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ԵՊՀ ՈՒԳԸ հրատարակումների կայք՝ ssspub.ysu.am

Yeter Simonyan[1], Andranik Keryan[2], Inga Bazukyan
YSU, Faculty of Biology, [1]BSc student, [2]PhD student
Supervisor: Ph.D. I. Bazukyan
E-mail: yetersl@gmail.com

ISOLATION OF LACTIC ACID BACTERIA WITH BROAD-RANGE ANTIMICROBIAL AND PROTEOLYTIC ACTIVITY FROM ARMENIAN TRADITIONAL DAIRY PRODUCTS

Lactic acid bacteria (LAB) are heterogeneous group of gram-positive microorganisms. They express broad range of biological activities such as antagonistic, proteolytic and lipolytic activities, the ability to synthesize different polysaccharides etc. [1]. They are involved in a large number of spontaneous food fermentations and may be used as starters in dairy productions. LAB are able to synthesize large variety of antimicrobial substances such as organic acids (lactic acid, acetic acid, etc.), hydrogen peroxide, diacetyl, carbon dioxide, bacteriocins and other low molecular weight compounds [2]. Bacteriocins are peptides with antibacterial action that are usually active against closely related strains. Many bacterial strains are able to synthesize bacteriocins but that synthesized by LAB are of particular interest [3]. They may be used in food production as biopreservatives thus replacing chemical preservatives. Bacteriocinogenic LAB can also be used for development of new probiotics.

Milk is an important source of proteins but at the same time it can be a source of human allergies. Cow milk proteins are resistant to high temperatures and maintain most of their activity after cooking. The reason of non-tolerance of milk and dairy products can also be lactose intolerance [4]. Many studies have revealed that caseins and β-lactoglobulin are the main allergens in cow milk. Different attempts have been made to reduce the allergenicity of dairy proteins, and various technological processes have been applied [5]. Possibilities of pharmaceutical therapies are unsatisfactory. Usuallythey are limited by use of antiallergenic, antihistamine and cortisone drugs. The allergies have become more serious and more frequent than before. One way to avoid the allergenicity of milk is the use of soybean milk instead. However, its consumption may lead to digestive problems. Soybean proteins important for human nutrition can be extracted but resultant sludgecontains free-form cytotoxic amino acids and other potentially harmful chemicals including cancer-causing chemicals in many cases [6]. The best solution to avoid potential complications is the use of LAB. In order to be able to grow in milk, they depend on expression of proteolytic system that degrades casein which is the main milk protein. They are also able to hydrolyze soybean proteins. Proteolytic system of LAB consists of cell-envelope proteinases, specific peptide uptake systems and intracellular peptidases [7].

Isolation and screening of microorganisms from natural sources have always been a powerful source of useful and genetically-stable strains for industrially-important products [8]. Armenia has well-defined vertical zoning which creates a vast variety of ecological-geographic conditions. This promoted the development of unique associations of LAB in traditional homemade dairy products such as matsoun and some types of cheeses[9]. The aim of this work was to isolate LAB strains with broad-range antimicrobial and proteolytic activity that may be useful in development of new probiotics or in dairy food production.

Materials and methods: Matsoun and cheese samples were collected from small farms from different villages of Armenia. Previously isolated strains stored at the Department of Microbiology, Plants and Microbes Biotechnology were also screened for proteolytic and antimicrobial activity. For isolation of LAB 1ml of matsoun, or 1 g of cheese were added to

9ml 0.96% saline solution to obtain 1:10 dilution and vortexed. Serial dilutions of the samples were made in saline solution. Dilutions were inoculated by pour plate technique [10] into milk agar for detection of proteolytic activity and cultivated at 37 °C for up to 48 h. Proteolytic activity was determined by the zone of hydrolysis around the colony. Colonies that showed proteolytic activity were picked and cultured into 10% skim-milk. Pure cultures were obtained by streaking onto MRS agar (HiMedia, India) and confirmed by microscope examination (Motic M10LB, Motic Europe, EU) of cells stained with methylene blue.

Determination of ability of LAB to hydrolyze skim-milk and soybean milk was carried out using method described by Bazukyan et al [11]. LAB were pre-cultivated overnight in MRS at 37 °C. 5% (v/v) of pre-cultures were inoculated into 10% skim-milk and 10% soybean milk and cultivated during 24 h at 37 °C. Then samples were mixed with loading buffer (3% β-mercaptoethanol, 20% glycerol, 50 mM Tris-HCl (pH 6.8), 1 crystal of bromphenol blue) at 1/1 volume ratio and boiled for 5 minutes. The reduction of proteins profile was analyzed by SDS-polyacrylamide gel-electrophoresis (SDS-PAGE) with 12% resolving gel and 4% stacking gel. Constant current of 20 mA was applied in a migration buffer (50 mM Tris, 0.384 M glycine, 0.1 M SDS). Samples were stained by 0.1% Coomassie brilliant blue G-250 dissolved in mixture of 300 ml ethanol, 50 ml acetic acid and 650 ml water for 1 h and detained in the same mixture without dye.

To investigate the proteolytic activity of LAB towards caseinsstrains were pre-cultivated for 72h at 37 °C on MCA plates (4.4% skim-milk, 0.8% sodium citrate, 0.1% yeast extract, 0.5% glucose, 1.5% agar). Then they were collected by washing in 100 mm potassium-phosphate buffer (pH 6.5) to a density of 20^{10} cells/ml. β-casein (5mg/ml), sodium caseinate (12 mg/ml) and lactoserum (5 mg/ml) dissolved in the same buffer were used as substrates. Cell suspension was mixed with substrates at 1/1 volume ratio and incubated at 37 °C for 24h. After incubation mixtures were centrifuged at 9000 ×g for 5 min, supernatants were mixed with loading buffer. Then samples were boiled for 5 min and analyzed by SDS-PAGE as mentioned above.

Selected strains were further screened for antimicrobial activity. For this purpose following microorganisms were used as test strains – gram-positive *Micrococcus luteus* WT, *Staphylococcus aureus* WDCM 5233 and *Bacillus subtilis*WT-A1, gram-negative *Pseudomonas aeruginosa*WT2, *Escherichia coli* VKPM-M17 and *Salmonella typhimurium* WDCM 1474, as well as yeasts - *Candida guilliermondii*HM-12 and *Debaryomyceshansenii*. Antimicrobial activity was studied using agar-well diffusion assay [12] with some modifications. Briefly, test strains were cultivated overnight in LB broth (10 g/l peptone, 5g/l yeast extract, 10 g/l NaCl, 5 g/l sucrose, 0.5 g/l $MgSO_4$). 100 µl of their cultural liquids was inoculated (pour plate) into LB agar (the same as LB broth with addition of 9 g/l agar). After solidification wells with diameter 6mm were cut aseptically in these plates. LAB strains were pre-cultivated overnight in 10% skim-milk and in MRS broth. 100 µl of their cultural liquid was added in these wells. Petri dishes were kept at room temperature for 1h for diffusion of antimicrobial substances, and then incubated at 37 °C for 24h. Then zones of growth inhibition were measured. A clear zone of inhibition of at least 2 mm was recorded as positive.

Results and discussion: From small farms of different provinces of Armenia samples of matsouns and cheeses were collected from spring 2012 to autumn 2013. These farms are located at the height 600-2120 meters above sea level. Products were prepared from cow, sheep and goat milks and their combinations. Interestingly, during summer period thermophilic lactobacilli were isolated more often compared with spring and autumn periods. More than 100 strains were isolated but only 13 of them showed proteolytic activity on skim-milk agar (Fig. 1).

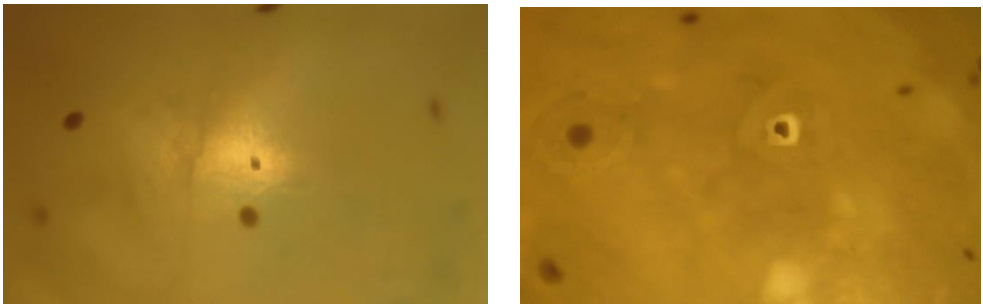

Figure 1. *LAB colonies with proteolytic activity on milk agar*

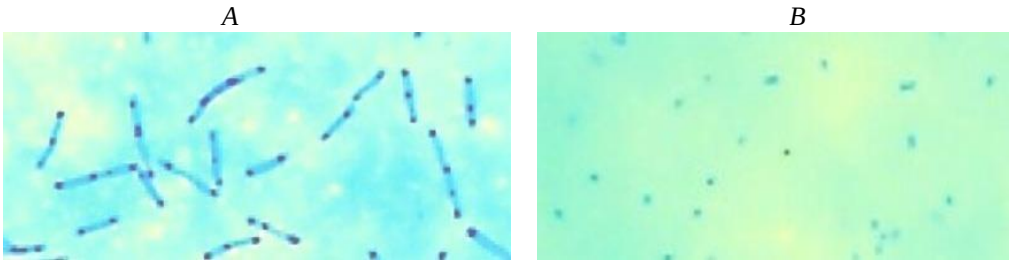

Figure 2. *Microscopic examination of methylene-blue stained cells, 1000 ×; A-Lactobacillus delbrueckiisubsp. lactisINRA-2010-4.2; B-T 3.1*

Pure cultures of all isolates were obtained and their proteolytic activity was studied by SDS-PAGE. Despite visual zones of hydrolysis around colonies, some isolates did not show significant proteolytic activity when analyzed by SDS-PAGE. None of the strains was able to hydrolyze soybean proteins and lactoserum. Maximal proteolytic activity was expressed by newly isolated strains T5.2, T5.3, T3.1, M6, M2, as well as by strains *Lactobacillus delbrueckii*subsp. lactis INRA-2010-4.2 and *Lactobacillus crispatus*INRA-2010-5.2, which were isolated previously.

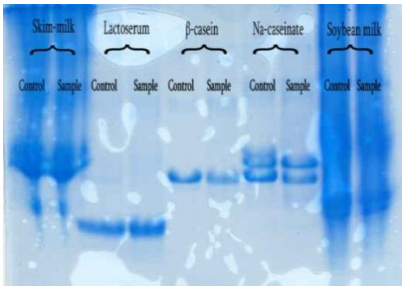

Figure 3. *Proteolytic activity of T5.3 strain analyzed by SDS-PAGE*

According to substrate specificity LAB have two main types of proteinases. The PI-type primarily degrades β-casein that is cleaved into more than100 different oligopeptides ranging from 4 to 30 amino acid residues. κ- and α_{S1}-caseins are cleaved to a lesser extent by the PI-type enzyme, whereas the PIII-type is able to cleave α_{S1}-, β–, and κ-caseins equally well [7]. All LAB strains used in this study possess PIII-type proteinases.

LAB strains that expressed proteolytic activity were also checked for their ability to synthesize antimicrobial compoundsduring growth in milk and MRS (Table 1).

Table 1.

Antimicrobial activity of LAB

Strain	Medium	*M. luteus*WT	*S. aureus*WDCM5233	*B. subtilis*WT-A1	*P. aeruginosa*WT-2	*E. coli*VKPM-M17	*S. typhimurium* WDCM 1474	*C. guilliermondii*HM-12	*D. hansenii*
M2	milk	0	0	0	0	0	0	0	0
	MRS	22	0	0	0	0	16	0	0
M6	milk	0	0	0	0	0	0	0	0
	MRS	23	0	0	0	0	13	0	0
T3.1	milk	0	0	0	0	0	0	0	0
	MRS	20	0	0	0	0	15	0	0
T5.2	milk	0	0	0	0	0	0	0	0
	MRS	21	0	0	0	0	14	0	0
T5.3	milk	0	0	0	0	0	0	0	0
	MRS	22	0	0	0	0	15	0	0
4.2*	milk	18	16	0	0	-0	16	0	0
	MRS	21	21	12	0	0	14	0	0
5.2**	milk	14	16	0	0	0	13	0	0
	MRS	21	16	12	0	0	10	0	0

*- *Lactobacillus delbrueckii*subsp. lactis INRA-2010-4.2;
**- *Lactobacillus crispatus*INRA-2010-5.2

All strains expressed antibacterial activity against gram-positive *M. luteus*and gram-negative *S. typhimurium*, but this activity was dependent from growth medium. All strains were able to synthesize antibacterial compounds against these two strains when grown in MRS, while only *Lactobacillus delbrueckii* subsp. *lactis* INRA-2010-4.2 and *Lactobacillus crispatus*INRA-2010-5.2 showed antibacterial activity when cultivated in skim-milk. Additionally, these two strains showed inhibitory activity against *B. subtilis* and *S. aureus* too. It should be noted that *Lactobacillus delbrueckii*s ubsp. *lactis* INRA-2010-4.2 and *Lactobacillus crispatus*INRA-2010-5.2 showed better antibacterial activity against gram-positive test strains when grown in MRS, but they inhibited gram-negative *S. typhimurium* more efficiently when cultivated in milk. These results are in accordance with similar literature data. It was shown that proteolytic LAB can produce peptides with antibacterial action against gram-negative microorganism, including *Salmonella* sp.[13].Thus, enhanced antimicrobial activity against gram-negative bacteria in skim-milk could be associated with activity of proteinases.

None of the tested strains had antimicrobial activity against gram-negative *P. aeruginosa* and *E. coli*, as well as against yeast strains *C. guilliermondii* and *D. hansenii*.

Thus, from over 100 tested strains 7 showed good proteolytic activity towards β-casein and Na-caseinate. They also expressed antibacterial activity against gram-positive and gram-negative test strains. These strains could be used in dairy production as starters, as well as in development of new probiotics.

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Եթեր Սիմոնյան, Անդրանիկ Քերյան, Ինգա Բազուկյան

ՀԱԿԱԲԱԿՏԵՐԻԱԿԱՆ ԵՎ ՊՐՈՏԵՈԼԻՏԻԿ ԱԿՏԻՎՈՒԹՅԱՄԲ ՕԺՏՎԱԾ ԿԱԹՆԱԹԹՎԱՅԻՆ ԲԱԿՏԵՐԻԱՆԵՐԻ ՄԵԿՈՒՍԱՑՈՒՄԸ ԱՎԱՆԴԱԿԱՆ ՀԱՅԿԱԿԱՆ ԿԱԹՆԱՄԹԵՐՔԻՑ

Բանալի բառեր՝ կաթնաթթվային բակտերիաներ, մեկուսացում, պրոտեոլիտիկ ակտիվություն, հակամանրէային ակտիվություն

Հայաստանի տարբեր մարզերի մածունի և պանրի նմուշներից անջատվել են կաթնաթթվային բակտերիաների ավելի քան 100 շտամներ: Ուսումնասիրվել է դրանց պրոտեոլիտիկ ակտիվությունը: Այս շտամներից 7-ը հիդրոլիզում էին կազեինը և նատրիումի կազեինատը: Դրանք օժտված էին նաև հակաբակտերիական ակտիվությամբ գրամ-դրական և գրամ-բացասական թեստ-օրգանիզմների նկատմամբ: Բոլոր ուսումնասիրված շտամների հակաբակտերիական ակտիվությունը դրսևորվում էր ՄՌԾ սննդամիջավայրում աճեցնելիս:

Етер Симонян, Андраник Керян, Инга Базукян

ВЫДЕЛЕНИЕ МОЛОЧНОКИСЛЫХ БАКТЕРИЙ С ШИРОКОЙ АНТИМИКРОБНОЙ И ПРОТЕОЛИТИЧЕСКОЙ АКТИВНОСТЬЮ ИЗ АРМЯНСКИХ ТРАДИЦИОННЫХ МОЛОЧНЫХ ПРОДУКТОВ

Ключевые слова: молочнокислые бактерии, изолирование, протеолитическая активность, антимикробная активность

В статье рассматривается более ста штаммов молочнокислых бактерий, полученных при изучении проб мацунов и сыров ИЗ различных регионов Армении. Изучена протеолитическая активность всех изолятов, 7 из которых гидролизовали β-казеин и казеинат натрия. Они также проявляли антибактериальную активность по отношению К грам-положительным и грам-отрицательным микроорганизмам. Антибактериальная активность всех изученных штаммов проявлялась при культивировании в MPC.

Yeter Simonyan, Andranik Keryan, Inga Bazukyan

ISOLATION OF LACTIC ACID BACTERIA WITH BROAD-RANGE ANTIMICROBIAL AND PROTEOLYTIC ACTIVITY FROM ARMENIAN TRADITIONAL DAIRY PRODUCTS

Keywords: lactic acid bacteria, isolation, proteolytic activity, antimicrobial activity

From matsoun and cheese samples collected from different provinces of Armenia over 100 strains of lactic acid bacteria have been isolated. Proteolytic activity of all isolates was studied. Seven strains were able to hydrolyze β-casein and sodium caseinate. They also showed antibacterial activity against gram-positive and gram-negative test strains. Antibacterial activity of all studied strains was expressed during cultivation in MRS.