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## BIOTECHNOLOGY OF OBTAINING A HYDROLYTIC ENZYMATIC AGENT WITH $\alpha$ -D- GALACTOSIDASE ACTIVITY

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**Resume.** The studies referred to in this article showed the principle possibility of use of hydrolytic enzymatic agent with  $\alpha$ -D-galactosidase activity for the enzymatic hydrolysis of soy oligosaccharides. Optimal parameters of the enzyme activity were determined (pH, temperature). It was shown that the use of various techniques of purification and concentrating of the enzyme preparation allows increasing of the activity of the agent 5.5 times. Enzyme activity was determined.

**Keywords:** hydrolytic enzymatic agent with  $\alpha$ -D-galactosidase activity, soya олигосахариды, enzyme activity, enzymatic agent Galactolongin G10x.

## БІОТЕХНОЛОГІЯ ОТРИМАННЯ ГІДРОЛІТИЧНОГО ФЕРМЕНТНОГО ПРЕПАРАТУ З $\alpha$ -D- ГАЛАКТОЗИДАЗНОЮ АКТИВНІСТЮ

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**Анотація.** Дослідженнями цієї роботи показано принципову можливість застосування гідролітичного ферментного препарату з  $\alpha$ -D-галактозидазною активністю для ферментативного гідролізу соєвих олигосахаридів, встановлено оптимальні параметри дії ферменту (рН, температура). Показано, що використання різних методів очищення і концентрації ферментного препарату дозволяє збільшити активність препарату в 5,5 разів. Встановлена активність ферменту.

**Ключові слова:** гідролітичний ферментний препарат з  $\alpha$ -D-галактозидазною активністю, соєві олигосахариди, активність ферменту, ферментний препарат Галактолонгін Г10х.

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### Introduction. The problem formulation

In the recent years, the development of the science of nutrition resulted in origin of a qualitatively new food production method based on the use of non-traditional raw material for the production of mass and treatment and preventive nutrition products.

There is sufficient evidence that consumption of soy products has a positive impact on human health [1].

Currently, soya products take a particular place in the nutrition structure, and new technologies using enzymes are developed. Carbohydrases ( $\alpha$ -D-galactosidase, EC 3.2.1.) which carry out the hydrolysis of oligo- and polysaccharides take an important place among the hydrolases which are used in this field.

### Literature review

The literature state that oligo- and polysaccharides having  $\alpha$ -1,6-glycosidic bonds, such as raffinose, stachyose, melibiose, galactobiose, manninotriose and others are the natural substrate for  $\alpha$ -D-galactosidase. Enzymatic hydrolysis is used for galactooligosaccharide cleavage. A few publications on the use of  $\alpha$ -D-galactosidase from plants and microorganisms to degrade oligosaccharides which are present in soy products are available [2].

Alpha-galactosidase is considered to be a highly specific enzyme. The ability to hydrolyze any substrate with  $\alpha$ -1,6-glycosidic bonds is an obvious feature of  $\alpha$ -D-galactosidases [3]. Alpha-galactosidase (3.2.1.22) which belongs to the glycosidases is the most prominent representative of this class of enzymes. The enzyme provides hydrolysis of  $\alpha$ -1,6-D-glycosidic bonds by cleavage of galactose residues starting from the non-reducing end of galactooligosaccharides, wherein the bond between C<sub>1</sub> of a galactose residues atom and a glycosidic hydrogen atom is cleaved [4].

### The main part

The objective of the study was the biotechnology of obtaining a hydrolytic enzymatic agent with  $\alpha$ -D-galactosidase activity.

The following tasks were solved to obtain the above objective: the deep cultivation of *Bifidobacterium longum*; purification and obtaining a hydrolytic enzymatic agent with  $\alpha$ -D-galactosidase activity; study of the conditions of enzymatic hydrolysis of soya galactooligosaccharides using  $\alpha$ -D-galactosidase.

The subject of the study was a hydrolytic enzymatic agent with  $\alpha$ -D-galactosidase activity.

Methods of study. The findings of the previous studies showed a possibility in principle to use a hydrolytic enzymatic agent with  $\alpha$ -D-galactosidase activity for enzymatic hydrolysis of soya galactooligosaccharides. Bifidobacteria which are an active producer of  $\alpha$ -galactosidases can be used as starter cultures, together with lactic acid bacteria in fermenting of soya products, as well as sources of  $\alpha$ -galactosidase enzyme [5].

Pure cultures of *Bifidobacterium longum*, *Bifidobacterium adolescentis*, *Bifidobacterium bifidum* from the collection of microorganisms of the Department of nutrition biochemistry, microbiology and physiology of Odessa National Academy of Food Technologies were used in the study.

The results of accumulation of biomass of the studied bifidobacteria cultures on various nutrient mediums are shown in Fig. 1-4.

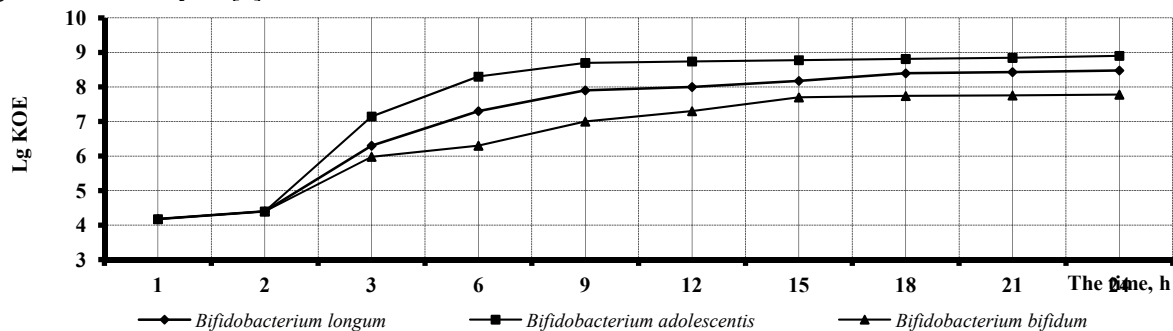


Fig. 1. The bifidobacterias growth dynamics on the corn-lactose medium

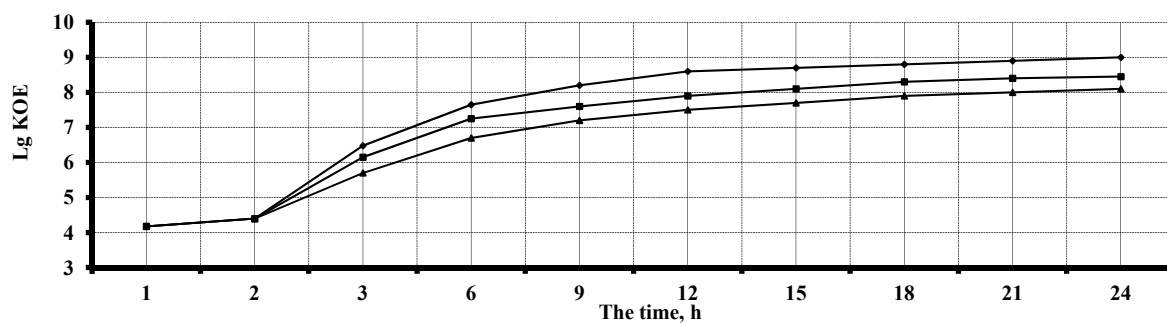


Fig. 2. The bifidobacterias growth dynamics on the Blaurock medium

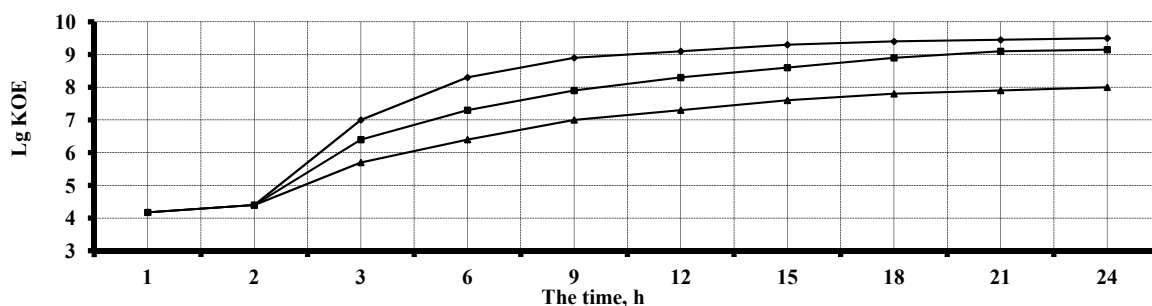


Fig. 3. The bifidobacterias growth dynamics on the MRS medium

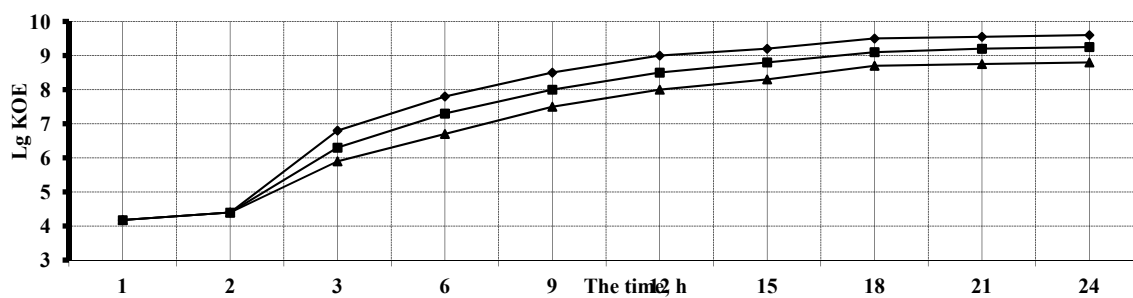


Fig. 4. The bifidobacterias growth dynamics on the soy serum medium

Bifidobacteria cultivation on the selected media showed practically similar culture growth rate with insignificant shortening of the logarithmic phase for MRS medium and higher technological parameters of

*B. longum* on MRS medium and on the soya serum. Therefore, *B. longum* culture was used in further studies to obtain  $\alpha$ -D-galactosidase.

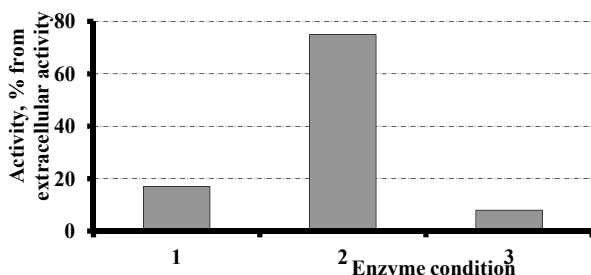
**Table 1 – The indicators of bifidobacteria cultivation on various growing medium (n = 5, p = 0,045)**

Indicators	The corn-lactose medium			The Blaurock medium			The MRS medium			The soy serum medium		
	<i>B. longum</i>	<i>B. adolescentis</i>	<i>B. bifidum</i>	<i>B. longum</i>	<i>B. adolescentis</i>	<i>B. bifidum</i>	<i>B. longum</i>	<i>B. adolescentis</i>	<i>B. bifidum</i>	<i>B. longum</i>	<i>B. adolescentis</i>	<i>B. bifidum</i>
$\mu$ , час <sup>-1</sup>	0,61	0,59	0,3	0,54	0,37	0,3	0,65	0,65	0,5	0,73	0,42	0,3
Lg KOE	7,3	8,3	6,3	7,65	7,25	6,7	7,8	7,3	6,7	8,3	7,3	6,4

Assessment of biotechnological parameters of cultivation (Table 1) shows that cell count and the specific growth rate are maximal for *B. longum* on MRS medium and on the soya serum.

Isolation of  $\alpha$ -galactosidases and especially their purification is quite a complicated task, since  $\alpha$ -galactosidases often form complexes with pigments, ballast proteins, carbohydrates and other structural components of the microbial cell. Alpha-galactosidases are water-soluble proteins able to get adsorbed on various non-soluble substrates of cytoplasmic structures of bifidobacteria cells [6]. Data on characteristics of localization of  $\alpha$ -galactosidases *B. longum* and determination of ratio of extracellular, intracellular and adsorbed enzyme forms are required to extract  $\alpha$ -galactosidase (Fig. 5). The presented results show that  $\alpha$ -galactosidases is an intracellular enzyme, therefore the enzyme was further obtained by cultivation of *B. longum* on MRS medium with following cell disintegration within 10 min. Maximum activity was 8.5–9.0 U/mg.

To obtain a cell-free extract, raw biomass was suspended in 0.07 M phosphate buffer (pH 7.4) containing 5 mMol 2-mercaptoethanol, then cells were mechanically destroyed in the disintegrator within 10 min, and the homogenate was centrifuged at 30000 min<sup>-1</sup> within 30 min. The obtained supernatant liquid with the solid content of 5.17±0.11 % was used as a cell-free extract.



**Fig. 5. The ratio of extracellular, intracellular and adsorbed  $\alpha$ -galactosidase**

The obtained culture liquid filtrate is a liquid system containing a large amount of water-soluble colloidal compounds and substances in addition to enzymes. Isolation of enzymes from this complicated system is a challenging task, because the system is very sensitive to external interference, therefore ensuring a high yield of the enzyme in the precipitate requires compliance with the conditions under which start protein molecules begin to aggregate and precipitate.

Hydrophobic protein groups are known to tend to concentrate inside a protein molecule, yet many of them are on the surface of the molecule. Protein solubility in various solvents is largely determined by the distribution of hydrophobic and hydrophilic residues on the surface of the molecule. Since water is the principal protein solvent, certain environment of the protein molecule can be created by altering certain properties of the water, adding various salts, organic solvents, etc. which would result in aggregation of the protein molecules and their precipitation. Enzyme precipitation with organic solvents is the most common purification technique. The protein precipitation effect is related to the fact that the water activity decreases in the presence of an organic solvent, which results in solvation of charged hydrophilic molecules of enzyme proteins. The water molecules are displaced from the surface of the protein molecule and substituted with the organic solvent molecules. At the same time, the solubility of protein decreases and are protein molecules are aggregated and precipitated [7].

The effect of the nature of 3 organic solvents (acetone, ethanol, isopropanol) and their concentration on the isolation of  $\alpha$ -galactosidases from the cultural liquid was studied.

According to the provided results, organic solvents added to the cultural liquid at 2:1 ratio increase the specific enzyme activity from 7.8 U/mg to 13.8 U/mg protein, reaching the purification degree of 1.3–2.3 times.

Using solvents ensure preservation of activity by 80–85 %. pH value of the medium has great importance at enzyme precipitation. The best conditions for isolation of

$\alpha$ -galactosidase irrespective of the solvent nature were recorded at pH 5.8 – 6.1 since the enzymes precipitate most completely in the isoelectric point.

**Table 2 – Results of precipitation  $\alpha$ - galactosidase by organic solvents**

The volume of solvent on the 1 volume of culture liquid		Activity, % from initial precipitate	Specific activity, U/mg protein in the precipitate	The degree of purification, times
The initial culture medium (pH 6.0)		100	6,0	1,0
Acetone	1,0	21,7	4,8	0,8
	1,5	29,9	9,6	1,6
	2,0	71,2	11,4	1,9
	2,5	80,3	13,2	2,2
	3,0	78,2	12,0	2,0
Isopropanol	1,0	28,3	6,0	1,0
	1,5	64,4	8,4	1,4
	2,0	80,2	13,8	2,3
	2,5	83,1	13,2	2,2
	3,0	74,8	12,0	2,0
Ethanol	1,0	25,4	5,4	0,9
	1,5	38,8	6,6	1,1
	2,0	40,9	7,8	1,3
	2,5	60,7	9,7	1,6
	3,0	86,3	13,8	2,3
	3,5	88,5	15,6	2,6
	4,0	84,1	13,2	2,2

The obtained results show that when enzyme was precipitated with ethanol at pH 5, the enzyme activity was about 90 % of the initial activity vs. other organic precipitators. Further increase of pH values does not result in any increase of the enzyme activity.

Enzymes and other proteins are able to adsorb onto the various insoluble compounds. Adsorptive techniques, in particular column chromatography allow to obtain high purity enzymes with high yield of the final product. Complex preparations of alcohol precipitated enzymes of  $\alpha$ -galactosidases of *B. longum* are complex mixtures consisting of target and related enzymes, ballast substances and pigments. The purification degree increases significantly when the gel filtration of the target enzyme is used.

The presented results (Table 3) show that due to the use of ethanol precipitation and ammonium sulfate desalting, the specific activity increases from 6.0 to 82.8 U/mg protein, while the purification degree is 13.8. Alpha-galactosidase yield at such conditions is 40.1 %.

When gel-chromatographic separation on columns with sephadex G-25 and G-100 was used, the specific activity was increased up to 134 U/mg at the first stage and up to 422.9 % at the second stage. Molecular mass of  $\alpha$ -galactosidase was 112 kDa. Further, purified agent with  $\alpha$ -galactosidase activity was separated using electrophoresis. Multiple types of electrophoresis are known which use different carriers, the most common of which is polyacrylamide gel (PAAG) which is characterized with chemical inertness, stability, absence of adsorption and electroosmosis. PAAG also has functions of molecular sieve which causes the

high resolving power of the technique. PAAG does not absorb UV light at 270 nm, therefore, the location of proteins after separation was determined using light absorbance [8].

The disc electrophoresis was used for the study of  $\alpha$ -galactosidase from *B. longum*. Electropherogram of the chromatographically separated preparation of  $\alpha$ -galactosidase showed a single band in the stained gel which is the evidence of homogeneity of the fraction of the preparation.

The ultrafiltration method was used to ensure a high degree of preserved enzyme activity. The cultural liquid filtrate obtained after disintegration and centrifugation was concentrated using vacuum evaporation to obtain a technical preparation. Concentration was performed using the rotor evaporator bod LUVA at 32 °C.

The enzyme sediment obtained after ethanol precipitation was dried. Drying was aimed at obtaining an enzymatic agent from the cultural liquid which would be stable in storage. The enzyme was dried in the vacuum oven at the parameters as follows: stabilizer concentration 0.06 %;  $t=32$  °C; Post.=125 Pa;  $\tau=10$  h.

At the next stage of the study described herein, the effect of temperature and pH on catalytic activity of  $\alpha$ -D-galactosidase was determined. Alpha-D-galactosidase produced by *B. longum* was determined to show activity within the pH range from 5.0 to 7.5 reaching the peak at pH about 6.0. The temperature range for maximum  $\alpha$ -D-galactosidase activity of the enzyme is quite wide – 33 to 48 °C, however, it is inactivated at higher temperature.

The conditions of enzymatic hydrolysis of soya galactooligosaccharides were studied. An aqueous soya extract containing 4.5 % oligosaccharides (1.2 % raffinose and 3.5 % stachiose) was used as a source of galactooligosaccharides. The fermentative hydrolysis degree was evaluated based on the accumulation of reducing substances (RS).

The separated extract of cultural liquid of *B. longum* grown on MRS medium within 24 hours with the following cell disintegration at the ultrasound disperser UZDN-A was used as an enzymatic agent.

Based on the results of hydrolysis of soya galactooligosaccharides it was determined that when

the enzyme concentration in the reaction medium grows to up to 8 – 9 U/mg, accumulation of RS increases from 3.5 % to 11.8%.

Complete hydrolytic galactooligosaccharide cleavage is recorded in 30 – 40 minutes of fermentolysis.

**Conclusions:** the performed studies allowed developing the biotechnology for obtaining a hydrolytic enzyme agent with  $\alpha$ -D-galactosidase activity. The conditions of enzymatic hydrolysis of soya galactooligosaccharides using  $\alpha$ -D-galactosidase were studied.

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## БИОТЕХНОЛОГИЯ ПОЛУЧЕНИЯ ГИДРОЛИТИЧЕСКОГО ФЕРМЕНТНОГО ПРЕПАРАТА С $\alpha$ -D- ГАЛАКТОЗИДАЗНОЙ АКТИВНОСТЬЮ

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**Аннотация.** Исследованиями данной работы показана принципиальная возможность применения гидролитического ферментного препарата с  $\alpha$ -D-галактозидазной активностью для ферментативного гидролиза соевых олигосахаридов, установлены оптимальные параметры действия фермента (pH, температура). Показано, что использование различных методов очистки и концентрирования ферментного препарата позволяет увеличить активность препарата в 5,5 раз. Была определена активность фермента.

**Ключевые слова:** гидролитический ферментный препарат с  $\alpha$ -D-галактозидазной активностью, соевые олигосахариды, активность фермента, ферментный препарат Галактолонгин Г10х.

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