

ISOLATION AND CHARACTERIZATION OF LACTIC ACID BACTERIA WITH HIGH PROTEOLYTIC ACTIVITY

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Abstract

The aim of this study was to isolate and identify lactic acid bacteria with high proteolytic activity from various natural sources, including fermented dairy and plant products. The proteolytic properties of the isolated strains were evaluated under various environmental conditions. LAB species were identified based on their morphophysiological, cultural, and biochemical characteristics using the Bergey’s Guide to Bacteria and the API 50 CH test. The effectiveness of the selected strains in milk fermentation and their impact on the organoleptic properties of finished dairy products were assessed. As a result of this study, 47 LAB strains were isolated from various fermented products, six of which exhibited high proteolytic activity. Based on morphophysiological and biochemical studies

and the API CH test, the selected strains were classified as *Lactobacillus delbrueckii*, *Lactobacillus plantarum*, *Lactobacillus salivarius*, *Lactocaseibacillus rhamnosus*, *Lactococcus lactis*, and *Streptococcus thermophilus*. Dairy products fermented with these LAB strains exhibited high microbiological, physicochemical, and organoleptic properties. The LAB strains and consortia obtained are proposed for use as starter cultures to develop new fermented dairy products, improve production technologies, and enhance product quality.

Keywords and phrases: Lactic acid bacteria, isolation and identification, proteolytic activity, milk fermentation, characteristics of fermented milk products.

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Համառոտագիր

Այս ուսումնասիրության նպատակն է տարբեր բնական աղբյուրներից, այդ թվում՝ ֆերմենտացված կաթնամթերքից և բուսական արտադրանքից, մեկուսացնել և նույնականացնել բարձր պրոտեոլիտիկ ակտիվությամբ կաթնաթթվային բակտերիաները: Մեկուսացված շտամների պրոտեոլիտիկ հատկությունները գնահատվել են տարբեր շրջակա միջավայրի պայմաններում: Բարձր ակտիվ պրոտեոլիտիկ ԿԹԲ շտամների տեսակների նույնականացումը կատարվել է դրանց մորֆոֆիզիոլոգիական, մշակութային և կենսաքիմիական բնութագրերի հիման վրա՝ օգտագործելով Բերջեյի մանրէների ուղեցույցը և API 50 CH թեստը: Ավելին, գնահատվել է ընտրված շտամների արդյունավետությունը կաթի խմորման գործընթացում և դրանց ազդեցությունը պատրաստի կաթնամթերքի օրգանոլեպտիկ հատկությունների վրա:

Արդյունքում տարբեր ֆերմենտացված արտադրանքներից մեկուսացվել է 47 ԿԹԲ շտամ, որոնցից վեցը բնութագրվում էին բարձր պրոտեոլիտիկ

ակտիվությամբ: Մանրամասն մորֆոֆիզիոլոգիական և կենսաքիմիական բնութագրումը, որին հաջորդել է API 50 CH նույնականացումը, ցույց է տվել, որ այս շտամները պատկանում են *Lactobacillus delbrueckii*, *Lactobacillus plantarum*, *Lactobacillus salivarius*, *Lacticaseibacillus rhamnosus*, *Lactococcus lactis* և *Streptococcus thermophilus* տեսակներին: Այս ԿԹԲ շտամներով ֆերմենտացված կաթնամթերքը ցուցաբերել է բարելավված ֆիզիկաքիմիական և օրգանոլեպտիկ հատկություններ: Ստացված ԿԹԲ շտամները և դրանց կոնսորցիումները առաջարկվում են որպես մեկնարկային կուլտուրաներ՝ նոր ֆերմենտացված կաթնամթերքի մշակման, արտադրական տեխնոլոգիաների կատարելագործման և արտադրանքի որակի բարելավման համար:

Բանալի բառեր և բառակապակցություններ՝ կաթնաթթվային մանրէներ, պրոտեոլիտիկ ակտիվություն, ֆերմենտացված կաթնամթերք, մեկուսացում և նույնականացում, մեկնարկային կուլտուրաներ, կաթի խմորում, օրգանոլեպտիկ հատկություններ:

ВЫДЕЛЕНИЕ И ХАРАКТЕРИСТИКА МОЛОЧНОКИСЛЫХ БАКТЕРИЙ С ВЫСОКОЙ ПРОТЕОЛИТИЧЕСКОЙ АКТИВНОСТЬЮ

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Аннотация

Целью данного исследования являлась изоляция и идентификация молочнокислых бактерий с высокой протеолитической активностью из различных природных источников, включая ферментированные молочные и растительные продукты. Протеолитические свойства изолированных штаммов оценивались в различных условиях окружающей среды. Видовую идентификацию МКБ проводили на основе их морфофизиологических, культуральных и биохимических характеристик в соответствии с «Определителем бактерий Берджи» и с использованием теста API 50 CH. Была оценена эффективность отобранных штаммов в процессе ферментации молока, а также их влияние на органолептические свойства готовых молочных продуктов. В результате проведенной работы из различных ферментированных продуктов было выделено сорок семь штаммов МКБ, шесть из которых отличались высокой протеолитической активностью. По данным морфофизиологических, биохимических исследований и API 50 CH теста отобранные штаммы были

относены к видам *Lactobacillus delbrueckii*, *Lactobacillus plantarum*, *Lactobacillus salivarius*, *Lactocaseibacillus rhamnosus*, *Lactococcus lactis* и *Streptococcus thermophilus*. Молочные продукты, ферментированные с использованием этих штаммов МКБ, отличались высокими микробиологическими, физико-химическими и органолептическими показателями. Полученные штаммы МКБ и их консорциумы предлагается использовать в качестве заквасочных культур для создания новых ферментированных молочных продуктов, улучшения производственных технологий и повышения качества продукции.

Ключевые слова и фразы: молочнокислые бактерии, выделение и идентификация, протеолитическая активность, ферментация молока, характеристика кисломолочных продуктов.

Intaduction

Lactic acid bacteria (LAB) play a crucial role in the fermented dairy industry due to their metabolic and technological activities [3, 11]. Since milk contains limited amounts of readily assimilable free amino acids and peptides, the growth and activity of LAB depend largely on their proteolytic system, including high protease and peptidase activities, which enable the hydrolysis of milk caseins and the formation of nutrients necessary for bacterial growth [3, 9, 11]. The proteolytic activity of LAB also determines the efficiency of milk coagulation, the organoleptic properties of fermented products—such as taste, aroma, texture, and acidity—as well as the formation of bioactive compounds [3, 10]. LAB strains with high proteolytic activity can be widely applied not only in fermented dairy products but also in the production of sourdough bread, sausages, silage, pickled products, beverages, and other fermented foods [6, 10].

The rapid development of global industry and advances in the understanding of enzyme structure and function have enabled the extensive application of enzymes in the production of high-quality consumer goods, thereby confirming their major industrial significance. Enzymes act as highly efficient biocatalysts that mediate all anabolic and catabolic pathways and significantly reduce the activation energy of biochemical reactions. Although enzymatic activity has been known and utilised by humans for centuries, recent progress in biotechnology has substantially expanded the industrial use of enzymes, with hydrolytic enzymes currently being among the most widely applied [11].

Within this context, lactic acid bacteria represent one of the most thoroughly studied groups of microorganisms [11]. Among their physiological characteristics, proteolysis is of particular importance and has been the subject of extensive research [3, 9, 11]. The proteolytic system of LAB is primarily associated with the utilisation of milk caseins and provides the cells with essential amino acids required for growth in milk-based environments [3,11]. Consequently, LAB and their proteolytic activity play a decisive role in the development of the organoleptic properties of fermented dairy products, including flavour, aroma, texture, and acidity [3, 9].

Beyond nutrient processing, cellular proteolysis is also involved in the regulation of protein quality and cellular homeostasis, ensuring the maintenance of appropriate levels of regulatory proteins and their removal when necessary [11]. The proteolytic system of LAB is a complex and well-organised network consisting

of cell-envelope proteinases, intracellular peptidases, and specific transport proteins [11]. Proteinases initially cleave casein into oligopeptides, which are subsequently transported across the cytoplasmic membrane and further degraded by intracellular peptidases into free amino acids and smaller peptides that can be directly utilised by the cell [1, 2].

The growing demand for proteolytic enzymes in food and biotechnology industries has intensified the search for new microbial sources of these enzymes, as well as the in-depth analysis of existing genetic resources [11]. At present, the majority of industrial enzymes are produced using recombinant microbial strains, making biocatalysts significantly more cost-effective than enzymes isolated directly from natural producers. Nevertheless, naturally occurring LAB strains remain a valuable source of diverse and highly efficient proteolytic systems [6, 11].

As a result, considerable research efforts have been devoted to understanding the activity, regulation, and physiological roles of the proteolytic system in lactic acid bacteria [8, 11]. These systems are of great importance for food fermentations and are widely exploited in the dairy, baking, brewing, and other fermentation-based industries [6, 10].

The work aims to isolate and identify strains with high proteolytic activity from various natural sources: fermented dairy products and plant foods.

Material and Methods

For the cultivation of LABs, solid and liquid LAPTg medium containing 10 g/L yeast extract, 15 g/L Bacto peptone, 10 g/L Bacto tryptone, 10 g/L glucose, and 1 mL/L Tween 80 were used; 15 g/L Difco agar was used for solidification. Skimmed milk powder.

Sampling was conducted from various natural sources, including fermented dairy products, plant-based foods, pickled foods, and other sources. To obtain pure cultures, 0.1 mL of the samples was inoculated into 2 mL of skimmed milk and spread plated on LAPTg agar, then incubated at 37°C for 48 h. The colonies capable of milk fermentation were used for further study.

The morphophysiological characteristics of the isolated LAB strains were evaluated, including Gram staining, microscopic examination of cells, and assessment of growth and stability under different temperature and pH conditions.

The proteolytic activity of LAB was determined using Eickman's milk agar medium. Whatman paper disks (5 mm) impregnated with culture medium were placed on agar, incubated for 24 h, and the diameter of clear zones formed around the disks as a result of casein proteolysis was measured.

Biochemical and cultural characteristics and identification of LAB were performed in accordance with Bergey's Manual of Systematic Bacteriology and using the API 50 CH test system (France) [4, 5].

The growth rate was determined by cultivation of LAB in LAPTg medium in 200 mL Erlenmeyer flasks under intensive agitation in a water bath shaker, with periodic measurement of optical density (OD) at a wavelength of 600 nm using a spectrophotometer.

The proteolytic activity of fermented dairy products was evaluated using the

method described by Shihata and Shah, with some modifications [3, 9].

LAB strains exhibiting pronounced proteolytic activity were tested in milk fermentation by assessing fermentation rate, bacterial titer, viscosity, pH, and titratable acidity [1, 2, 7].

The organoleptic properties, taste, and aroma of fermented lactic acid dairy products were evaluated in accordance with standard sensory evaluation guidelines.

Statistical Analysis

All data are expressed as mean \pm standard error of the mean (SEM) for the respective parameters. Statistical comparisons among groups within each experiment were performed using one-way analysis of variance (ANOVA). Statistical significance was established at $p \leq 0.05$.

Results

In the first phase of the study, approximately 47 Gram-positive, catalase-negative lactic acid bacteria (LAB) were isolated from various fermented products using the LAPTg selective medium. All isolated strains possessed milk fermentation activity. Morphometric studies revealed that 31 of these LAB were rod-shaped bacteria, while the remainder were solitary or chain-shaped cocci. All isolated strains fermented milk at different rates, indicating variability in their proteolytic activity. Using Eickman's milk agar, six LAB strains demonstrating the highest proteolytic activity were selected for further study. The zones of proteolysis around the disks impregnated in supernatants and culture fluids of the strains are presented in Figure 1.

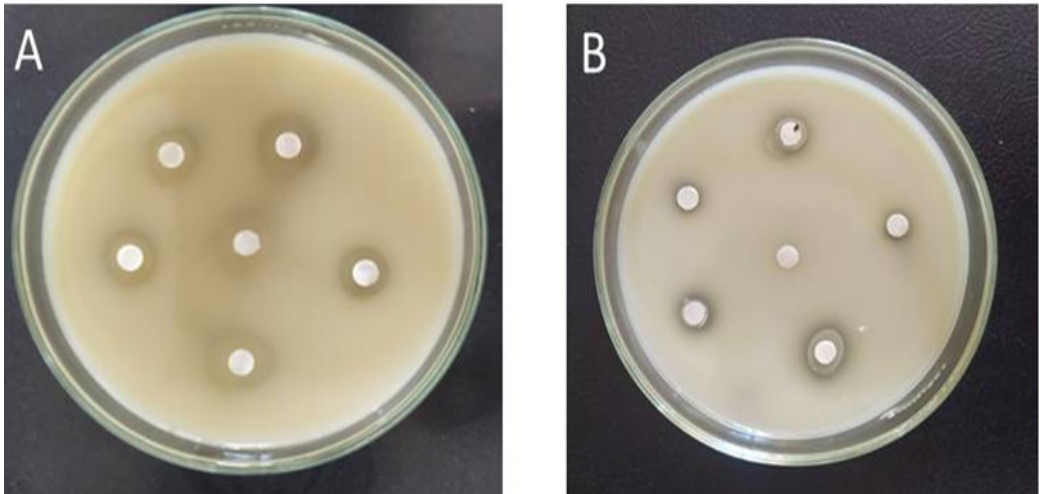


Figure 1. Proteolysis zones on Eickman's milk agar caused by culture fluids (A) and supernatants (B).

As shown in Fig. 1, compared to the supernatants (22–25 mm), the culture fluids formed larger proteolytic digestion zones (28–37 mm), likely due to continuous cell activity.

The proteolytic activity of the lactic acid bacteria supernatant was also assessed spectrophotometrically using Folin's reagent and is presented in Figure 2.

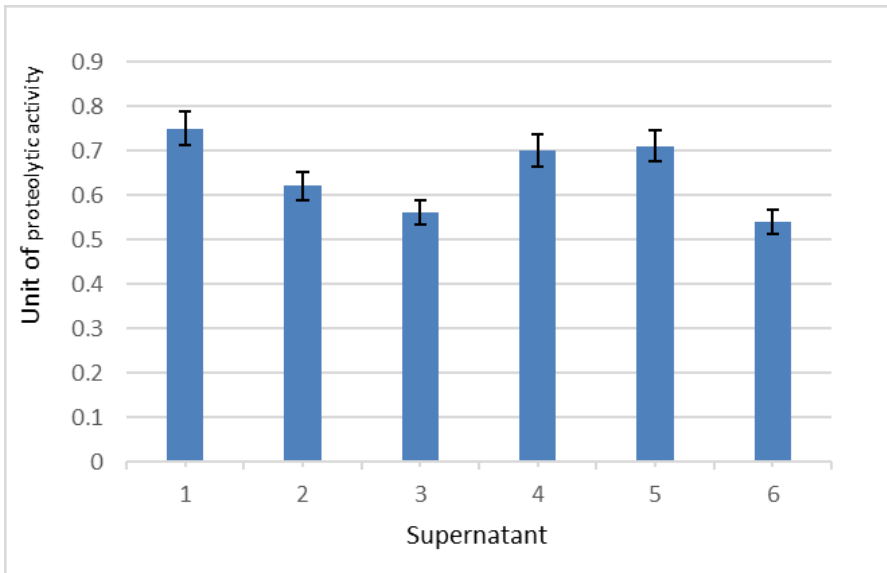


Figure 2. Proteolytic activity of strains determined by the spectrophotometric method

According to the data presented in Fig. 2, strains 1, 4, and 5 exhibit higher proteolytic activity than the others.

LABs identified according to Bergey's Manual were also identified using the API 50 test (Fig. 2).

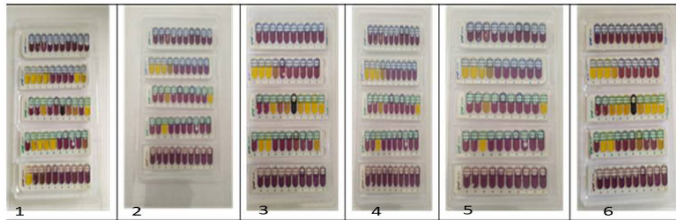


Figure 3. API 50 CH test profile.

According to the Bergey's Manual and API 50 CH test, the isolated strains with high proteolytic activity were identified as follows: species 1 - *Lactobacillus delbrueckii*, 2 - *Lactobacillus plantarium*, 3 - *Lactobacillus salivarius*, 4 - *Lacticaseibacillus rhamnosus*, 5 - *Lactococcus lactis*, 5 - *Streptococcus thermophilus*. The data obtained using Bergey's Manual of Systematic Bacteriology confirmed the results of the API 50 test [4, 5].

The cultural characteristics of the isolated LAB strains were studied.

The proteolytic activity of the isolated LAB cultures was evaluated at different growth temperatures (37°C, 45°C, and 50°C) for 48 hours, as well as under refrigeration conditions (4°C) for 14 days.

Table 2.

Proteolysis zones on Eickman's milk agar depending on ambient temperature.

LAB	Zones of proteolysis, mm.			
	4°C	37°C	45°C	50°C
<i>L. lactis</i> LH1	17±0.5	33±0.2	35±0.1	34±0.2
<i>L. delbrueckii</i> LH2	16±0.2	30±0.2	32±0.2	31±0.2
<i>L. plantarium</i> LH3	14±0.1	26±0.5	29±0.2	27±0.2
<i>L. salivarius</i> LH4	18±0.5	34±0.2	36±0.2	35±0.5
<i>L. rhamnosus</i> LH5	19±0.2	35±0.2	37±0.5	34±0.1
<i>St. thermophilus</i> LH6	8±0.5	26±0.2	28±0.1	28±0.2

It was found that the proteolytic activity is highest at a temperature of 45°C, where the size of the proteolysis zones reaches 33–37 mm. Interestingly, in the refrigerator, despite the absence of bacterial growth and metabolism, the formation of proteolysis zones is observed starting from the 5th day, the size of which reaches 8–19 mm by the 14th day.

The growth rate of lactic acid bacteria cultures in LAPTg medium at 37°C under aeration was also determined. The growth curves are shown in Fig. 4.

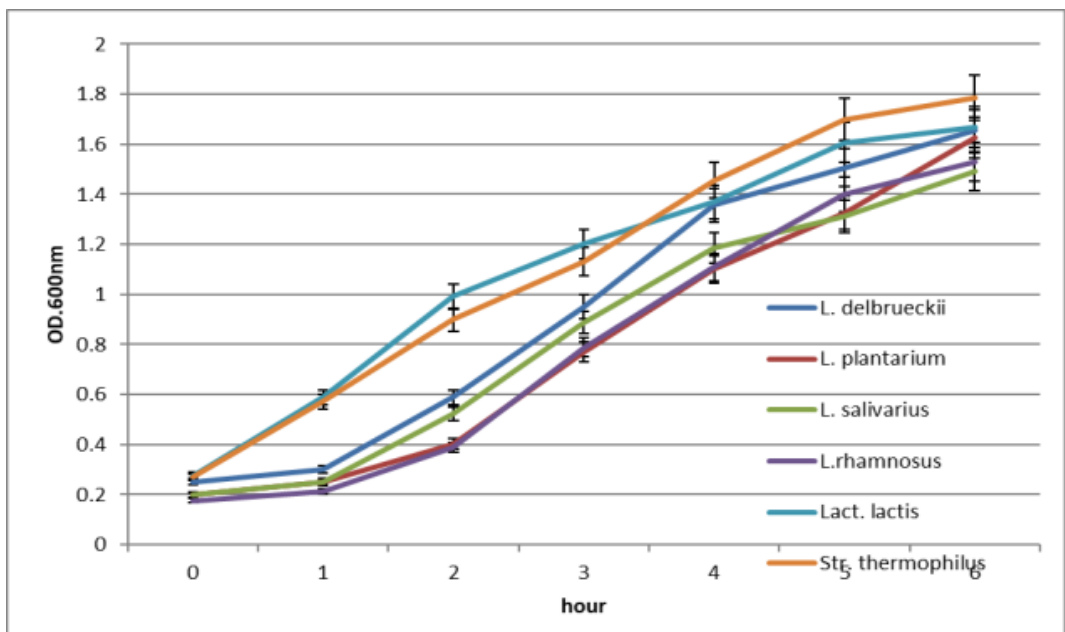


Figure 4. Growth rate of LAB strains in LAPTg medium with agitation at 200 rpm, at 37°C.

As shown in Fig. 4, coccoid cultures of *L. lactis* and *S. thermophiles* exhibit higher growth rates than lactobacilli and reach maximum biomass within 7 hours. Among lactobacilli, *L. delbrueckii* exhibits the highest growth rate.

The effects of the selected strains on the process of milk fermentation and on the physicochemical and organoleptic properties of the resulting dairy products were studied (Tab. 3).

Table 3.

Technological, microbiological, physicochemical, and organoleptic properties of fermented milk with LABs products

LABs	Milk curdling, h	pH	°Th	Viscosity, mPa · s	Titer CFU/mL	Organoleptic properties
<i>L. lactis</i> LH1	8	5.2	62	69.25	6.8 x 10 ⁸	average
<i>L. delbrueckii</i> LH2	6	4.8	85	59.06	8.5 x 10 ⁹	good
<i>L. plantarium</i> LH3	4	4.5	98	71.50	9.1 x 10 ⁹	excellent
<i>L. salivarius</i> LH4	4.5	4.7	95	77.66	8.4 x 10 ⁹	excellent
<i>Lactocaseibacillus rhamnosus</i> LH5	9	4.7	90	57.28	7.9 x 10 ⁹	good
<i>St. thermophilus</i> LH6	8	5.1	65	52.71	7.2 x 10 ⁸	average

As shown in the table, all the LAB obtained are capable of rapidly coagulating milk, although coccal cultures are slower than cupric cultures in terms of coagulation speed. The resulting preparations exhibit high viscosity, a homogeneous structure, and a high titer of viable bacteria. As a result of organoleptic evaluation, *L. delbrueckii* LH2, *L. plantarium* LH3 and *L. rhamnosus* LH5 received the highest score in terms of taste and aroma harmony. Thus, the proteolytic activity of LABs in particular determines the rate of milk coagulation, the organoleptic properties of fermented products: taste, smell, structure, texture, acidity, as well as the formation of bioactive compounds.

The LAB with high proteolytic activity can be used in monocultures as well as in symbiotic systems in the production of fermented dairy products and other fermented foods, including sourdough bread, sausages, silage, pickles, beverages, etc.

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