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Isolation and identification of new strains of lactic acid microorganisms from various traditional lactic acid products of the Central region of Kazakhstan

The article describes the process of isolation and identification of lactic acid microorganisms obtained from various types of traditional lactic acid products of the Central region of Kazakhstan. In the course of the work, 10 samples were taken. 5 individual isolates were isolated from them, including 2 isolates from ayran (CK-1, CK-4), 2 isolates from sour cream (CK-3, CK-5) and 1 isolate from koumiss (CK-2). While studying cultural and morphological properties, large long rods with the length of 0.5–1.2 μm long have been spotted, located singly or assembled in chains. They didn't form spores. They are facultative anaerobes. Gram-positive. Catalase-negative. The colonies are convex with a solid edge, opaque and not pigmented. The optimum growth temperature is 37 ºC. Genetic identification based on the analysis of the 16S *rRNA* nucleotide sequence has been performed. A fragment of the 16S *rRNA* gene was amplified by PCR method. After the terminal fragments had been removed, nucleotide sequences with the length of more than 650 base pairs were obtained, which were identified in GeneBank using the BLAST algorithm. Based on a comparison of the nucleotide sequences of the 16S *rRNA* gene fragment, the sequences of the corresponding genome regions were obtained. As a result of the work on the study of cultural and morphological characters and comparison of the analysis of the nucleotide sequence of the 16S *rRNA* gene, it was ascertained that isolates CK-1, CK-3, CK-4, CK-5 isolated from ayran and sour cream belong to the species *Lactobacillus plantarum*, and isolate CK-2 isolated from koumiss belongs to the species *Lactobacillus paracasei.*

Keywords: lactic acid products, lactic acid microorganisms, isolates, *Lactobacillus spp.,* DNA, PCR, identification, 16S *rRNA*.

Introduction

The food industry uses thousands of strains of hundreds of species of microorganisms that were primarily isolated from natural sources based on their beneficial properties, and then improved using various methods. In connection with the expansion of the production and the range of products, more and more representatives of the world of microorganisms are involved in the microbiological industry [1, 2].

An important way to replenish the collection fund with new valuable microbial genetic resources is a directed isolation from nature and the study of microorganisms with valuable properties, individual genes that control the synthesis of industrially valuable substances [3].

The development of the agricultural and food industries of biotechnology is economically significant. Lactic microorganisms with probiotic propertiesplay an important role in these sectors [4].

Unfortunately, the intensification of production and the reduction in the production of products cooked at home leads to a decrease in biodiversity. Therefore, the urgent issue is the development and the replenishment of collections of lactic acid microorganisms isolated from traditional fermented milk products [5].

One of the important tasks of collections of microorganisms is the accurate taxonomic identification of microorganisms. Currently, most collections use classic methods for identification. However, in the developed collections, a polyphase approach is used, which consists in combining all possible data of both a phenotypic and genetic nature in order to obtain reliable identification [6].

The aim of this work was the isolation and identification of lactic acid bacteria from various traditional lactic acid products of the Central region of Kazakhstan.

Materials and research methods

Samples of home-made lactic acid products (airan, koumiss, sour cream) selected in the Central region of Kazakhstan (Karaganda region, Bukhar-Zhyrau district, IE «Suleimenov AK») were used as research materials.

Obtaining accumulative cultures and isolating pure culture

In order to obtain an accumulative culture of lactic acid strains, a nutrient medium of the following composition g/l was used: skimmed milk powder — 87; yeast autolysate — 3 ml. Cultivation was carried out at 37 ºC for 24 hours.

Pure cultures were isolated by ten-fold dilutions followed by plating on Petri dishes with MRS agar medium. The grown isolated colonies were seeded by the loop into tubes on the surface of a beveled solid medium and cultivated at a temperature of 37 ºC for 48 hours. The purity of the cultures was checked by the absence of extraneous growth in the MPB liquid nutrient medium.

Study of the cultural and morphological properties of isolated isolates

Visual observation of cultures of the isolated isolates was carried out using a phase contrast microscope.

Analysis of the nucleotide sequence of the 16S rRNA gene in isolated isolates

DNA isolation. 1 ml of the bacterial culture was centrifuged for 10 minutes at 12,000 rpm, the supernatant was removed. The precipitate was suspended in 500 ml of TE buffer, 20 µl of lysozyme was added and incubated for 1 hour at 37 ºC. 30 µl of 10 % SDS and 3 µl of proteinase K were added to the suspension. The contents of the tubes were incubated for 12 hours at 37 ºC. To remove fragments of the cell wall, residual proteins, and polysaccharides, 100 µl of 5M NaCl and 80 µl of a CTAB solution (10 % CTAB in 0.7 M NaCl) were added, vortexed, and incubated for 10 minutes at 65 ºC. Then, 700 µl of chloroform/isoamyl alcohol (24/1) was added and centrifuged for 10 minutes at 12,000 rpm. The aqueous phase was transferred into a new tube. The purification procedure was repeated with chloroform/isoamyl alcohol (24/1) and centrifuged for 10 minutes at 12,000 rpm. The supernatant was collected in new tubes. 0.6 of the volumes of isopropanol were added. A microtube containing the reaction mixture was incubated for 30 minutes, and then centrifuged for 10 minutes at 12,000 rpm. The precipitate formed was washed with 70 % ethanol and centrifuged for 10 minutes at 12,000 rpm. The purified DNA sample was dissolved in 100 µl of a single TE buffer and placed in a thermostat at 60 °C for 30 minutes.

Amplification of a 16S rRNA gene fragment

The PCR reaction was performed with 8f5'universal primers — AgAgTTTgATCCTggCTCAg-3 and 806R-5'ggACTACACgggTATCTAAT [7] in a total volume of 20 µl. The PCR mixture contained 150 ng. DNA, 1 unit Maxima Hot Start Taq DNA Polymerase, 0.2 mM of each dNTP, 1 PCR buffer, 2.5 mM $MgCl_2$, 10 pmol of each primer. The PCR amplification program included prolonged denaturation of 95 ºC for 7 minutes; 30 cycles: 95 ºC — 30 seconds, 55 ºC — 40, 72 ºC — 1 minute; final elongation of 7 minutes at 72 ºC.

The analysis of PCR products was carried out on a 1 % agarose gel containing 3 µl of bromophenol blue in TAE buffer. Electrophoresis was carried out at 120 V for 25 minutes. The sizes of the molecules of the analyzed DNA samples were determined by comparing their electrophoretic mobility of the markers — DNA fragment of known molecular weight.

Nucleotide sequence determination

The PCR products were purified from unbound primers by the enzymatic method using exonuclease I and alkaline phosphatase [8].

The sequencing reaction was carried out using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applide Biosystems) according to the manufacturer's instructions, followed by fragment separation on a 3730xl DNA Analyzer (Applide Biosystems).

Nucleotide sequence analysis

The nucleotide sequences of the 16S *rRNA* gene of the identified strain were analyzed and combined into a common sequence in SeqMan software (Applide Biosystems).

Results and discussion

Isolation of lactic acid microorganisms

In the course of the work, 10 samples were selected from traditional home-made lactic acid products. 5 separate isolates were isolated from those samples, including: 2 isolates from ayran (CK-1, CK-4), 2 isolates from sour cream (CK-3, CK-5) and 1 isolate from koumiss (CK-2).

The morphology of isolated microorganisms is as follows — large long rods with the length of 0.5–1.2 μm long, elongated fixed rods, located singly or assembled in chains. They did not form spores. They are facultative anaerobes. Gram-positive. The colonies are convex with a solid edge, opaque and not pigmented. The optimum growth temperature is 37 ºC [9].

As a result of studying the cultural and morphological properties, the isolated isolates were identified as representatives of the genus *Lactobacillus spp.*

Nucleotide sequence analysis

DNA concentration was measured using a *Nano-Drop* spectrophotometer at a wavelength of 260 nm. The results are given in Table 1.

Table 1

The source of isolation and DNA concentration

Sample	The source of isolation	Concentration, ng/ul	A260/280
CK-1	Ayran	547.4	1.93
CK-2	Koumiss	498.0	1.94
CK-3	Sour cream	621.4	1.99
CK-4	Ayran	460.5	1.99
CK-5	Sour cream	389.1	2.0

As it can be seen from Table 1, at the samples with DNA extraction, the concentration varies from 389.1 to 621.4, the value of 260/280 from 1.93 up to 2.0.

As a result, DNA of the required quality and quantity was extracted from the samples.

A fragment of the *16S rRNA* gene with a molecular weight of about 800 bp was amplified by PCR method. PCR amplification products were used to determine the nucleotide sequence.

After the terminal fragments had been removed, nucleotide sequences with the length of more than 650 bp were obtained, which were identified in Gene Bank using the BLAST algorithm.

Based on a comparison of the nucleotide sequences of the 16S *rRNA* gene fragment, sequences of the corresponding genome regions were obtained. The identification results are given in Table 2.

Table 2

Identification of lactic isolates based on sequence analysis of 16S *rRNA* genes

Sample	Size of sequenced DNA, bp	Bacteria with homologous gene sequences16S *rRNA*	Identity, %	Registration number in GenBank
CK-1	507	*Lactobacillus plantarum* OSB	100.00	MK351320.1
CK-2	338	*Lactobacillus paracasei* BS303	97.04	MF521890.1
CK-3	552	*Lactobacillus plantarum* L15	100.00	MK713565.1
CK-4	205	*Lactobacillus plantarum* S67	99.51	KR011009.1
CK-5	213	*Lactobacillus plantarum* HBUAS51184	99.06	MH665802.1

According to the results of a comparative analysis of the 16S *rRNA* gene, isolates CK-1, CK-3, CK-4, CK-5 were identified as the representatives of the species *Lactobacillus plantarum* and the isolate CK-2 as *Lactobacillus paracasei*.

Thus, the analysis which we have performed based on a comparison of the 16S *rRNA* gene sequence has confirmed the cultural-morphological classification of the isolated strains.

The results obtained will subsequently be used to get a molecular genetic passport for strains of microorganisms.

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Орталық Қазақстан өңірінің түрлі дәстүрлі сүт қышқылды өнімдерінен сүт қышқылды микроорганизмдердің жаңа штаммдарын бөліп алу және идентификациялау

Мақалада Орталық Қазақстан өңірінің түрлі дәстүрлі сүт қышқылды өнімдерінен бөлініп алынған сүт қышқылды микроорганизмдердің бөлінуі және идентификациясы сипатталған. Жұмысты орындау барысында 10 үлгі іріктелініп алынды. Олардан 5 жекеленген изоляттар бөлінді, оның ішінде: 2 изолят (СК-1, СК-4) айраннан, 2 изолят қаймақтан (СК-3, СК-5) және 1 изолят (СК-2) қымыздан. Культуралды-морфологиялық қасиеттерін зерттеу барысында ұзындығы 0,5–1,2 мкм ірі ұзын, дара немене тізбектес орналасқан таяқшалар кездесті. Спора пайда болған жоқ. Факультативтік анаэробтар. Грамоң. Каталазатеріс. Колониялар тұтас шеті дөңес, мөлдір емес және пигменттелмеген. Оңтайлы өсу температурасы 37 ºС. 16S *rRNA* нуклеотидті бірізділікті талдау негізінде генетикалық идентификациялау жүргізілді. ПТР әдісімен *16S rRNA* генінің фрагменті амплифицияланды. Соңғы фрагменттерді жойғаннан кейін ұзындығы 650 астам нуклеотидті тізбектер алынды, олар GeneBank-те BLAST алгоритмі бойынша сәйкестендірілді.16S *rRNA* генінің фрагментінің нуклеотидтік тізбектерін салыстыру негізінде геномның тиісті учаскелерінің сиквенстері алынды.Жүргізілген культуралдық-морфологиялық талдау негізінде, сондай-ақ 16S *rRNA* генінің нуклеотидтік бірізділік талдау нәтижелерін салыстыру барысында айран және қаймақтан алынған СК-1, СК-3, СК-4, СК-5 изоляттары *Lactobacillus plantarum,* ал қымыздан алынған СК-2 изоляты *Lactobacillus paracasei* түріне жататындығы анықталды.

Кілт сөздер: сүт қышқылды өнімдер, сүт қышқылды микроағзалар, изоляттар, *Lactobacillus spp*., ДНҚ, ПТР, идентификация, 16S *rRNA*.

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Выделение и идентификация новых штаммов молочнокислых микроорганизмов из различных традиционных молочнокислых продуктов Центрального региона Казахстана

В статье описан процесс выделения и идентификации молочнокислых микроорганизмов, полученных из различных традиционных молочнокислых продуктов Центрального региона Казахстана. В ходе выполнения работы было отобрано 10 образцов. Из них было выделено 5 отдельных изолятов, в том числе 2 изолята из айрана (СК-1, СК-4), 2 изолята из сметаны (СК-3, СК-5) и 1 изолят из кумыса (СК-2). При изучении культурально-морфологических свойств встречались крупные длинные палочки длиной 0,5–1,2 мкм, расположенные единично или собранные в цепочки. Спор не образовывали. Факультативные анаэробы. Грамположительные. Каталазоотрицательные. Колонии выпуклые, с цельным краем, непрозрачные и не пигментированы. Оптимальная температура роста 37 ºС. Проведена ге-

нетическая идентификация на основании анализа нуклеотидной последовательности *16SrRNA*. Методом ПЦР был амплифицирован фрагмент гена *16SrRNA*. После удаления концевых фрагментов были получены нуклеотидные последовательности протяженностью более 650 п.н., которые были идентифицированы в GeneBank по алгоритму BLAST. На основе сравнения нуклеотидных последовательностей фрагмента гена *16SrRNA* были получены сиквенсы соответствующих участков генома. В результате проделанной работы по изучению культурально-морфологических признаков и сравнению анализа нуклеотидной последовательности гена 16S*rRNA* было установлено, что изоляты СК-1, СК-3, СК-4, СК-5, выделенные из айрана и сметаны, относятся к виду *Lactobacillus plantarum*, а изолят СК-2, выделенный из кумыса, — к виду *Lactobacillus paracasei*.

Ключевые слова: молочнокислые продукты, молочнокислые микроорганизмы, изоляты, *Lactobacillus spp*., ДНК, ПЦР, идентификация, 16S*rRNA.*

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