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EXTRACELLULAR LECTIN PRODUCED BY *BACILLUS SUBTILIS* STRAIN IMV B-7014 DEPENDING ON THE CULTURE CONDITIONS

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The aim. *To develop a new nutrient medium for the biosynthesis of extracellular lectin strain Bacillus subtilis IMB B-7014; to study changes in biomass and pH values, accumulation of protein metabolites in the culture fluid (CF) and in its foamed fraction under separate batch cultivation of the R and S morphotypes of the strain in the developed medium.* ***Methods.*** *Microbiological, biochemical, immunological. The selection of the source of inorganic nitrogen (ammonium citrate, ammonium nitrate, ammonium sulfate, glycine) was performed using the base medium of the following composition, g / l: galactose – 10.0; yeast extract – 2.0; K_2HPO_4 – 0.70; KH_2PO_4 – 0.30; NaCl – 0.50; $MgSO_4 \cdot 7H_2O$ – 0.50; $CaCl_2 \cdot 6H_2O$ – 0.10; $FeSO_4 \cdot 7H_2O$ – 0.005; $CoCl_2 \cdot 6H_2O$ – 0.005; $MnSO_4 \cdot 4H_2O$ – 0.005; pH 7.0. Lectin activity (LA) of the CF of both morphotypes of the strain evaluated by the level of hemagglutination activity of its supernatant with rabbit erythrocytes. The molecular weight of the protein elements was determined in a denaturing system (SDS-PAGE).* ***Results.*** *The highest values of LA of the CF observed using ammonium sulfate. Differences in the dynamics of changes in optical density and pH of the CF during batch cultivation of the R and S morphotypes of the strain not observed, whereas the LA of the R morphotype CF was higher. Major and sub-major protein components with a molecular weight of about 50 and 73 kDa, respectively, found in the CF and in its foamed fraction of both strain morphotypes. A unique component with a molecular weight of 64.5 kDa was present in the foam. A component with a molecular weight of 84 kDa was present on the electrophoregrams of the CF R and S morphotypes in an insignificant amount, but was absent in the foam fraction. A sub-major component with a molecular weight of 43 kDa of a foam fraction found in the CF S and R morphotypes in trace amounts. The foam fraction characterized by a large presence of protein components due to their transition from the CF.* ***Conclusions.*** *The LA of the CF R morphotype was higher than for the S morphotype. The foam fraction contained more protein components than the CF.*

Keywords: Bacillus subtilis, R and S strain morphotype, nitrogen source, extracellular lectin, culture fluid, foam, protein components.

Lectins are a heterogeneous group of proteins and glycoproteins that can bind carbohydrates selectively and inversely without any changes in their chemical structures [1–3]. They are play a leading role in the processes of carbohydrate-protein recognition, present in all biological systems and have a wide range of medical and biological activities. In particular, the study of

extracellular lectins (EL) of saprophytic aerobic bacteria of the genus *Bacillus* was started more than 30 years ago at the Zabolotny Institute of Microbiology and Virology of NAS of Ukraine.

These EL are thermostable metal-independent glycoproteins with molecular weight (MW) in the range of 40–55 kDa, resistance to pH fluctuations of the medium and the detergents' action, and a long-term storage. They are inducers of synthesis of gamma-interferon, active inhibitors of adsorption and reproduction of influenza viruses, herpes, hepatitis C and HIV and selectively act on tumors of various origins [4, 5–9]. Due to these properties, the EL have broad possibilities for their use in various fields of medicine and biology [2, 3, 5–10].

According to the method of obtaining of EL from *Bacillus subtilis* strain IMV B-7014 these bacteria were cultivated in the Gause N2 liquid medium specifically modified for the synthesis of EL by replacing glucose (10 g/L) by galactose using shaking flasks. This medium contain high-value sources of organic nitrogen: Hottinger's tryptic meat digest and peptone [2]. However, the process of growing a producer in a laboratory fermenter is accompanied by a significant loss of foamed culture fluid (CF) caused by the continuous injection of air into its thickness (deep aeration) and is very problematic. The use of some antifoaming agents (defoamers) in the fermentation process gives the opportunity to control the foam level but subsequently prevents EL sedimentation with ammonium sulfate from the cell-free CF. As a result, after centrifugation, it was formed an upper oily layer of defoamer molecules which are tightly linked to the EL.

It is known that some microorganisms are capable of forming different morphological types during the growth. These morphotypes may differ from each other in their biological characteristics as well. This phenomenon is known as polymorphism and is especially common among bacteria [11]. It is believed that the polymorphism is a response of the bacterial population to constant fluctuations of the environment conditions and thus is an important adaptation strategy [12, 13]. Such variations increase in response to the restriction or alteration of bacterial culture conditions: temperature, pH, oxygen level, components of the growth medium can induce changes in the cell and colony morphology [14]. Thus, the phenotypic feature of the strain *B. subtilis* IMV B-7014 during growth in a solid medium is the dissociation on R (rough) and S (smooth) morphotypes. The technology of obtaining of the EL involves the use of the R type morphological variant. Alteration of the medium composition and the conditions of cultivation of this strain leads to the rising of the dissociation and the frequency of the S morphotype formation.

The aim of this study were to develop the composition of the new nutrient medium to reduce the cost of obtaining the final product – the EL of *Bacillus subtilis* strain IMV B-7014 and to study the features of it synthesis and accumulation by different strain morphotypes during their periodic cultivation in a new optimized medium in laboratory fermenters.

Materials and methods. In this work, the saprophytic strain *B. subtilis* IMV B-7014 deposited in Ukrainian collection of microorganisms of IMV NASU was used. This strain was isolated from a gastrointestinal tract of

healthy newborn calve. The inoculum was obtained by growing the strain on the Gromyko (MPA: wort-agar = 1:1) agar medium slopes at 37 °C for 24 hours and was standardized to the optical density of 1×10^8 cells / ml.

The growth intensity and the ability to synthesize the EL by the strain B-7014 were investigated by its cultivation in a new semisynthetic liquid medium containing relatively cheap sources of nitrogen such as ammonium citrate, ammonium nitrate, ammonium sulfate, glycine, diammonium phosphate and urea. The growth medium consists of (g/L of tap water): nitrogen source – 0.5 / 1.0 / 2.0 (by the amount of nitrogen); galactose – 10.0; yeast extract – 2.0; K_2HPO_4 – 0.70; KH_2PO_4 – 0.30; NaCl – 0.50; $MgSO_4 \cdot 7H_2O$ – 0.50; $CaCl_2 \cdot 6H_2O$ – 0.10; $FeSO_4 \cdot 7H_2O$ – 0.005; $CoCl_2 \cdot 6H_2O$ – 0.005; $MnSO_4 \cdot 4H_2O$ – 0.005. The medium was prepared as the separate stock solutions of galactose, potassium dihydrogen phosphate and potassium hydrophosphate. The other components were dissolved in the rest of the water. The solutions were sterilized 30 min at 121°C and combined before using. The pH of the prepared media after sterilization was 7.0 ± 0.1. In all cases the modified Gause N2 liquid medium with galactose [2] served as control. Cultivation of the strain B-7014 was carried out on rotary shakers (160 rpm) in Erlenmeyer flasks (V = 750 ml, a working volume of inoculated liquid medium 100 ml) at 37°C for 24 hours as well as in laboratory fermenters.

The colonies of R and S morphotypes of the producer were obtained during scattering of B-7014 strain on Petri dishes containing Gromyko agar medium. The growth of these morphological morphotypes of the bacteria and the synthesis of EL were investigated under batch conditions at 37 °C for 18–21 hours in two laboratory fermenters Biotec (Sweden) (a total volume is 4.0 L and a work volume is 2.5 L of a medium). The medium was heated to 37 °C and inoculated with 6% (v/v) bacteria suspension grown in the control medium [2] in Erlenmeyer flasks under conditions mentioned above. The cultivation was carried out at the aeration of $0.2\ L \cdot L^{-1} \cdot min^{-1}$ and the stirrer speed 350 rpm (oxygen transfer rate sulfite number – $0.45 \pm 0.02\ g\ O_2 \cdot L^{-1} \cdot h^{-1}$). This regime has been previously developed in order to avoid significant losses of CF to the foam.

Emissions of the foamed CFs of the R and S strain morphotype that occurred during fermentation were collected and accumulated in 5-liter bottles for the further analysis.

The CF was sampled from the fermenters every 3 hours for the pH (I-160M ionometer) and optical density (photocolorimeter KFK-2, λ – 540 nm, l – 3 mm) determination. The intensity of the EL synthesis was estimated by the level of hemagglutinating activity (HAA) of the CF supernatant (2500 g, 15 min) with rabbit and sheep erythrocytes. HAA was determined as the last dilution during which hemagglutination reaction (HAR) was still observed. It was expressed as HAR $titer^{-1}$ or $\log_2$ of HAR $titer^{-1}$, where 1 $\log_2$ $titer^{-1}$ HAR is equal to 2 hemagglutinating units (HAU), 2 $\log_2$ HAR $titer^{-1}$ = 4 HAU, 3 $\log_2$ HAR $titer^{-1}$ = 8 HAU, etc. [15]. The MW of the protein elements in the samples of EL isolated from the CFs of R and S morphotypes of the strain and the foam fraction from the R morphotype was determined by the method of polyacrylamide gel electrophoresis in the presence of SDS (SDS-PAGE).

Results. It was found that the investigated strain most effectively synthesized EL in the presence of ammonium sulfate in the amount of 1.0 g of nitrogen per 1 liter of medium, and the intensity of foam formation in this medium was lower than in the control medium (Fig. 1).

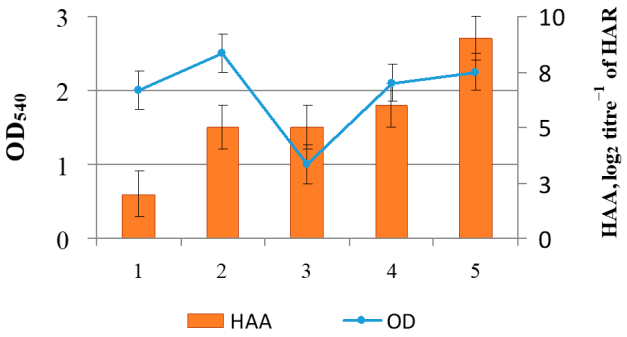

Fig. 1. CF hemagglutinating activity of *B. subtilis* IMV B-7014 on semisynthetic media with different sources of nitrogen (1g N/L): 1 – ammonium nitrate, 2 – ammonium citrate, 3 – glycine, 4 – ammonium sulfate, 5 – modified Gause N2 with galactose (control).

The application of a new growth medium with an optimized aeration regime in the cultivation process allowed to reduce significantly the loss of CF in the foam.

We found that the R and S morphotypes of this strain did not show any differences in the dynamics of biomass accumulation and pH values during their cultivation. At the same time, there was a tendency to increase the HAA level in the supernatant of the R morphotype (Fig. 2).

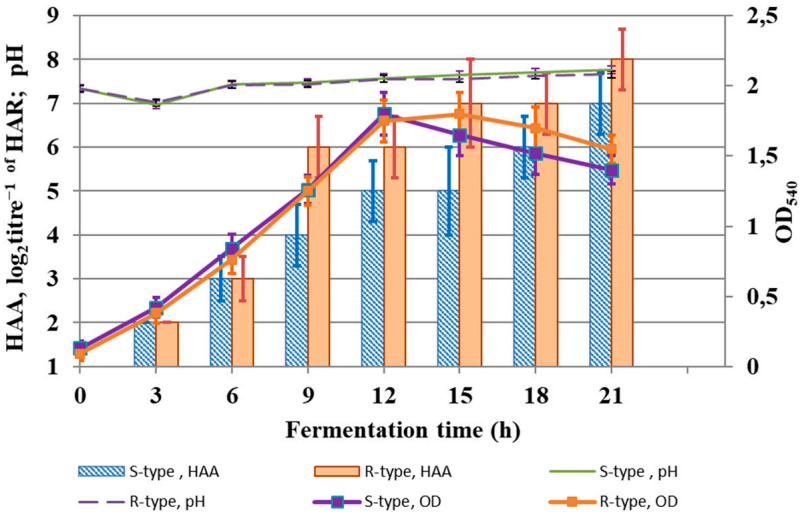

Fig. 2. Dynamics of changes in optical density (OD_{540}), pH and HAA of the CF during the cultivation of R and S morphotypes of *Bacillus subtilis* IMV B-7014 on a semisynthetic medium with ammonium sulfate (1 g/L as nitrogen)

Later, an electrophoretic study of molecular forms in EL samples obtained from the supernatants of the CF of the R and S morphotypes of the strain after their cultivation in laboratory fermenters, as well as from the accumulated emission of the R morphotype foam after it settling was carried out. It was not possible to isolate the EL sample from the settled emissions of the S morphotype foam due to its very limited amount.

Subsequently, it was investigated electrophoretic spectra of EL molecular forms of the *B. subtilis* IMV B-7014 R and S morphotypes and the peculiarities of their distribution between the CF and the foam fractions formed during the cultivation of the producer in the semisynthetic medium in laboratory fermenters (Fig. 3, 4). For comparative analysis of the EL composition were used densitograms obtained from the digital image of gels.

It was detected 10–16 protein bands with MW from 20 to 85 kDa. There were major, sub-major and trace components. The protein bands with MW less than 19 kDa were believed to be a front of protein elution. Only one major component with a molecular weight in the range of 50.5–52.5 kDa was identified on the electrophoregrams of the EL isolated from the CF variants obtained after the submerged cultivation of the S and R morphotypes of the colonies, as well as the R morphotype foam fraction.

The MW of the sub-major component also detected in all EL fractions was about 73 kDa. While a compound with MW of about 43 kDa was found in sub-major quantities in the foam fraction, being present only in trace amounts in S and R morphotypes colonies. In addition, a unique component

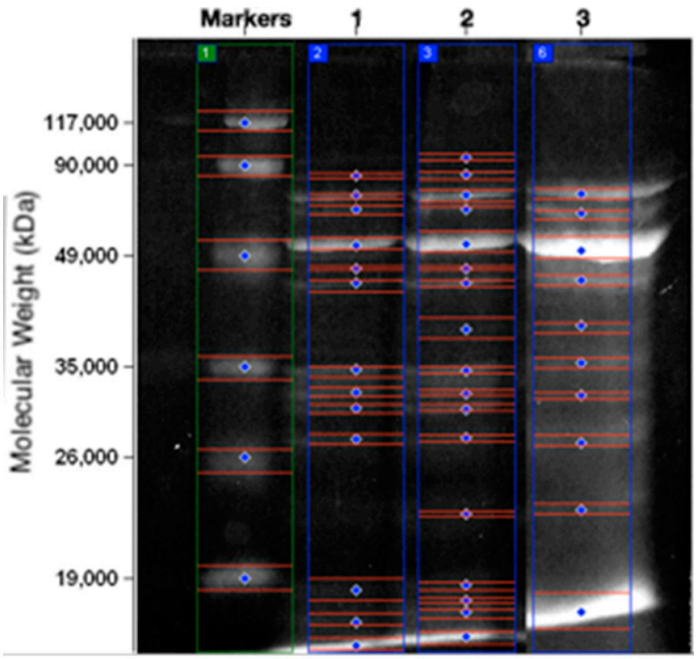

Fig. 3. Molecular forms of extracellular lectin isolated from the CF variants obtained after the submerged cultivation of the R morphotype (1), S morphotype (2) colonies and from the R morphotype CF foamed fraction (3) of *Bacillus subtilis* strain IMV B-7014. Electrophoresis in SDS-PAAG system

(MW 64.5 kDa) was found in the foam electrophoregram which was not detected in the electrophoregram of EL from S and R morphotypes colonies. The trace amounts of a separate component with MW about 28 kDa and a complex with MW 31–35 kDa were also detected on the EL electrophoregrams from all fractions (S, R morphotypes of colonies and the foam). The component with MW about 84 kDa was present on the electrophoregrams of the S and R morphotype colonies in a small amount.

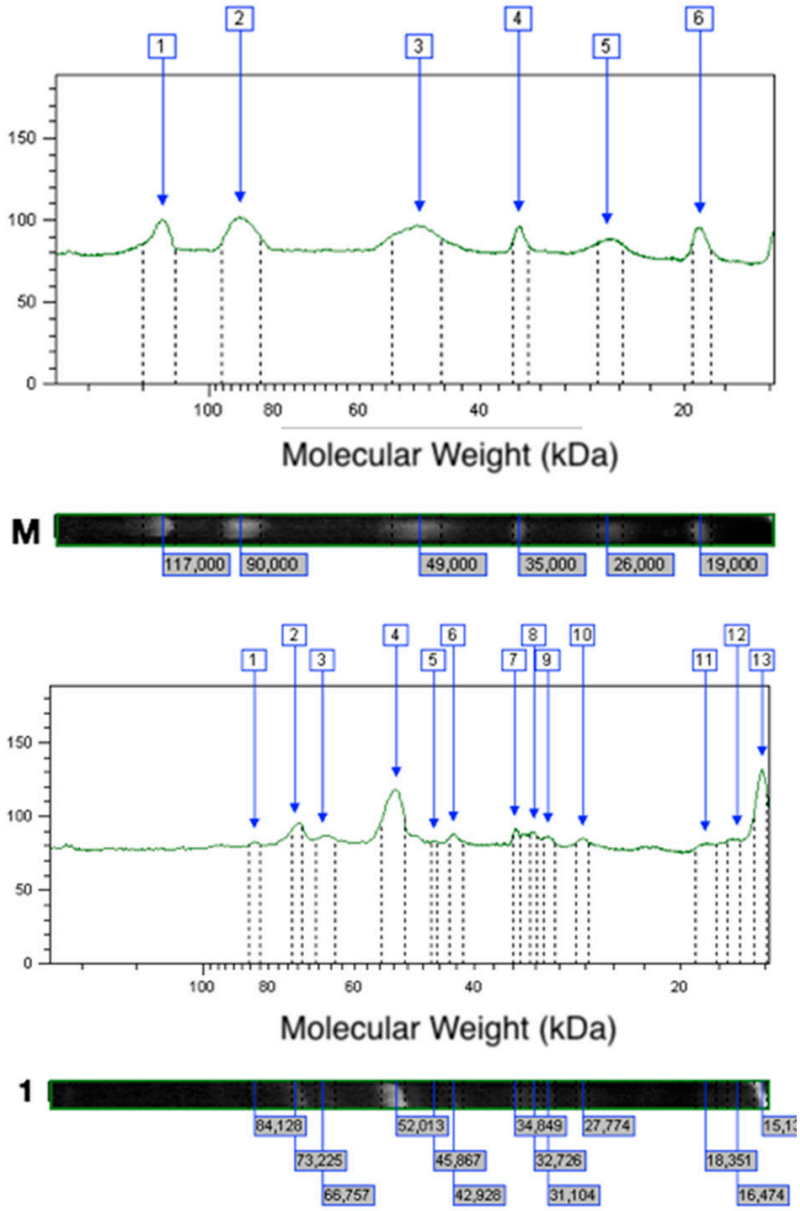

Fig. 4. Legend on the next page 9.

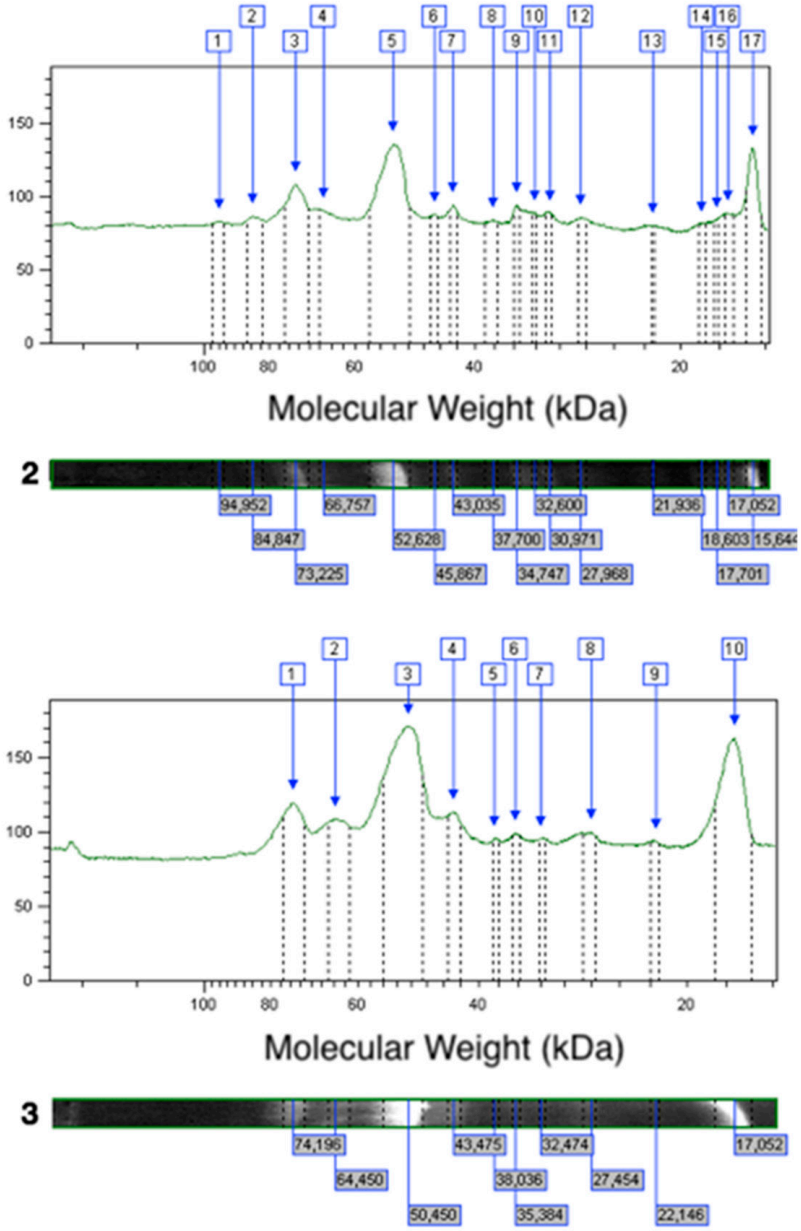

Fig. 4. Densitograms of molecular forms of extracellular lectin from CF of different morphotypes of *Bacillus subtilis* IMV B-7014: M – markers, 1 – R morphotype, 2 – S morphotype and 3 – foam (R morphotype)

Discussion. Thus, a submajor component with MW near 64.5 kDa was detected in the foam fraction while it was absent in CF of S and R morphotypes colonies. This appearance may be explained by transition to the foam and association of low MW components, which might be responsible for the creation of carbohydrate-binding centers of EL. On the contrary a component with MW of 84 kDa was present in the S and R morphotypes colonies but was not registered in the foam fraction. At the same time, the 43 kDa compound was detected in all three variants of EL showing different quantities, being

in small amount in CF of S and R morphotypes colonies and in considerably higher quantity in the foam fraction. It can be assumed that present in the CF of the S and R morphotypes 43 kDa component, possibly aggregating with the formation of 84 kDa compound there, could pass to the foam without the formation of the aggregate reducing the potential of the carbohydrate binding properties of the given EL. Consequently, the disappearance of lectin activity during foaming can be explained by the transition of molecular forms of the EL from the CF to the foam.

Phenomena of polymorphism is well studied for lactic acid bacteria because of their high industrial value. In particular, it was shown for *Lactobacillus farciminis* CNCM I-3699 the presence of colonies of S and R morphotypes in the bacterial population with the ratio depending on the cultivation conditions [16]. The performed enzymatic and molecular genetic studies did not reveal significant or even insignificant differences in these two strain variants, but microscopic studies revealed significant differences in the morphology of R and S types of cells. The subsequent biochemical analysis of capsular polysaccharides explained this difference [14].

The revealed differences in the cell morphology and R and S morphotypes of colonies determine their aggregation and adhesion properties. It was shown the presence of different morphotype-specific surface proteins for *Lactobacillus brevis* ATCC 14869 (Slp): SlpB and SlpD for the R variant and SlpB for the S variant of the strain [17]. The presence of the exopolysaccharide layer on the cell surface was shown for S-type of the strain *L. farciminis* CNCM I-3699. It caused a decrease in aggregation and adhesion properties of the S type colonies compared to the R variant of this strain [16]. Thus, the reduction of the lectin activity in the CF of the S morphotype of *B. subtilis* IMV B-7014 compared to the R morphotype may have a similar explanation.

Thus, the composition of the semisynthetic growth medium containing ammonium sulfate as a nitrogen source for synthesis of the EL by *B. subtilis* IMV B-7014 strain was developed.

The producer cultivation on this medium under optimized aeration and stirring regimes in laboratory fermenters allowed to reduce a loss of foamed CF and EL consequently. It allowed to obtain comparable level of the EL synthesis close to the values obtained on the control Gause medium.

There were no differences in biomass and pH revealed during the growth of R and S morphological types of this strain. The lectin activity in absolute values for the CF of R morphotype bacteria had a tendency to exceed the S morphotype lectin activity. The foam fraction had larger quantity of the major and the minor components. The components with MW about 43 and 65 kDa probably play a key role in the formation of the carbohydrate binding properties of the EL, since in the event of their transition to the foam fraction there is a sharp drop in the level of the hemagglutinating activity of the EL considered here.

УТВОРЕННЯ ПОЗАКЛІТИННОГО ЛЕКТИНУ R- ТА S-ФОРМАМИ ШТАМУ *BACILLUS SUBTILIS* ІМВ В-7014 В ЗАЛЕЖНОСТІ ВІД УМОВ КУЛЬТИВУВАННЯ

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Р е з ю м е

Мета. Розробити нове живильне середовище для біосинтезу позаклітинного лектину штаму *Bacillus subtilis* ІМВ В-7014; вивчити зміни значень біомаси і pH, накопичення білкових метаболітів у культуральній рідині (КР) і в її спіненій фракції при роздільному періодичному культивуванні R і S морфотипів штаму у розробленому середовищі. **Методи.** Мікробіологічні, біохімічні, імунологічні. Підбір джерела неорганічного азоту (цитрат амонію, нітрат амонію, сульфат амонію, гліцин) проводили з використанням базового середовища наступного складу, г/л: галактоза – 10,0; дріжджовий екстракт – 2,0; K_2HPO_4 – 0,70; KH_2PO_4 – 0,30; NaCl – 0,50; $MgSO_4 \cdot 7H_2O$ – 0,50; $CaCl_2 \cdot 6H_2O$ – 0,10; $FeSO_4 \cdot 7H_2O$ – 0,005; $CoCl_2 \cdot 6H_2O$ – 0,005; $MnSO_4 \cdot 4H_2O$ – 0,005; pH 7,0. Лектинову активність (ЛА) КР морфотипів штаму оцінювали за рівнем гемаглютинуючої активності її супернатанту з еритроцитами кроля. Молекулярну масу білкових елементів визначали в денатуруючій системі (SDS-PAGE). **Результати.** Найвищі значення ЛА КР відмічали при використанні сульфату амонію. Відмінностей в динаміці змін оптичної щільності і pH КР при періодичному культивуванні R і S морфотипів штаму не спостерігали, в той же час ЛА КР R морфотипу була вище. Мажорний і субмажорний компоненти з молекулярною масою близько 50 і 73 кДа відповідно виявлені в КР та у її пінній фракції обох морфотипів штаму. У піні був присутній унікальний компонент з молекулярною масою 64,5 кДа. Компонент з молекулярною масою 84 кДа був присутній на електрофореграмах КР S і R морфотипів у незначній кількості, але був відсутній у фракції піни. Субмажорний компонент піни з молекулярною масою 43 кДа був виявлений у КР S і R морфотипів у слідових кількостях. Фракція піни характеризувалась більшою присутністю всіх основних білкових компонентів внаслідок їх переходу з КР. **Висновки**. Значення ЛА КР R форми штаму було вищим, ніж у S форми. Фракція піни містила більше основних білкових компонентів, ніж КР.

Ключові слова: *Bacillus subtilis*, R та S морфотип штаму, джерело азоту, позаклітинний лектин, культуральна рідина, піна, білкові компоненти.

ОБРАЗОВАНИЕ ВНЕКЛЕТОЧНОГО ЛЕКТИНА R- И S-ФОРМАМИ ШТАММА *BACILLUS SUBTILIS* IMB B-7014 В ЗАВИСИМОСТИ ОТ УСЛОВИЙ КУЛЬТИВИРОВАНИЯ

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Резюме

Цель. Разработать новую питательную среду для биосинтеза внеклеточного лектина штамма *Bacillus subtilis* ИМВ В-7014; изучить изменения значений биомассы и pH, накопления белковых метаболитов в культуральной жидкости (КЖ) и в ее вспененной фракции при раздельном периодическом культивировании R и S морфотипов штамма в разработанной среде. **Методы.** Микробиологические, биохимические, иммунологические. Подбор источника неорганического азота (цитрат аммония, нитрат аммония, сульфат аммония, глицин) проводили с использованием базовой среды следующего состава, г/л: галактоза – 10,0; дрожжевой экстракт – 2,0; K_2HPO_4 – 0,70; KH_2PO_4 – 0,30; NaCl – 0,50; $MgSO_4 \cdot 7H_2O$ – 0,50; $CaCl_2 \cdot 6H_2O$ – 0,10; $FeSO_4 \cdot 7H_2O$ – 0,005; $CoCl_2 \cdot 6H_2O$ – 0,005; $MnSO_4 \cdot 4H_2O$ – 0,005; pH 7,0. Лектиновую активность (ЛА) КЖ морфотипов штамма оценивали по уровню гемагглютинирующей активности ее супернатанта с эритроцитами кролика. Молекулярную массу белковых элементов определяли в денатурирующей системе (SDS-PAGE). **Результаты.** Наивысшие значения ЛА КЖ отмечали при использовании сульфата аммония. Различий в динамике изменений оптической плотности и pH КЖ при периодическом культивировании R и S морфотипов штамма не наблюдали, тогда как ЛА КЖ R морфотипа была выше. Мажорный и субмажорный компоненты с молекулярной массой около 50 и 73 кДа соответственно обнаружены в КЖ и в ее пенной фракции обоих морфотипов штамма. В пене присутствовал уникальный компонент с молекулярной массой 64,5 кДа. Компонент с молекулярной массой 84 кДа присутствовал на электрофореграммах КЖ R и S морфотипов в незначительном количестве, но отсутствовал во фракции пены. Субмажорный компонент пены с молекулярной массой 43 кДа выявлен в КЖ S и R морфотипов в следовых количествах. Фракция пены характеризовалась большим присутствием всех основных белковых компонентов вследствие их перехода из КЖ. **Выводы.** Значение ЛА КЖ R морфотипа штамма было выше, чем у S морфотипа. Фракция пены содержала больше основных белковых компонентов, чем КЖ.

Ключевые слова: Bacillus subtilis, R и S морфотип штамма, источник азота, внеклеточный лектин, культуральная жидкость, пена, белковые компоненты.

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