cordycepin***, ***Adenosin*** and its derivatives in the extract was increased 5.25 times compared to the standard production methods.
8. A prescription composition was formulated and a technology was developed for the production of a functional product - "***FibrinoZIME***" with a physiological focus on the degradation of fibrin and amyloid protein in order to clear it from the body and reduce the pathogenic effects caused by the accumulation of plaques in the tissues.
9. For the first time in the new functional product - "***FibrinoZIME***" the combined action of various enzymes with fibrinolytic action, differing in molecular mass (from 10 to 110 kDa) was used, which ensures an increase in the biologically active action due to the ability of the enzymes peptides to pass through all biological barriers in the human body and thus participate in the recovery process in a large number of socially significant diseases related to the formation of plaques in the tissues.
10. The method of lyophilization of the starting raw materials for the new product was applied, which guarantees high quality and long-term storage.

Applied Contributions

1. A technological scheme has been developed for the production of the fruiting body and extract of ***Cordyceps militaris*** with an increased content of ***Cordycepine***.
2. The semi-industrial production parameters of *Cordyceps militaris* under real conditions in a medicinal mushroom farm with extract production plant located in Jinhua City, China were established.

IX. DISSERTATION RELATED PUBLICATIONS

Scientific publications in publications that are referenced and indexed in world-renowned databases of scientific information.

1. **Solakov, N.**, 2018. Optimization of the method for the extraction of biologically active substances from fresh vegetable raw materials by enzymatic hydrolysis and cryogenic concentration, *Journal of Mountain Agriculture on the Balkans*, Vol.21, №. 6, pp.68-80, ref.5, ISSN 1311-0489 (Print).
2. **Solakov Nikolay**, Milena Kostova, Kamelia Loginovska, Zlati Markov, Ana Caroline de Oliveira, and Yordan Muhovski. 2022. "Investigation of

Adenosine Precursors and Biologically Active Peptides in Cultured Fresh Mycelium of Wild Medicinal Mushrooms, " *Applied Sciences* 12, no. 20: 10618. <https://doi.org/10.3390/app122010618>

Научни публикации в нереферирани списания с научно рецензирани или редактирани колективни томове.

1. **Solakov N., A. Valchkov**, 2020. Optimization of the process of lyophilization of microbial culture of *Bacillus subtilis* NBIMCC 2353 by ultrasound. *International Journal of Development Research*, Vol. 10, Issue, 11, pp. 42163-42166, November, 2020 ISSN-L2230-9926.

X. PARTICIPATION IN SCIENTIFIC CONFERENCES

1. Participation in the 21st scientific conference with international participation "EcoMountain-2018" RIMSA-Troyan.
2. Participation in a National scientific conference with international participation "90 years of creativity, growth, development in agricultural science", October 18-19, 2018, BIASES - town of Smolyan.
3. Scientific conference for students and young scientists with international participation "45 years of higher education - Stara Zagora" on 05.09 - 05.11.2019 Thrace University, Stara Zagora, Faculty of Engineering and Technologies, Yambol.
4. Scientific conference with international participation "Livestock science - challenges and innovations" - IAS - Kostinbrod, held online on 05.11.2020.

XI. PARTICIPATION IN SCIENTIFIC PROJECTS

1. **SSA - Project No. TN 10**: "Obtaining fermented lyophilized products based on bioactive protein hydrolysates", 2021-2023.
2. **SSA - HTAI Project - No. 146** "Obtaining healthy foods based on biologically active plant and animal components", 2018-2020.
 - **Task 1**: "Creation of a functional colostrum-based bioproduct by biotechnological route"
 - **Task 2**: "Development of technology for obtaining lactic acid drinks and soft cheeses from milk whey"
3. **SSA - HTAI Project - TN_2** "Alternative biotechnological approaches to improve the protection and productivity of agricultural crops", 2019-2022.
4. **SSA - Project G 155** "Conservation and use of genetic resources in cattle breeding, sheep breeding and goat breeding for the production of milk and meat in the mountainous regions of Bulgaria", 2019-2021.
5. **FNI, MES - Project No. KP-OB-M26/7** "Cryogenic method with ultra-lyophilization to preserve the biological activity of extracts from microbial sources", 2018-2020.

6. **FNI, MES - Project No. KP - 06 PN39/15** "Development and improvement of new approaches for qualitative and quantitative evaluation of radiation treatment based on EPR spectroscopy", 2019-2021.
7. **MES, National Scientific Program "Healthy foods for a strong bioeconomy and quality of life", 2019-2022.**
 - **Component 2:** Plant health and safety in food systems
 - **RP 2.3.** Topic: "Alternative approaches using biostimulants to stabilize yields and production with high biological value in leguminous crops"
 - **Component 3:** Food quality for a better quality of life
 - **RP 3.1.** Topic: "Innovative technology for the production of lyophilized symbiotic concentrates"

XII. TRAINING AND AWARDS

1. **Certificate** for successfully completed scientific and practical course on internal audit for HACCP system on topics: "HACCP system - development and implementation; internal audit of food safety management systems; general principles and implementation of EU legislation in the area of ensuring food traceability;; labeling, energy value of foods", organized by the Food Industry Union and held in the House of Technology, Sofia on 2-4.09.2019.
2. **Diploma** in Innovation Competition, Scientific Activity and Development Section for "Biologically Active Enzyme Product **FibrinoZIME**" from the International Agricultural Exhibition Agra 2020.
3. **Certificate No. 01/20.06.2022.** for a scientific product accepted by the Permanent Commission for Innovations and Technologies at the SSA for "Functional lyophilized product **Fucostem**".