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Bacillus amyloliquefaciens subsp. *plantarum* PROBIOTIC STRAINS AS PROTEASE PRODUCERS

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Proteases from probiotic strains of the genus *Bacillus*, just like the antibiotics, bacteriocins and other hydrolytic enzymes, are one of the main factors that determine their biological activity. The aim of this work was to study the synthesis and biochemical properties of proteases from two strains *Bacillus amyloliquefaciens* subsp. *plantarum* UCM B-5139 and UCM B-5140 that included in the probiotic Endosporin. The cultivation of strains was carried out in flasks under rotating for two days. The influence of physicochemical parameters of the reaction medium on proteolytic activity was studied on partially purified protease preparations. Lytic activity was determined by turbidimetric method. On the second day of cultivation *B. amyloliquefaciens* subsp. *plantarum* UCM B-5139 and UCM B-5140 synthesized the metal-dependent peptidase and serine protease, respectively. The optimum conditions of their action were the following: temperature 37–40 °C and pH 6.5–7.0. Isolated proteases are able to lyse the living cells of *Staphylococcus aureus* and *Candida albicans*. Thus we demonstrated that *B. amyloliquefaciens* subsp. *plantarum* UCM B-5140 and UCM B-5139, included in the probiotic veterinary preparation Endosporin, produced proteolytic enzymes that hydrolyze the native insoluble proteins (elastin, fibrin and collagen). These enzymes belong to the group of neutral metal-dependent and serine proteases. They are active under physiological conditions against gram-positive bacteria and yeasts. The application of these proteases in biotechnology is considered.

Key words: *Bacillus amyloliquefaciens* subsp. *plantarum*, probiotics, proteases, lytic activity.

Bacteria of the genus *Bacillus* belong to large heterogeneous group of gram-positive spore-forming microorganisms that are used in medicine, and in many chemical and industrial processes because of the wide range of their physiological characteristics and the ability to secrete a variety of useful metabolites.

It was proved the efficiency of strains of spore-forming bacteria as probiotic agents to normalize gut microbiota, and to stimulate the immune system cells of the recipient organism [1, 2]. The activity of probiotics on the base of bacilli towards staphylococci, enterococci and yeast can be considered as positive effect compared with probiotics based on bifidobacteria and lactobacilli.

This is determined by antimicrobial activity associated with the production of large amounts of antibiotics (polymyxin, bacitracin, gramicidin C, subtilin, edein, microbacillin et al.), as well as the synthesis of bacteriocins and bacteriolytic enzyme — glycosidases, amidases, proteases. After the pre-clinical tests of two new probiotic preparations Vitasporin and Irilis [3] it was shown that the process of spore germination of *Bacillus subtilis*, *B. cereus* and *B. licheniformis* was accompanied by intensive production of antibiotics, lysozyme, amino acids, vitamins and proteolytic enzymes. It was also established [2] that the combine action of catalase and subtilisin of bacilli can stimulate the growth of bacteria of the genus *Lactobacillus*.

The Institute of Microbiology and Virology of National Academy of Sciences of Ukraine developed the bacillary probiotic Endosporine elaborated for the prevention and treatment of dysbiosis, intestinal infections, purulent wounds and postpartum endometritis in farm animals [4, 5]. This probiotic consists of two strains: *B. amyloliquefaciens* subsp. *plantarum* UCM-5139 and UCM B-5140. These investigated bacilli cultures expressed antagonistic properties against a broad spectrum of gram-positive and gram-negative bacteria, and some types of fungi isolated from different econiches. As it has been found in previous investigations [6] the strains possess elastase, gelatinase and caseinolytic activity and can synthesize the complex of

bacteriolytic and yeast lysing enzymes. Since proteolytic enzymes of probiotic strains, along with antibiotics and bacteriocins are factors that determine their biological activity the aim of this study was the synthesis of proteases by strains *B. amyloliquefaciens* subsp. *plantarum* UCM-5139 and UCM B-5140 and the study of their physical and chemical properties as well as substrate specificity.

Materials and Methods

The object of the study were strains of *Bacillus amyloliquefaciens* subsp. *plantarum* UCM B-5140 and UCM B-5139. Subject of investigation were extracellular peptidases produced by these strains. Strains of *B. amyloliquefaciens* subsp. *plantarum* were grown in a liquid nutrient medium which had the following composition (g/l): KH_2PO_4 — 1.6; $MgSO_4 \cdot 7H_2O$ — 0.75; $ZnSO_4 \cdot 7H_2O$ — 0.25; $(NH_4)_2SO_4$ — 0.5; maltose — 1.0; gelatin — 10.0; yeast autolysate — 0.15; pH 6.5–6.7 [7]. Cultivation was carried out in 700-mL Erlenmeyer flask containing 150 ml of medium at 42 °C and 200 rpm. The inoculum was prepared in the same medium and plated in flasks in an amount of 10^5–10^6 CFU / ml. The cells were separated from the culture by centrifugation at 5 000 g, for 30 min. Dry ammonium sulfate salt (final concentration 90%) was added to the supernatant of culture fluid. The mixture was incubated for 24 hours at 4 °C, centrifuged at 5 000 rpm, and the resulting precipitate was collected. To separate enzyme complex the method of gel filtration was used: column (1.8×40 cm) of neutral TSK-gel — Toyopearl HW-55 (Toyosoda, Japan). Elution was carried out with 0.01 M Tris-HCl-buffer, pH 7.5. Fractions with proteolytic activity were pooled. Protein content at all purification steps were recorded on SF-26 at a wavelength of 280 nm.

To carry out inhibition analysis the following reagents were used: phenylmethylsulfonyl fluoride (PMSF), dithiothreitol (DTT), 1-ethyl-3-[3-dimethylaminopropyl] carbodiimide (EDC), N-ethylmaleimide (NEM), sodium ethylenediaminetetraacetate (EDTA). To study the influence of reagents, pH and temperature on the enzyme activity we used peptidases preparations obtained from gel filtration. Inhibitor (in a final concentration 10^{-3} M) was added to the enzyme (1 mg / ml), incubated for 60 min at room temperature, then activity was determined according to the described method. The effect of pH and temperature on the peptidase activity was studied at temperatures ranging from 4 to 80 °C and a pH of from 3.0 to 11.0, pH range was created by the universal 0.05 M phosphate buffer (UPB).

The protein content was determined by the method of Lowry [8]. Caseinolytic activity was determined by the method of Anson with Petrova's modification [9], elastase activity was evaluated colorimetrically according to the intensity of staining solution at enzyme hydrolysis of elastin stained with Congo red [10], fibrinolytic — by Masada [11], collagenase — by Mandl [12]. The lytic activity of the strains of bacilli was established by turbidimetric method [13].

All experiments were performed at least in triplicate (n = 3–5). Data were presented as average magnitude ± standard error. Statistical analysis was performed using the Student t-test, evaluating the reliability of the results on the significance level of at least 95% ($P \leq 0.05$).

Results and Discussion

Bacteria of the genus *Bacillus*, depending on environmental conditions, can synthesize a number of different classes of enzymes that enables them to exist on a variety of substrates. Due to this property bacilli are widely used in industry for production of such enzymes as α-amylase, neutral and alkaline proteases, which are applied in medical and veterinary practice (protosubstilin, subtilisin, etc.) [14]. *Bacillus* proteases are also used to treat the patients with diseases of the gastrointestinal tract, acute and chronic poisoning, inflammation of various etiologies and certain tumors. Special attention is paid to the enzymes having fibrinolytic activity, providing the clot lysis [15]. Besides, bacillar proteases, possessing affinity to hydrophobic residues of amino acids in particular alanine, can cause the lysis of certain microbial cells and certain yeast-like fungi. Such a protease can cleave, for example, Gly-Ala bond in peptidoglycan [16]. The protease preparations are used in veterinary to cleave the proteins of animal feed that improves their absorption. These preparations include, for example, protosubstilin, which is obtained by drying of the supernatant of submerged cultivated *B. sublilis*.

It is therefore expedient to study the protease activity of strains used in the composition of veterinary drugs for the treatment and prevention of gastrointestinal diseases and chronic inflammatory

processes in animals. The studied here *B. amyloliquefaciens* subsp. *plantarum* UCM B-5140 and UCM B-5139 (components of probiotic preparation Endosporin) belong to this group of strains. It is established (Fig. 1), that the culture liquid of two examined strains *B. amyloliquefaciens* subsp. *plantarum* UCM B-5140 and UCM B-5139 grown on a medium containing gelatin, were characterized by a wide spectrum of proteolytic activity: total proteolytic (caseinolytic, PA) elastolytic (EA) and the fibrinolytic (FA). The maximum level of enzyme activity was observed on the 2nd day of cultivating.

The complex enzyme preparations were obtained from the culture supernatant after 2 days of cultivating by precipitation with ammonium sulfate of 90% saturation. Comparative study of their proteolytic activity showed (Fig. 2), that they possess the entire spectrum of activity determined in the culture liquid.

Preparations obtained from *B. amyloliquefaciens* subsp. plantarum UCM B-5140 has a higher specific activity towards insoluble protein substrates (elastin, fibrin and collagen). It should be noted that both strains showed no keratinolytic activity, although there is an evidence that *B. amyloliquefaciens* is able to synthesize a protease having keratinase activity [17]. The elastase activity of *B. amyloliquefaciens* subsp. *plantarum* UCM 5140 is comparable to that one of *B. thuringiensis* IMV B-7324 and *B. subtilis* 316m, which were studied earlier [7, 18].

The partial purification of peptidases from the enzyme preparations of *B. amyloliquefaciens* subsp. *plantarum* UCM B-5140 (Fig. 3) and *B. amyloliquefaciens* subsp. *plantarum* UCM B-5139 (Fig. 4) was carried out by gel filtration on column TSK-gel Toyopearl HW-50 (Toyosoda, Japan).

It was found that there was a substantial amount of proteins with different molecular weights in the preparations of the investigated strains. Analysis of the fractions obtained from the gel filtration showed that each of the two complex preparations contains only one peak of proteolytic activity (red line on the elution profile). It concentrated all the studied activity, among which the elastolytic activity was dominant. This result was very surprising because the bacilli are generally synthesized a wide range of proteases, belonging to different types (serine and metal-dependent peptidase). For example, *B. amyloliquefaciens* FSE-68, isolated from Korean fermented food synthesized two proteases (metal- and serine type) on the first day of cultivation. These proteases possessed similar substrate specificity towards Leu and Phe residues [19]. Perhaps this result is due to physiological and biochemical characteristics of these particular strains.

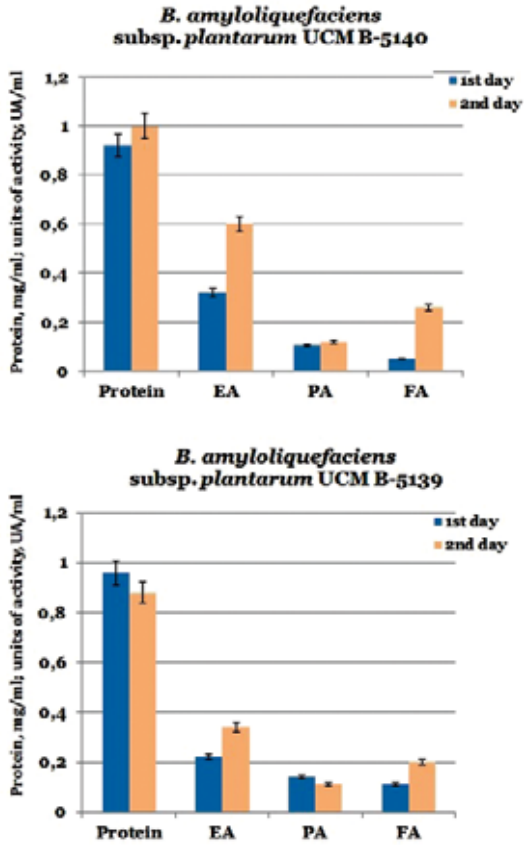

Fig. 1. **The dynamics of the synthesis of peptidases by strains of *B. amyloliquefaciens* subsp. *plantarum* UCM B-5139 and UCM B-5140 during cultivating**

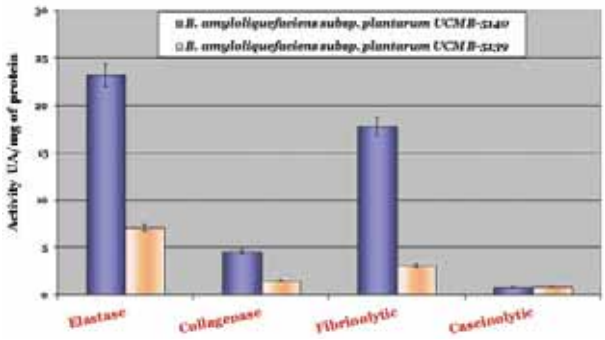

Fig. 2. **Hydrolysis of the native protein complex by enzyme preparation from *B. amyloliquefaciens* subsp. *plantarum* UCM B- 5139 and UCM B-5140**

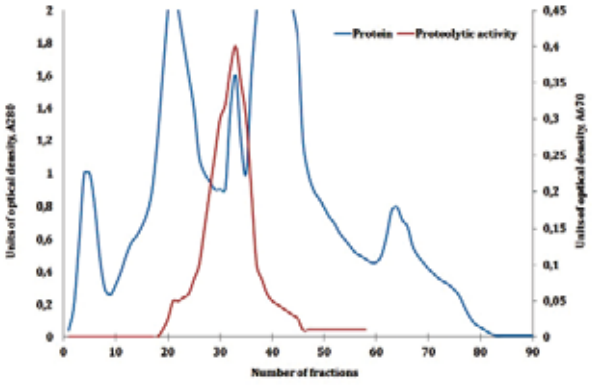

Fig. 3. **The elution profile of the complex enzyme preparation from *B. amyloliquefaciens* subsp. *plantarum* UCM B-5140 on TSK HW-55**

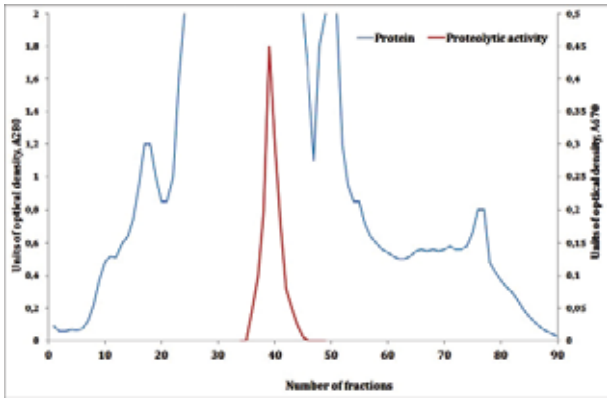

Fig. 4. **The elution profile of the complex enzyme preparation from *B. amyloliquefaciens* subsp. *plantarum* UCM B-5139 on TSK HW-55**

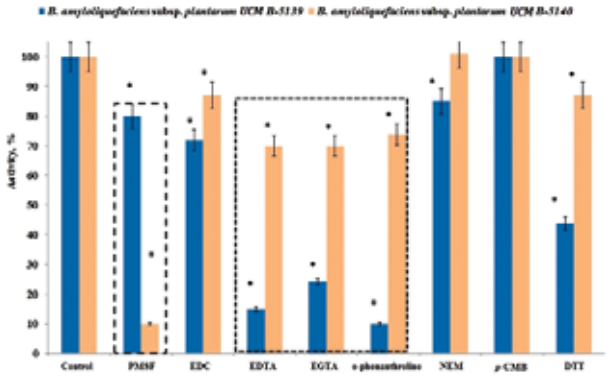

Fig. 5. **The influence of group-specific inhibitors on the protease activity obtained from *B. amyloliquefaciens* subsp. *plantarum* UCM B-5140 and UCM B-5139**

Here and after * — difference is significant in comparison with the control *($P \leq 0.05$)*.

Investigations of the effect of group-specific reagents on the activity of protease *B. amyloliquefaciens* subsp. *plantarum* UCM B-5139 showed (Fig. 5) that metal chelators inhibited this activity by 76–90%. This may indicate the presence of metal in the active site of the enzyme, so the enzyme may belong to metal-dependent proteases.

Simultaneously PMSF irreversibly inhibited protease activity of *B. amyloliquefaciens* subsp. *plantarum* UCM B-5140 by 90%. Since it is an inhibitor of nearly all serine proteases with trypsin-like specificity, and it is covalently bound to His or Ser of the active site, we consider that the investigated protease belongs to the group of serine protease type.

Metal chelators slightly decreased (by 23–30%) the activity of protease *B. amyloliquefaciens* subsp. *plantarum* UCM B-5140 that may indicate the metal-dependent enzyme. As it is well known [20], metals such as calcium, are involved in stabilizing of the molecular structure.

We studied the effect of DTT on the protease activity. This agent provides efficient recovery of intramolecular and intermolecular disulfide bonds between cysteine residues. It was shown a significant inhibitory effect (56%) on the enzyme from *B. amyloliquefaciens* subsp. *plantarum* UCM B-5140, and a slight one (23%) in case of the enzyme from *B. amyloliquefaciens* subsp. *plantarum* UCM B-5139. It was also detected the inhibitory effect of N-ethylmaleimide (NEM) towards the protease from the strain of UCM B-5140, that suggests the presence of thiol group on the surface of the enzyme. Chemical modification of these groups results in a conformational change of the molecule and makes the catalytic site less accessible to the substrate.

Optimal conditions of protease activity such as pH optimum, optimal temperature of the medium are very important enzyme characteristics. It was found that the two strains (убрать) peptidases from two strains of *B. amyloliquefaciens* subsp. *plantarum* are active in the pH range from 4.0 to 8.0 (Fig. 6), and the optimum pH is achieved by a native substrate hydrolysis at pH 6.0–6.5.

Thus, both enzymes belong to the type of the neutral protease. Study of the temperature effect (Fig. 7) showed that these peptidases (enzymes убрать) are active in the range of 20–60 °C, with an optimum of the enzymatic activity at 37–40 °C, and at 60 °C approximately 10–15% of the initial enzyme activity is stored.

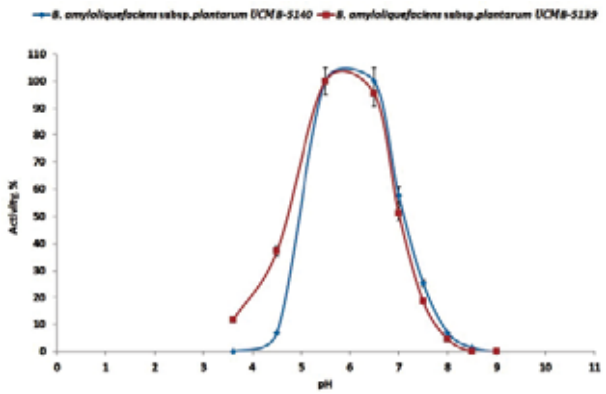

Fig. 6. **The effect of pH on the protease activity obtained from *B. amyloliquefaciens* subsp. *plantarum* UCM B-5140 and UCM B-5139**

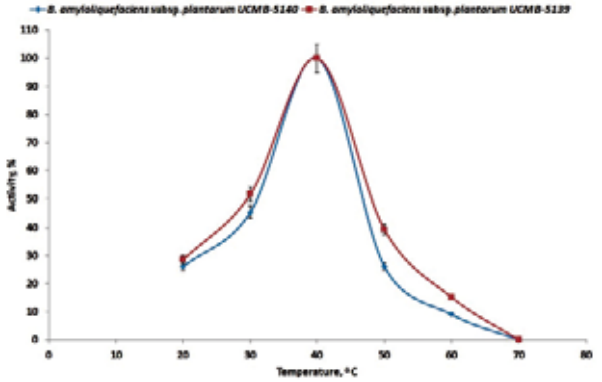

Fig. 7. **The effect of temperature on the protease activity obtained from *B. amyloliquefaciens* subsp. *plantarum* UCM B-5140 and UCM B-5139**

As it was previously found [6] the studied strains *B. amyloliquefaciens* subsp. *plantarum* UCM B-5140 and UCM B-5139 possess antagonistic activity against a broad spectrum of microorganisms such as Staphylococcus aureus, *Escherichia coli*, *Proteus morganii*, *Pseudomonas aeruginosa*, *Acinetobacter* sp., *Pseudomonas syringae* pv. *atrofaciens*, *Agrobacter tumefaciens*, *Clavibacter michiganensis* subsp. *michiganensis*, as well as against certain types of fungi. Such activity, except for the synthesis of antibiotics, may be determined by the presence of bacteriolytic enzymes, in particular proteases with specificity for Ala and Gly. The presence of elastase activity (Fig. 2) at the proteases of investigated strains can indirectly indicate the ability of these enzymes to hydrolyze Ala-Ala and Ala-Gly bonds. A similar substrate specificity was observed in case of bacteriolytic proteases, which can cleave peptidoglycan bonds in the cellular wall of microorganisms [16]. Bacteriolytic activity of the partially purified proteases from strains of *B. amyloliquefaciens* subsp. *plantarum* in UCM B-5140 and B-5139 was investigated towards the live cells of microorganisms from different taxonomic groups: Gram-positive (*S. aureus*), Gram-negative (*E. coli*) and yeast (*Candida albicans*).

It was found that the protease from the strain of *B. amyloliquefaciens* subsp. *plantarum* UCM B-5140 has a greater lytic activity against *S. aureus* cells (a significant decrease of the density of the cell suspension, 19%) than to the yeast cells (13%). At the same time the enzyme *B. amyloliquefaciens* subsp. *plantarum* UCM B-5139 possesses higher (вместо greater) yeast lytic activity (degree of lysis of living cells was 20%) compared to the lytic activity against staphylococcal cells (11%). The effect of lysis of *E.coli* cells was negligible, due probably to the structural features of the membranes of Gram-negative bacteria. Based on studies of lytic activity of bacilli it is possible to conclude that one of the factors determining the previously established [6] high antagonistic activity of these strains is their ability to synthesize proteolytic enzymes.

Thus, we have demonstrated the presence

Table 1. **Lytic activity spectrum of the partially purified protease from *B. amyloliquefaciens* subsp. *plantarum* UCM B-5140**

Time of incubation, min	The degree of lysis (%) of living cells from test-cultures		
	S. aureus	*E. coli*	*C. albicans*
15	6±0.22	0	0
30	11±0.53*	0	6±0.22
60	14±0.67*	3±0.14	9±0.36*
120	19±0.96*	6±0.23	13±0.59*

Note: suspension of the live untreated cells was used as the control — (0%).

Table 2. **Lytic activity spectrum of the partially purified protease from *B. amyloliquefaciens* subsp. *plantarum* UCM B-5140**

Time of incubation, min	The degree of lysis (%) of living cells from test-cultures		
	S. aureus	*E. coli*	*C. albicans*
15	0	0	3±0.14
30	0	2±0.08	6±0.23
60	5±0.25	5±0.25	11±0.52*
120	11±0.55*	5±0.22	20±0.98*

of proteolytic enzymes that cleave native insoluble proteins (elastin, fibrin and collagen) at the strains of *B. amyloliquefaciens* subsp. *plantarum* UCM B-5140 and UCM B-5139, which are the component of the veterinary probiotic Endosporin. These enzymes belong to the groups of metal-dependent and serine neutral proteases, which are able to function effectively under physiological conditions. They possess lytic activity against gram-positive bacteria and yeasts.

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ПРОБІОТИЧНІ ШТАМИ *Bacillus amyloliquefaciens* subsp. *plantarum* ЯК ПРОДУЦЕНТИ ПРОТЕЇНАЗ

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Протеолітичні ензими пробіотичних штамів роду *Bacillus*, так само як антибіотики, бактеріоцини та інші гідролітичні ензими, є одним із головних чинників, які визначають їхню біологічну активність, тому метою роботи було вивчення синтезу і властивостей протеаз двох штамів — *Bacillus amyloliquefaciens* subsp. *plantarum* УКМ В-5139 та УКМ В-5140, що входять до складу пробіотика Ендоспорин. Культивування штамів проводили глибинним способом упродовж двох діб. На частково очищених препаратах протеаз вивчали залежність протеолітичної активності від фізико-хімічних параметрів реакційного середовища. Літичну активність визначали турбідиметричним методом. Штами *Bacillus amyloliquefaciens* subsp. *plantarum* УКМ В-5139 і УКМ В-5140 на другу добу культивування синтезують метало- і серинову протеазу, відповідно. Оптимальні умови їхньої активності — за температури 37–40 °С і pH 6,5–7,0. Виділені протеази здатні лізувати живі клітини *Staphylococcus aureus* та *Candida albicans*. Показано наявність у штамів *B. amyloliquefaciens* subsp. *plantarum* УКМ В-5140 та УКМ В-5139, що входять до складу пробіотика ветеринарного призначення Ендоспорин, протеаз, здатних гідролізувати нативні нерозчинні протеїни (еластин, фібрин, колаген). Ці ензими у фізіологічних умовах виявляють літичну активність стосовно дріжджів та грампозитивних бактерій і можуть бути використані у біотехнології.

Ключові слова: *Bacillus amyloliquefaciens* subsp. *plantarum*, пробіотики, протеїнази, літична активність.

ПРОБИОТИЧЕСКИЕ ШТАММЫ *Bacillus amyloliquefaciens* subsp. *plantarum* КАК ПРОДУЦЕНТЫ ПРОТЕИНАЗ

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Протеолитические энзимы пробиотических штаммов рода *Bacillus* наряду с антибиотиками, бактериоцинами и другими гидролитическими энзимами являются одним из главных факторов, определяющих их биологическую активность, поэтому целью работы было изучение синтеза и свойств протеаз двух штаммов — *Bacillus amyloliquefaciens* subsp. *plantarum* УКМ В-5139 и УКМ В-5140, входящих в состав пробиотика Эндоспорин. Культивирование штаммов проводили глубинным способом в течение двух суток. На частично очищенных препаратах протеаз изучали зависимость протеолитической активности от физико-химических параметров реакционной среды. Литическую активность определяли турбидиметрическим методом. Штаммы *B. amyloliquefaciens* subsp. *plantarum* УКМ В-5139 и УКМ В-5140 на вторые сутки культивирования синтезируют металло- и сериновую протеазу, соответственно. Оптимальные условия их активности — температура 37–40 °С и pH 6,5–7,0. Выделенные протеазы способны лизировать живые клетки *Staphylococcus aureus* и *Candida albicans*. Показано наличие у штаммов *B. amyloliquefaciens* subsp. *plantarum* УКМ В-5140 и УКМ В-5139, входящих в состав пробиотика ветеринарного назначения Эндоспорин, протеаз, способных расщеплять нативные нерастворимые протеины (эластин, фибрин, коллаген). Эти энзимы обладают литической активностью в отношении дрожжей и грамположительных бактерий и могут быть использованы в биотехнологии.

Ключевые слова: *Bacillus amyloliquefaciens* subsp. *plantarum*, пробиотики, протеиназы, литическая активность.