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Lactobacillus AS PRODUCERS OF EXTRACELLULAR TANNASE

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The aim of the work was to find strains of lactic acid bacteria capable to synthesize extracellular tannase enzyme — the key enzymehe hydrolyzing tannins which are plant food constituent. One of the main product of tannins hydrolyzis is gallic acid- the compound with proven antioxidant and onco-protective. As a result of lactobacteria screening, two biocompatible strains of lactic acid, namely *L. rhamnosus* LB3 and *L. delbrueckii* subsp. *delbrueckii* with a high level of enzyme productivity, were selected. The maximum accumulation of tannase, corresponding to 0.031 ± 0.002 U/ml for *L. rhamnosus* LB3 and 0.03 ± 0.002 U/ml for *L. delbrueckii* subsp.*delbrueckii*, was observed after 48 h of cultivation. Both strains showed rapid growth and performance of tannase in MRS medium in the presence of glucose or lactose as a carbon source. It was shown that gallic acid, which was a necessary component of the medium as a target enzyme inducer, did not affect the accumulation of lactobacilli biomass. The selected strains are of interest as producers of a bicomponent probiotic with antioxidant properties and require further investigation.

Key words: lactobacillus, probiotics, tannase, antioxidants, carbon sources.

Along with the science development new properties and mechanisms for implementing the probiotic biotherapeutic potential are discovered. The study of anticarcinogenic activity of probiotic strains is particularly noteworthy.

The mechanisms of oncoprotective action of lactic acid bacteria, which are widely present in the composition of functional food products and probiotic-containing medications, have not been fully studied, however they are associated with such properties as:

– the ability to modify fecal enzymes, which believed to be involved in carcinogenesis of the colon [1];

– cellular absorption and removal of mutagenic substances or reduction of the mutagenic effect of chemicals, by its transformation [1–5];

– tumor suppression by stimulating the immune response, in a way of increasing the activity of natural killers (NK cells) [3];

– antagonistic activity in relation to pathogenic microorganisms that may have an indirect carcinogenic effect (e.g. *H. pylori*) [6–8];

– induction of apoptosis in myeloid leukemia cells;

– production of substances which induce apoptosis [9];

– biodegradation of natural substances with the formation of antioxidant compounds [10].

The implementation of the last mechanism is well illustrated by the example of the natural polyphenolic compounds of tannins splitting. The main common natural source of tannins is plants. Many types of tannins are found in a range of food products such as tea, coffee etc. The presence of tannins in foods gives it a bitter taste, which makes them less appealing for consumption. In addition, tannins at a certain concentration have toxic, bacteriostatic and carcinogenic properties and irreversibly form compounds with proteins, as well as with other molecules such as starch, cellulose and minerals [10, 11]. Tannase is known to be the main enzyme which is involved in the decomposition of tannins, in particular halo-tannins. It is

produced by a number of microorganisms which belong to fungi and bacteria [12, 13]. However, from that point of view, lactic acid bacteria are the most important among all probiotic cultures.

The high interest in tannase is due to the fact that the main product of tannins hydrolysis is gallic acid, which is known for its antioxidant properties [12, 14, 15].

Modern medical field pays close attention to the substances with antioxidant properties, since it is believed that one of the causes of oncological diseases is oxidative tissue damage. There are reports on the capability of gallic acid to protect human cells from oxidative damage and cause an anti-apoptotic effect, along with a pronounced cytotoxic action in relation to cancer cells [16].

Thus, the data from the literature testifies the promising usage of the drugs based on the tannin-positive bacteria of the genus *Lactobacillus* for both food industry and medical practice, as a source of antioxidant complexes to protect the body from the negative effects of free radicals. Therefore, the search for new rational and effective natural sources of antioxidants among probiotic strains is relevant and feasible.

Materials and Methods

The objects of the research were bacteria strains of the genus *Lactobacillus* from the collection of the Department of Industrial Biotechnology (Faculty of Biotechnology and Biotechnics of the National Technical University of Ukraine "Igor Sikorsky Kyiv Polytechnic Institute") and the strain with proven tannase activity from the collection of Microbial Type Culture Collection and Gene Bank of the Institute of Microbial Technology (Chandigarh, India). A complete list of strains is presented in Table 1.

Incubation conditions. Lactic acid bacteria (LAB) strains were transferred to sterile MRS broth (Man-Rogosa Sharpe, Himedia) and incubated at +37 °C for 24 hours. The medium was previously sterilized at 0.5 MPa during 20 min and the medium pH after sterilization procedure was 6.2–6.6. The investigated *Lactobacillus* cultures were stored at +4 °C in a semi-liquid MRS medium (with 2% of agar).

Tannase activity determination. Extracellular tannase activity of LAB strains was estimated according to previously described technique [17].

Estimation of biomass accumulation. The rate of biomass accomulation was measured after 24 hours and 48 hours of incubation using spectrophotometry (UNICO Spectrophotometer 1201) at wavelengths of 560 nm.

Examination of LAB strains biocompatibility. The biocompatibility of the strains was determined by the method of *in vitro* joint cultivation [18] with minor modifications (strokes and droplets method). The biocompatibility of test cultures was estimated by the sizes of growth zones. Accounting was performed after 24 and 48 hours incubation at +37 °C.

Acid production determination. Acid production by the strains was examined by the titration [19]. The result was estimated by the volume of alkali spent on neutralization of acid in the medium and expressed in degrees Terner (°T).

Statistical Analysis. All experiments were performed in at least three repetitions using appropriate control samples. The digital data obtained during the research was processed by statistical analysis methods with using the Student's *t*-test for small samples at 95–99% levels of significance. Calculation and diagrams construction were done using the computer program Excel (Microsoft Office 2010) [20].

Results and Discussion

10 LAB strains from the microbial collection of the Department of Industrial Biotechnology (Faculty of Biotechnology and Biotechnics, NTUU "Igor Sikorsky Kyiv Polytechnic Institute") were screened for their ability to produce extracellular tannase. The strain *Lactobacillus plantarum MTCC 2621* from the Indian collection of MTCC IMTECH which has proven high-grade tannase activity was chosen as reference microorganism.

As a result of the screening, 3 tannase active strains of the genus *Lactobacillus* were selected: *L. rhamnosus LB3, L. bulgaricus LB51 and L. delbrueckii subsp. delbrueckii* with tannase activity at 48 hours of incubation 0.031 ± 0.002 U/ml, 0.013 ± 0.001 U/ml and 0.03 ± 0.002 U/ml, respectively.

As a result of the data analysis, it was found that the level of enzyme biosynthesis by all selected strains was significantly higher on the second day of incubation in comparison to the estimated tannase activity on the first day After 48 hours of incubation the activity of the reference strain had reached a maximum level of enzymatic activity (0,051 ± 0,002 U/ml), which was 2.29 times higher than the activity on the first 24 hours of growth. Tannase activity on the 48 hour of incubation had

Table 1. ***Lactobacillus*** **strains**

Full name of strain	The sources of origin
L. murinus LE IMB B-7037	Non-commercial dairy products *
L. rhamnosus LB3 IMB B-7038	Non-commercial dairy products *
L. acidophilus (C)	Non-commercial dairy products *
L. rhamnosus (C)	Institute Rossell INC, Canada
L. bulgaricus LB51	Zabolotny Institute of Microbiology and Virology of the National Academy of Sciences of Ukraine
L. delbrueckii subsp. *bulgaricus LB86 ВКПМ-В-5788*	Plant of microbiological synthesis preparations "Enzyme"
L. delbrueckii subsp. *delbrueckii DSM20074*	DSMZ, Germany
L. murinus DSM 20452	DSMZ, Germany
L. plantarum	Lactobacterin *
L. plantarum MTCC 2621	MTCC IMTECH, India

Note: * — the strains were isolated at the department of the industrial biotechnology at the faculty of biotechnology and biotechnics of the National Technical University of Ukraine "Igor Sikorsky Kyiv Polytechnic Institute"; DSMZ — Deutche Summlung von Mikroorganismen und Zellkulturen; MTCC IMTECH — Microbial Type Culture Collection and Gene Bank of Institute of Microbial Technology.

increased for *L. rhamnosus LB3* by 1.63 times; for the strain *L. delbrueckii* subsp. *delbrueckii* by 1.76 times. The level of tannase activity of the strains is presented in Table 2.

Thus, in a result of screening, 3 LAB strains with tannase activity were selected to be considered as potential producers of a new multistrain probiotic with antioxidant action. From the other hand, whenever a multicomponent probiotic product is created, the ability of the strains to exist with each other should be considered.

The microbiota that colonises biological systems and organs of macroorganism is known to be multicomponent. Probiotic strains interact in the own microflora biocenosis of the macroorganism, as well as with other microorganisms from different taxonomic groups. It is known that the ability to inhibit a growth and a reproduction of pathogenic and opportunistic microorganisms is one of the most important criterias for assessing the effectiveness of probiotic strains. An antagonistic activity of the normal microflora is one of the mechanisms of colonization resistance of the macroorganism.

However, it is very important that strains action to be synergistic or at least indifferent in relation to the host organism native microflora. In this regard, investigated strains that demonstrated the ability to synthesize extracellular tannase, as well as the reference strain, have been studied for biocompatibility with each other. The study was conducted using perpendicular strokes and a modified droplet method (common cultivation method) [18].

The results of the study showed that strains *L. rhamnosus LB3* and *L. delbrueckii subsp. delbrueckii* are biocompatible; while strains *L. bulgaricus LB51* and *L. plantarum 2621* do not demonstrate biocompatibility with any of the other investigated strains (Table 3). It worth to be noticed, that both methods of determining the biocompatibility of lactobacillus showed correlative results.

According to the results of biocompatibility test, 2 biocompatible strains (*L. rhamnosus LB3* and *L. delbrueckii subsp. delbrueckii*) were selected for further research as potential components of bi-component probiotic. For the *L. bulgaricus LB51* strain, it was decided not to conduct further studies due to the relatively low tannase activity and incompatibility with other strains. *L. plantarum 2621*, which has the highest tannase activity may possibly have the interest as a mono-probiotic with antioxidant properties.

While creating the technology of a biotechnological product, the focus is primarily put on the development of the technological parameters that provide an increase in the output of the target product. These parameters include optimization of nutrient components

Table 2. **Tannase activity of investigated *Lactobacillus* strains**

LAB Strain	Tannase activity, U/ml			
	24 hours-incubation	% from control*	48 hours incubation	% from control*
L. murinus LE	0	–	0	–
L. rhamnosus LB3	0.019 ± 0.001	79.2	0.031 ± 0.002	60.8
L. acidophilus (C)	0	–	0	–
L. rhamnosus (C)	0	–	0	–
L. bulgaricus LB51	0.009 ± 0.001	37.5	0.013 ± 0.001	25.5
L. delbrueckii subsp. *bulgaricus LB86*	0	–	0	–
L. delbrueckii subsp. *delbrueckii*	0.017 ± 0.001	70.8	0.030 ± 0.002	58.8
L. murinus	0	–	0	–
L. plantarum	0	–	0	–
L. plantarum 2621 (reference strain)	0.024 ± 0.001	100	0.051 ± 0.002	100

*Note:** — the percentage of the level of ectracellular tannase synthesis by the reference strain at the seame time of incubation.

Table 3. **The biocompatibility of tannase active *Lactobacillus* strains**

Strains	*L. rhamnosus LB3*	*L. bulgaricus LB51*	*L. delbrueckii* subsp. *delbrueckii*	*L. plantarum 2621*
L. rhamnosus LB3	++	– / +	+ / +	+ / –
L. bulgaricus LB51	+ / –	++	– / –	– / +
L. delbrueckii subsp. *delbrueckii*	+ / +	– / –	++	– / –
L. plantarum 2621	– / +	+ / –	– / –	++

Note: "+" — growth of the culture; "–" — lack of growth of the culture;
"*/*" — "growth characteristics of the strains located in the left vertical column of the table/ the growth characteristic of the strains located in the horizontal column".

composition of the medium and incubation conditions. It is known that the ingredients composition of the medium and the conditions of incubation are key factors influencing the growth rate of the microorganism-producer and the formation of metabolites. Very often the high cost of microbial preparation production, particuralry biomass obtaining stage, is a major restrictive factor of scaling it up to industrial production. Typically, such components of the medium as the inducer (tannic or gallic acids) and the carbonaceous compounds that directly affect the growth and enzymatic activity of the microorganism-producer, are the most costly for production [11, 21].

Despite of a growing scientific interest to the research of the repression and induction processes of microbial tannase, there still Today, there are still many controversies about the repression and induction of tannase, and the literature data is not full enough, therefore it makes a clear the necessity for thorough research of the mechanisms governing the biosynthesis of tannase.

For a long time, it was believed that the tannase enzyme is possible only in the presence of tannic acid in the medium. However, it was discovered later that the activity of microorganisms-producers has often been manifested also under cultivation on media with different carbon sources — monosaccharides, disaccharides, polysaccharides, even without adding an inductor [22].

It worth to be noticed, that one of the crucial growth factors is the type of the secondary carbon sources adding to the medium, such as

glucose, galactose, mannose, lactose, fructose, etc. Adding these components reduces the lag phase and promotes the growth of the microorganisms. From this point of view, glucose is known as the most considerable compound among the carbon sources. There is the literary evidence that lower glucose concentration contributes to the induction of tanase, whereas higher concentration causes even catabolic repression of the enzyme's biosynthesis [23]. Such a phenomenon can be explained by the emergence of an imbalance in the ratio of carbon and nitrogen in the medium that causes osmotic stress and as a result inhibits the enzyme biosynthesis [24, 25].

As it was mentioned earlier, the medium which is used to produce microbial products does not always ensure the maximum accumulation of biomass. That is very important to be taken into account in the production of probiotics because one of the most important technological parameters of the production of the biotechnological products is the level of accumulation of the target product. In the case of probiotics production, the target product is the biomass of the culture.

Therefore, in order to determine the prospect for the industrial application, the growth analysis of investigated LAB strains was held using the medium MRS (Himedia) and as well as modified MRS medium for microorganisms cultivation. The diagrams with results of the experiment of the growth of strains *L. plantarum 2621*, *L. rhamnosus LB3*, *L. delbrueckii subsp. delbrueckii* are shown in Fig. 1.

The diagram shows that LAB strains *L. rhamnosus LB3* and *L. delbrueckii subsp. delbrueckii* have significantly higher growth intensity compared to the reference strain *L. plantarum 2621*. The obtained data demonstrate that the intensity of biomass accumulation of all investigated strains grown on the industrially produced ready-made MRS medium (Himedia) and on the modified medium prepared from individual components has no statistically significant difference. Such results provide the basis for further research using the modified MRS medium in terms of economic expediency.

Taking into account the existing literature data and the aim of our research, it was decided to study the growth of LAB strains in a modified medium MRS with different carbon-containing components as well as in the absence or adding of an inductor in the medium (gallic acid at a concentration of 2%). Mono and disaccharides, such as glucose, galactose, lactose, fructose and sucrose, at a concentration of 20 g/l, were used as carbon sources. The results of the experiment are presented in the diagrams (Fig. 2–4).

As results of experiment have shown, the rate of biomass accumulation and tannase activity of all investigated strains depends on the type of carbon source contained in nutrient medium. It is important to note that the maximum performance of all investigated microorganisms was demonstrated while using glucose. Glucose has provided both the highest level of biomass accumulation and the

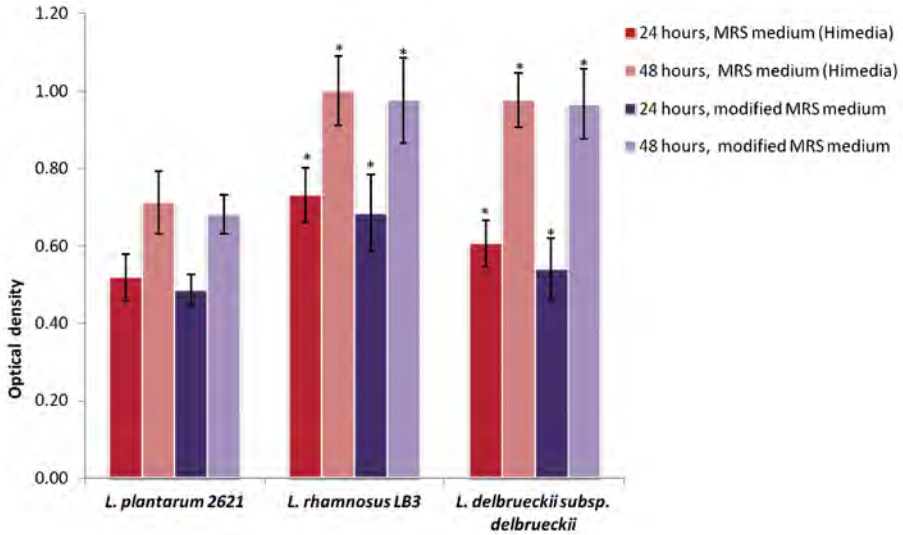

Fig. 1. **Comparative characteristic of growth intensity of the strains with tannase activity on MRS medium (Himedia) and modified MRS medium:**
values represented mean ± SEM. Values for strains *L. rhamnosus* LB3 and *L. delbrueckii* subsp.*delbrueckii* were compared with reference strain *L. plantarum* 2621. $*P < 0.05$ compared to the reference strain on the same medium at the same time of experiment

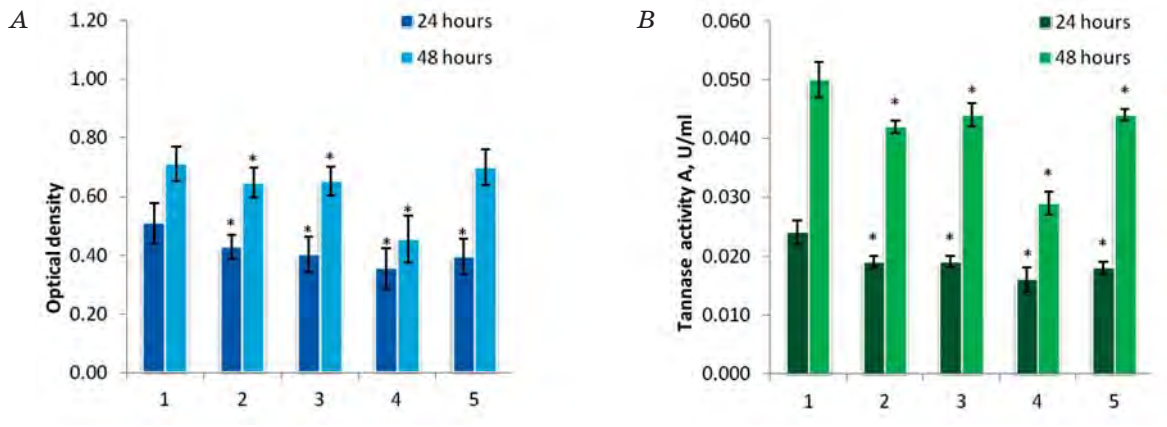

Fig. 2. **The biomass accumulation (*A*) and tannase activity (*B*) of strain *L. plantarum 2621* on the medium with the different carbon sources:**

Here and after: 1 — glucose; *2* — lactose; *3* — galactose; *4* — fructose; *5* — sucrose

**P* < 0.05 compared to the values for the strain growth on the medium with glucose (1) at the same time of experiment

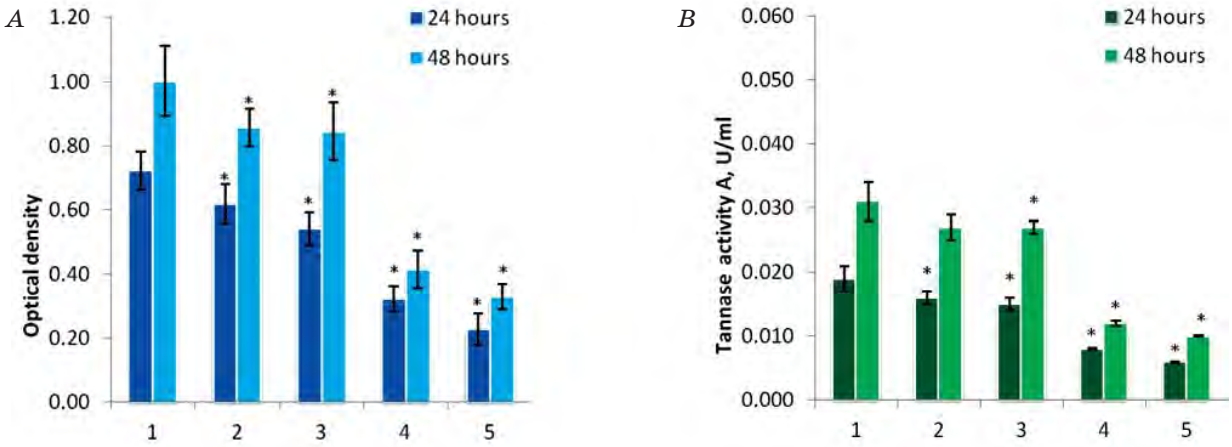

Fig. 3. **The biomass accumulation (*A*) and tannase activity (*B*) of strain *L. rhamnosus* LB3 on the medium with the different carbon sources**

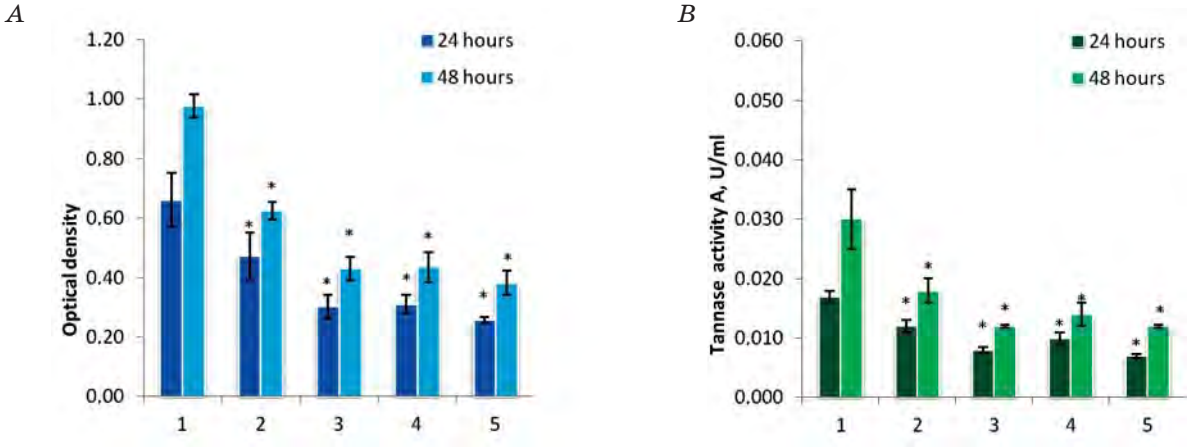

Fig. 4. **The biomass accumulation (*A*) and tannase activity (*B*) of strain *L. delbrueckii* subsp. *delbrueckii* on the medium with the different carbon sources**

Table 4. ***Lactobacillus*** **strain growth rate on medium with different sources of carbon in the presence and absence of gallic acid**

The biomass accumulation of *L. plantarum 2621*, OD										
gallic acid (+/–)	*glucose*		*lactose*		*galactose*		*fructose*		*sucrose*	
	24 h	48 h	24 h	48 h	24 h	48 h	24 h	48 h	24 h	48 h
+	0.51± 0.04	0.72± 0.02	0.43± 0.08	0.65± 0.02	0.40± 0.01	0.65± 0.03	0.36± 0.03	0.45± 0.03	0.40± 0.02	0.36± 0.02
–	0.47± 0.02	0.72± 0.03	0.41± 0.04	0.64± 0.03	0.40± 0.02	0.65± 0.03	0.34± 0.02	0.42± 0.02	0.47± 0.03	0.39± 0.05
The biomass accumulation of *L. rhamnosus LB3*, OD										
gallic acid (+/–)	*glucose*		*lactose*		*galactose*		*fructose*		*sucrose*	
	24 h	48 h	24 h	48 h	24 h	48 h	24 h	48 h	24 h	48 h
+	0.72± 0.01	1.01± 0.02	0.62± 0.02	0.86± 0.05	0.54± 0.03	0.85± 0.04	0.33± 0.03	0.41± 0.03	0.23± 0.01	0.30± 0.02
–	0.71± 0.03	1.01± 0.03	0.56± 0.07	0.80± 0.02	0.53± 0.04	0.78± 0.04	0.27± 0.02	0.41± 0.03	0.25± 0.04	0.34± 0.03
The biomass accumulation of *L. delbrueckii subsp. delbrueckii*, OD										
gallic acid (+/–)	*glucose*		*lactose*		*galactose*		*fructose*		*sucrose*	
	24 h	48 h	24 h	48 h	24 h	48 h	24 h	48 h	24 h	48 h
+	0.67± 0.02	0.97± 0.04	0.48± 0.02	0.62± 0.04	0.30± 0.02	0.43± 0.03	0.38± 0.02	0.44± 0.04	0.26± 0.04	0.38± 0.02
–	0.64± 0.03	0.97± 0.04	0.48± 0.05	0.65± 0.04	0.31± 0.03	0.40± 0.02	0.36± 0.03	0.42± 0.03	0.24± 0.02	0.38± 0.03

maximum level of tannase activity. Lactose may be considered as an alternative source of carbon for *L. rhamnosus LB3* and *L. plantarum 2126*. Biocompatible strains *L. rhamnosus LB3* and *L. delbrueckii subsp. delbrueckii* showed the lowest tannase activity while cultivated on sucrose-containing medium, and strain *L. plantarum 2621* on the medium with fructose.

It is known that the tannase activity of lactobacilli induces by gallic acid, which is one of the tannin decomposition products. However, gallic acid has antioxidant properties and, as the result, could effect on the growth of probiotics microorganisms. The comparative characteristics of growth intensity of the studied microorganisms on the medium with different sources of carbon under the conditions of the presence of gallic acid and in its absence are presented in Table 4.

As can be seen from the results presented in the table, the presence of the inductor did not affect LAB growth (there was no statistically significant difference between values of LAB growth), but during the experiment, none of the examined strains showed tannase activity in its absence. Therefore, the obtained results suggest that for the investigated Lactobacilli strains, the presence of the inductor in the cultivation medium is a prerequisite for the implementation of the tannase activity but, on the other hand, does not affect the accumulation of biomass of bacterial cultures.

Thus, as a result of screening, two tannase-positive and biocompatible strains *L. rhamnosus LB3* and *L. delbrueckii subsp. delbrueckii* were selected and considered as promising microorganisms-producers for creating a new bi-component probiotic with antioxidant properties. Both strains showed the most rapid growth in the presence of glucose in the cultivation medium. At the same time, according to the results of the study lactose can be considered as a carbon source in the composition of nutrient medium because of a pretty high level of biomass accumulation demonstrated by selected lactic acid bacterias. Gallic acid which is essential for tannase enzyme synthesis induction, did not reduce the growth rate of any strain, therefore, it can be used as a component of the medium for probiotic strains cultivating.

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Lactobacillus ЯК ПРОДУЦЕНТИ ПОЗАКЛІТИННОЇ ТАНАЗИ

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Метою роботи був пошук штамів молочнокислих бактерій, здатних синтезувати позаклітинний ензим таназу, необхідний для розщеплення харчових танінів. Одним з продуктів ензиматичної трансформації танінів є галова кислота, яка, в свою чергу, виявляє антиоксидантні та онкопротекторні властивості. У результаті скринінгу відібрано два біосумісні штами молочнокислих бактерій — *L. rhamnosus* LB3 та *L. delbrueckii* subsp. *delbrueckii* з високим рівнем продуктивності ензиму. Максимум накопичення танази, що відповідав рівню 0,031 ± 0,002 U/ml для *L. rhamnosus* LB3 і 0,03 ± 0,002 U/ml для *L. delbrueckii* subsp. *delbrueckii*, спостерігали через 48 год культивування. Для обох штамів показано швидкий ріст та продуктивність танази на середовищі MRS у присутності глюкози або лактози як джерела вуглецю. Виявлено, що галова кислота, яка була необхідним компонентом середовища як індуктор цільового ензиму, не впливала на накопичення біомаси лактобактерій. Відібрані штами становлять інтерес як продуценти двокомпонентного пробіотику з антиоксидантними властивостями та потребують подальшого дослідження.

Ключові слова: лактобактерії, пробіотики, таназа, антиоксидант, джерела вуглецю.

Lactobacillus КАК ПРОДУЦЕНТЫ ВНЕКЛЕТОЧНОЙ ТАННАЗЫ

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Целью работы был поиск штаммов молочнокислых бактерий, способных синтезировать внеклеточную танназу — энзим, необходимый для расщепления пищевых таннинов. Одним из продуктов энзиматической трансформации таннинов является галловая кислота, которая обладает антиоксидантными и онкопротекторными свойствами. В результате скрининга отобраны два биосовместимых штамма молочнокислых бактерий — *L. rhamnosus* LB3 и *L. delbrueckii* subsp. *delbrueckii* с высоким уровнем производительности энзима. Максимум накопления танназы, соответствующий уровню 0,031 ± 0,002 U/ml для *L. rhamnosus* LB3 и 0,03 ± 0,002 U/ml для *L. delbrueckii* subsp. *delbrueckii*, наблюдали через 48 ч культивирования. Для обоих штаммов показаны быстрый рост и производительность танназы на среде MRS в присутствии глюкозы или лактозы в качестве источника углерода. Выявлено, что галловая кислота, которая была необходимым компонентом среды как индуктор целевого энзима, не влияла на накопление биомассы лактобактерий. Отобранные штаммы представляют интерес как продуценты двухкомпонентного пробиотика с антиоксидантными свойствами и требуют дальнейшего исследования.

Ключевые слова: лактобактерии, пробиотики, танназа, антиоксиданты, источники углерода.