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APPLICATION OF CRYOBIOTECHNOLOGIES FOR DEVELOPMENT OF LYOPHILIZED POLYENZYME COMPLEXES

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Abstract

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Good nutrition is key factor for human health. Conditions of secondary insufficiency of the gastric gland and a decreased production of digestive enzymes lead to changes in the gastric-intestinal metabolism, which imposes the intake polyenzyme products as food supplement. The created on the basis of freeze-drying complex polyenzyme product with its composition, containing the main groups digestive enzymes (chymosin, α – amylase, bromelain, lipase), incorporated in a hydrocolloid matrix and in combination with plant biologically active components is appropriate for prophylaxis in cases of gastric-intestinal tract discomfort and disturbed digestion. The obtained product was qualified by organoleptic, biochemical, physical-chemical and microbiological characteristics. The retaining of the catalytic activity of the enzyme substances and the chemical composition of the incorporated biologically active substances has been established.

Key words: polyenzyme product, freeze-drying, digestion

Introduction

The contemporary way of living (nutrition, influence of the environment, stress, chronic diseases, alcohol, smoking, drugs intake) is the main reason for the physiological and pathologic changes in the digestive system and respectively the disturbance of the gastric-intestinal metabolism. The typical modern menu consists predominantly of heat treated foods and contains too much fats, starch and dairy products. This forces the organism to synthesize its own digestive enzymes in unusually big quantities which can lead to decrease of its capacity to produce metabolite enzymes and to a general worsening of health, faster ageing and degeneration. The infiltration of fats and fibrous tissue in the pancreas with ageing leads to malfunction of its exocrine activity and decrease of the digestive enzymes production. In case of missing or decreased enzyme secretion of the of the pancreas a substitute therapy with pancreatic enzymes as medicinal means is applied while in the case of secondary or relative insufficiency of the pancreas the intake if polyenzyme products as a food additive is recommendable (Layer and Keller, 2003; Shamburek and Farrar, 1990). The enzyme bioproducts are widely applied in cases of chronic degenerative conditions of the digestive system, of enzyme insufficiency, of irregular, disturbed or unbalanced nutrition with more fats, canned foods, of conditions with a feeling of fatigue after eating, o digestion problems (heaviness, swelling, pains, gases) and others (Gullo et al., 1986).

As commercial products the enzyme preparations should be catalytically stable with preserved initial characteristics and sufficiently long shelf life.

The objective of the study was by applying a cryobiotechnological approach to create a new lyophilized polyenzyme product, containing at the same time digestive enzymes and biologically active substances. During lyophilization (freeze-drying) two way of preservation are combined – freezing and drying under vacuum. The essence of the process is the detraction of the water substance from the hard matrix of the, moisture-containing materials through sublimation in vacuum. Because of the high quality of the end product – preserving of its

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biological properties, increased structural stability, many times decreased weight and long shelf life, lyophilization is a widely applied practice in the production of enzyme biopreparations (Day and Stacey, 2007; Diankova et al., 2002).

Materials and Methods

The following materials and methods were used in the course of the investigation:

Enzymes – Microbiological chymosin produced from *Mucor miehei* (DSM), Fromase, 500 U/g EC 3.4.23.4.; Fungal α-amylase produced from *Aspergillus oryzae* („Biovet" Peshtera), "Amilosin P" 2000 U/g, EC 3.2.1.1.; Lipase produced by *Rhizopus arrhizus* („Biovet" Peshtera), "Lipase" 500 U/g EC 3.1.1.3.; Plant protease produced from *Ananas comosus* (Merck) "Bromelain" 2 mAnson U/mg., EC 3.4.22.32.

Polysaccharidehydrocolloids-Na-carboxymethylcellulose (Na-CMC) (Fence Ltd); Guar gum (SNG India private Ltd.).

Other -Vitamin E – DL- α – tocopherol acetate, Fluka; Garden sage *(Salvia officinalis* L.*)*, Drug-store network; Black bilberry (*Vaccinium myrtillus* L.*),* Drug-store network.

Physical-chemical

Thin layer chromatographic analysis (TLC). For the extract from black bilberry fruits: movable phase - mixture of ethanol: acetone: form: water; immovable phase - silicagel, plate Kisegel 60 (Merck). For the garden sage extract: movable phase - mixture of chloroform; benzol; immovable phase – silicagel, plate Kisegel 60 (Merck); development - reactive vanillin-sulphuric acid. As standards were used thujone and thymol (Merck).

Biochemical

Determining of the amiolytic activity **(**Ashly et al., 2011). Determining of the proteolytic activity: modified method if Anson with casein substrate (http://www.sigmaaldrich.com**).** Determining of the lipolytic activity: using olive oil substrate (Veeraragaran, 1990).

Microbiological

Microbiological analysis for sterility of the end products – fluid thioglycollate medium and soybean casein digest medium. Determining of the microbial number, CFU/g (depth method of Koch).

Results and Discussion

Based on preliminary carried out cryogenic investigations, related to the establishment of cryo- and cryoprotective effect of some hydrocolloid types under different regimes of cryogenic treatment end freeze-drying of protease and amylase enzymes a technological scheme for the production of a new lyophilized polyenzyme product has been developed. The complete technological process of production unites five main stages in the following sequence: Stage I - preparatory (obtaining of plant extracts and preparation of the hydrocolloid matrices); Stage II – incorporation of the enzymes in the hydrocolloid matrix; Stage III – freeze-drying; Stage IV – mincing of the lyophilized material and mixing with the filler and vitamin E; Stage V –Dosing and filling in the gelatin capsules.

The created on the base of the new technology complex enzyme products contains digestive enzymes (chymosin, α – amylase, bromelain and lipase) plant biologically active components and vitamin E.

In the biotechnological process for obtaining of the product as a matrix for incorporation of the enzyme molecules were used the hydrocolloids of carbohydrate type Na – carboxymethylcellulose and guar gum.

The mechanical method for incorporation of enzyme molecules in a gel of hydrocolloid carrier was applied. This method allows keeping the maximum that enzymes stability in the process of freeze-drying.

The freeze-drying was carried out in a vacuum sublimation installation of the company -"Hochvakuum-TG –16.50" with contact plates heating.

On Figure 1 are presented the data, recorded by the measuring sensors, following the course of the lyophilization process.

From technological point of view, the freeze-drying process included several consequent stages:

- Reaching of the designated regime parameters, fast freezing in a freeze-drying chamber up to -50°C (Figure 1A);
- Vacuum processing to deep vacuum – 2.10^{l} Pa (Figure 1B);
- Primary drying at negative temperatures – the ice crystals sublime under the effect of initially intensive and then moderate heating under high vacuum conditions. During that period 90-95% of the total moisture is separated (Figure 1C);
- Secondary drying – desorption of the residual moisture at positive temperatures and under high vacuum conditions (Figure 1D);
- End of the process – at reaching of the designated residual moisture the vacuum is broken and the product is cooled and packed (Figure 1E).

After lyophilization the components were minced with the aim to equalize the dimension of the particles, after which they were mixed with vitamin E and the preliminary prepared excipient. The so prepared material was dozed and put in gastro-resistant gelatin capsules.

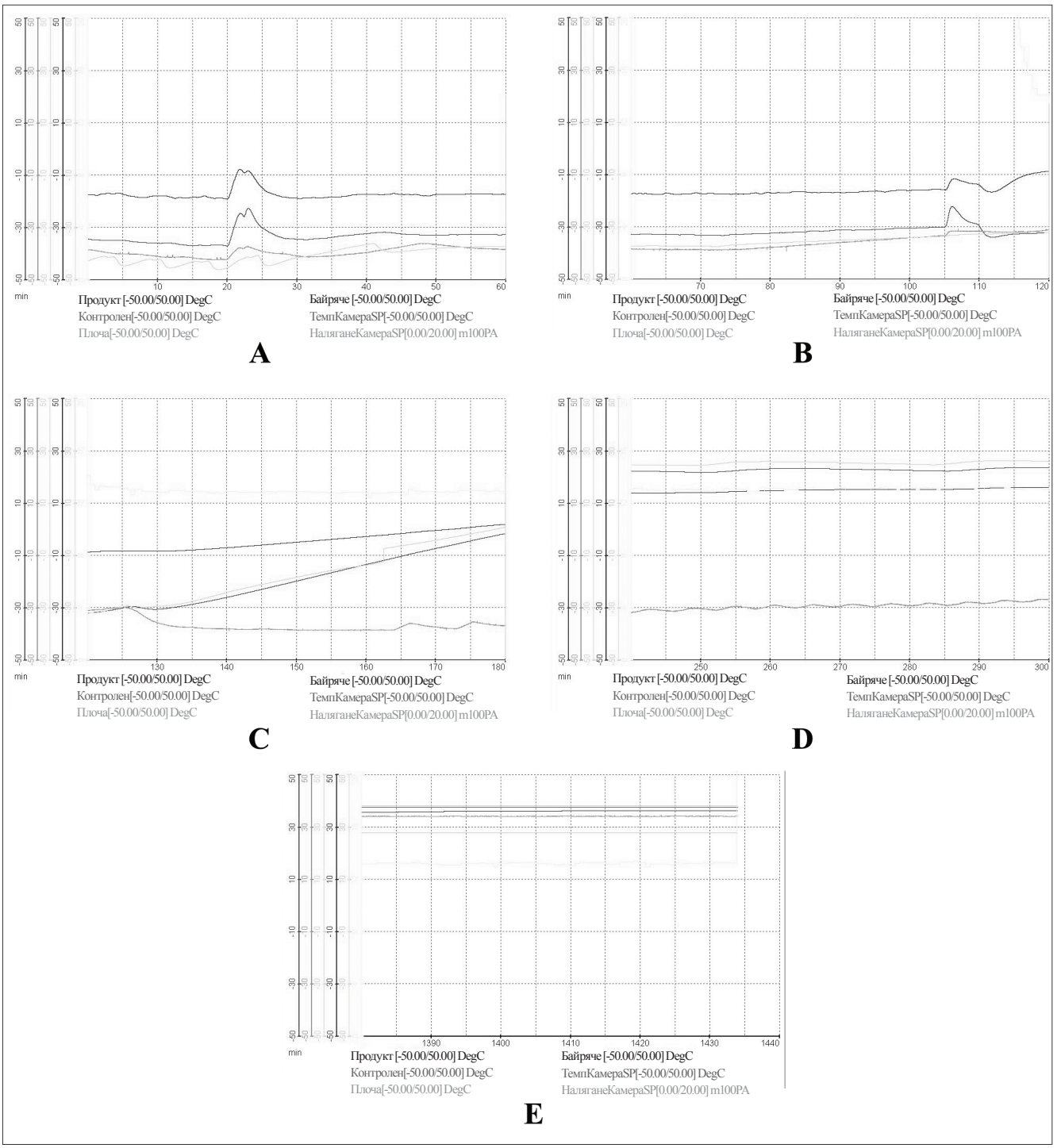


Fig. 1. Record from the automatic controls during the process of lyophilization A-beginning of the process –freezing in a freeze-drying chamber; B- vacuum processing to deep vacuum 2.10^{1} Pa; C – freeze-drying at negative temperatures; D – secondary drying – desorption of the residual moisture at positive temperatures and higher vacuum; E – end of the process – breaking of the vacuum, reaching of the limit final residual moisture

The obtained bioproduct was characterized by organoleptic, biochemical, physical-chemical and microbiological indices.

Organoleptically the product is characterized by homogenous powder-like consistency, cream to light pink color, taste and aroma specific for the composition.

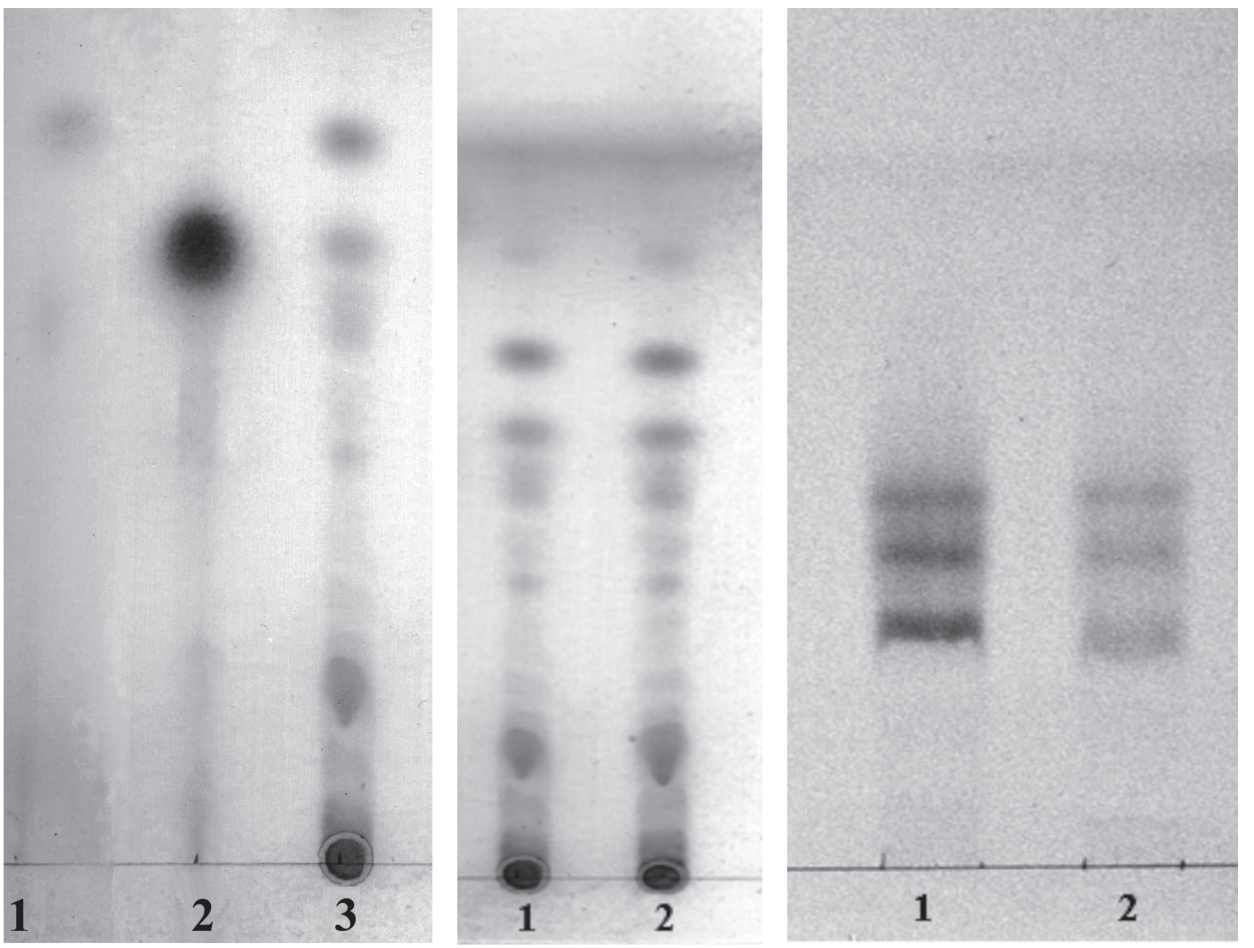
The obtained lyophilized polyenzyme preparation was with low residual moisture content up to 6%, which corresponds to the preliminary programmed values for this index. This fact proves the reliability of the applied freeze-drying technological process and the optimum chosen parameter for its running.

Every capsule of the product contains enzymes with minimum activity: amylase - 100 U/g; protease - 20 U/g; lipase – 200 U/g.

By TLC analysis was investigated the identity of the biologically active plant extracts included in the product.

For the garden tea extract the main active components are terpenes. On Figure 2A1 are presented the results from the TLC-analysis of the extract compared to standards of thymol and thujone and on Figure 2A2 the results from the TLC-analysis before and after the incorporation in the lyophilized polyenzyme product/. Spots were recorded which correspond to the terpene compounds, from both standards. An almost full coincidence between the compositions of the two extracts was also established.

The extract from the fruits of black bilberry contains anthocianidines, which are aglycones of the anthocyanins to which is due the violet color. The main anthocyanins char-



A1 A2 B

Fig. 2. Results from TLC for investigation for identity:
A1: 1-thymol; 2- thujone; 3- garden sage extract A2 garden sage extract; B – black bilberry extract

Table 1
Microbiological characteristics of the product

Microbiological characteristics	Norm	Established result
Total number CFU/g	10 3 CFU/g	Not traced
Pathogenic coli	not to be isolated	not isolated
Salmonella sp./10g	not to be isolated	not isolated
Staphylococcus aureus / 0.1 g	not to be isolated	not isolated
Candida albicans	not to be isolated	not isolated
Fungi and yeast	not to be isolated	not isolated

acteristic for the plant are derivatives of the delphinidin /delphinidin-3-glucoside, delphinidin-5-glucoside/.

On Figure 2B are presented the results from the TLC-analysis of the two extracts. An almost full coincidence between the composition of the extract before and after its incorporation in the lyophilized polyenzyme product was established.

This gives a reason to conclude that the plant extracts included in the obtained polyenzyme product are with preserved chemical composition.

The new complex enzyme product was analyzed for microbial purity. The results are shown in Table 1.

The presented results show that the product doesn't contain pathogenic microflora and meets the standard requirements for microbial purity. The absence of seedling with pathogenic microorganisms proves that the complete technological process was realized in accordance with the sanitary norms and requirements.

Conclusion

The new lyophilized product is a complex polyenzyme preparation with additionally included plant biologically active components and vitamin E. With its enzyme composition (chymosin, α – amylase, bromelain and lipase) is appropriate for prophylaxis in cases of gastric-intestinal tract discomfort and disturbed digestion, especially after intake of an abundant quantity of food rich in fats and proteins. The applied biotechnological approach – incorporation of the enzymes in a hydrocolloid matrix, high speed freezing and a subsequent freeze-drying is appropriate for producing of lyophilized polyenzyme complexes. The reliability of the used cryobiotechnological methods in relation to the microbial purity, the catalytic activity and the structural stability in the production of lyophilized enzyme bioproducts has been proved.

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