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**PCR AMPLIFICATION OF SAKACIN P STRUCTURAL GENE IN
Lactobacillus sakei T54 ISOLATED FROM IRANIAN CHEESE****T.B.MIRHADIZADI, N.A.ABDULLAYEVA, A.A.GULIYEV**
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Strain used in this study was isolated from homemade cheese manufactured by household in Tebriz city of Iran. Phenotypic identification was carried out by Gram staining, catalase production test and microscope examination of the cell morphology. 16S rRNA fragment sequencing resulted in identification of strain as *Lactobacillus sakei*. Strain T54 showed the strong antilisterial activity against *Listeria innocua*. The activity was totally disappeared after 1 h incubation with proteolytic enzymes, which indicate the proteinaceous nature of active substance. Several primers specific for bacteriocins of *Lactobacillus sakei* were used for screening of bacteriocin genes in the genome of studied strain. PCR amplification resulted in detection of sakacin P structural gene in the genome of *Lactobacillus sakei* T54. *Lactobacillus sakei* T54 is a producer of bacteriocin which is most probably sakacin P. and could be used as natural bioconservant in food industry.

Key word: lactic acid bacteria, antilisterial activity, Iranian cheese, PCR.

Introduction

Lactic acid bacteria (LAB) are known for their production of antimicrobial compounds, including bacteriocins and bacteriocinlike peptides [2]. Bacteriocins of LAB are defined as ribosomally synthesized proteins or protein complexes usually antagonistic to genetically closely related organisms [2, 8]. They are generally low molecular weight proteins that gain entry into target cells by binding to cell surface receptors. Their bactericidal mechanisms vary and may include pore formation, degradation of cellular DNA, disruption through specific cleavage of 16S rRNA, and inhibition of peptidoglycan synthesis [2, 4, 5].

Sakacins are bacteriocins produced by *Lactobacillus sakei* strains, that show high inhibitory activities towards *Listeria monocytogenes*, a common food-borne pathogen isolated from milk and milk products, meat and meat

products, and vegetables. Because of this capability, strains able to produce sakacins have been often investigated for their use as bioprotection agents in food fermentations [15]. Several types of sakacins have been identified and characterized: sakacin A [6], sakacin M [12], sakacin P [13], sakacin G [11], and sakacin X [16]. Among these bacteriocins, the molecule that has been most intensively investigated is sakacin P. Sakacin P (sakP) is a 43-amino-acid, hydrophobic, class IIa bacteriocin produced by *Lactobacillus sakei* [3]. The expression is regulated by a three-component system consisting of an induction factor, a histidine kinase and a response regulator. The induction factor for sakP synthesis is a bacteriocin-like peptide secreted by the production organism itself and serves as the signal for transcriptional activation of the genes necessary for bacteriocin production [1].

The objective of this study was to identify the type of bacteriocin produced by *Lb. sakei* strain T54 isolated from Iranian cheese, with the aim of using this strain as a bio-preservative culture in food industry.

Material and methods

Isolation and Identification. Strain used in this study was isolated from homemade cheese manufactured by household in Tebriz city of Iran. Phenotypic identification was carried out by Gram staining, catalase production test and microscope examination of the cell morphology. Purified isolate was reconstituted in sterile skim milk (12.5%, w/v) supplemented with 30% (w/v) glycerol and stored at -80°C . Before using strain was propagated twice in de Mann-Rogosa-Sharpe (MRS) media (Biokar diagnostics, France) at 37°C . Genotypic identification was carried out by 16 S rRNA fragment sequencing with universal primers fD1 (5'-AGAGTTTGATCCTGGCTCAG-3') and rD1 (5'-TAAGGAGGTGATCCAGGC-3') [17]. Genomic DNA was isolated by DNeasy purification kit (Qiagen, Hilden, Germany) from a 3 mL overnight culture inoculated with a single colony according to the manufacturer and stored at -20°C until use. DNA sequencing was carried out by MilleGen sequencing service (Labège, France). The blast algorithm (http://blast.ncbi.nlm.nih.gov/Blast.cgi?CMD=web&PAGE_TYPE=BlastHome) was used to determine the most related sequence relatives in the NCBI nucleotide sequence database.

Antimicrobial activity assay. The antimicrobial activity of cell-free supernatants was determined by well diffusion method [10] with slight modification. Cell-free supernatants of overnight (16-18h) cultures were obtained by centrifugation at 10000g for 15 min at 4°C , adjusted to pH 6.5 with 1N NaOH. To investigate the antibacterial activity by well diffusion assay, 100 μl -culture of one of the test bacteria, grown to the early stationary growth phase in nutrient medium, was added to 20 ml of soft nutrient agar (0,8%, w/v). Wells were made in the lawn of hardened soft agars in Petri

dishes. Aliquots (50 µl) of supernatants of overnight culture were poured in the wells. The plates were left for 40 min at room temperature in sterile conditions before incubating them at the adequate temperature of growth of the test microorganism. A clear zone of inhibition of at least 2mm in diameter was recorded as positive. As a test organism the strain of *Listeria innocua* was used.

Effect of enzymes. To test biochemical nature of active substance, the supernatant of the strain culture was treated with the following enzymes (final concentration 0.1mg/ml and 1 mg/ml): protease TypeX (from *Bacillus thermoproteolyticus* rokko) and α-Chymotrypsin Type II (from bovine pancreas) and trypsin in 0.05 M Tris HCl (pH 7); Protease Type VIII (from *Bacillus licheniformis*) in 0.05 M Tris HCl(pH 8); Lipase Type VII(from *Candida rugosa*, Sigma) in 0.05 M Tris HCl (pH 8); Catalase (from bovine liver, Sigma) in 0.01 M phosphate buffer (pH 7); Amylase (Sigma) in 0.01 M Tris HCl (pH 7). Reaction mixtures were incubated at 37⁰C for 2 h. After 2 h of incubation at 37⁰C enzyme activity was stopped by heating in 100⁰C for 10 min. Untreated supernatant and mixture of media with enzyme-buffer in the same concentration (also incubated at 37⁰C for 2 h) were used as controls. The remaining activity was tested by the agar-well diffusion assay against indicator strain.

PCR amplification and sequencing of the bacteriocin genes. The primers used for the screening of bacteriocin genes are listed in Table 1.

Table 2.3

Primers used for detection of bacteriocin genes

Target gene	Primer (5' – 3')	Product size (bp)	Ann. T(°C)	Refer.
Sak GA1	F: TTA GAA CTA CAC TGA TCG TG R: TGG AAG AAT GAG TAC TTG TT	186	38	[14]
Sak GA2	F: CGT TAC AAC AGA ACT TCA AG R: TGG AAG AAT GAG TAC TTG TT	186	38	[14]
Sak P	F: ATGGAAAAGTTTATTGAATTA R: TTATTTATTCCAGCCAGCGTT	186	40	[9]
Sak Q	F: CAAGATCGTTAACCAGTTT R: CCGTTGTTCCCATAGTCTAA	1238	44	[7]
Sak X	F: GCTATGAAAGGTATTGTCGGG R: TAAGATTTCCAGCCAGCAGC	156	46	[7]

For all sets of primers, a reaction mixture, in a final volume of 25 µl, containing 10 mM Tris–HCl, 50 mM KCl, 1.5 mM MgCl₂, pH 8.3, 200 nM deoxyribonucleotide triphosphates, 200 nM each primer, 0.125 U Taq polymerase (Applied Biosystems, Milan, Italy), and 100 ng DNA was prepared. DNA thermal cycler model (Techno, Barloworld scientific, Cambridge, UK) was used for the amplification, and 35 repetitions of 95°C for 1 min,

appropriate annealing temperature depending on primer for 45 s, and 1 min at 72°C were performed. An initial denaturation at 95°C for 5 min and a final extension at 72°C for 7 min were carried out as well. Agarose gels (2% [wt/vol] in Tris borate ethylenediamine tetraacetic acid, pH 8, containing 0.5 µg/ml ethidium bromide, Sigma, Milan, Italy) were used to visualize the PCR products under UV light.

Results and discussion

The source of the natural isolate T54 was a semi-hard white cheese manufactured from cow's milk by addition of 1% (v/v) homemade rennet to pasteurized milk. Cheese ripened for 1.5 month in the presence of 3% NaCl.

Isolate was phenotypically identified as catalase negative Gram-positive bacilli. After several steps of purification by streaking on MRS agar, the pure culture was used for detection of antimicrobial activity. Strain T54 showed the strong antilisterial activity against *Listeria innocua* (Fig. 1) and was preliminarily identified as bacteriocin producer.

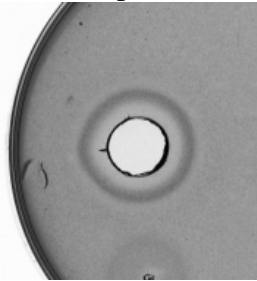


Fig. 1. Agar plate indicating the antimicrobial activity against *Listeria innocua*

In order to determine the nature of antimicrobial substance, the cell free supernatant from the studied strain was treated with catalase, α-amylase, lipase and proteolytic enzymes (Fig. 2).

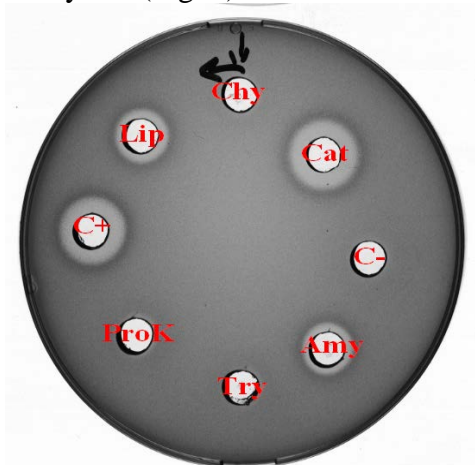


Fig. 2. The effect of enzymes on antimicrobial activity

The activity was totally disappeared after 1 h incubation with proteolytic enzymes, which indicate the proteinaceous nature of active substance. From this we can suppose that it is bacteriocin. Catalase, lipase and α -amylase have no effect on the antimicrobial activity. Antimicrobial activity didn't decrease in the presence of catalase, which excludes inhibition by hydroxide peroxide.

The obtained isolate was identified to species level by 16S rRNA fragment sequencing. For this reason the DNA from fresh cultures of obtained isolates was extracted (Fig.3 a) and used as template for 16S rRNA fragment amplification. PCR amplification resulted in detection of amplicon with expected size of 16S rRNA on the agarose gel (Fig. 3b). The results of sequencing showed that isolate T 54 belong to *Lactobacillus sakei* species.

Several primers specific for bacteriocins of *Lactobacillus sakei* were used for screening of bacteriocin genes in the genome of studied strain (Table 1). PCR amplicon of the expected size (186 bp) was obtained only with the pair of primers that targets the structural gene of sakacin P (*sak P*) (Fig. 4).

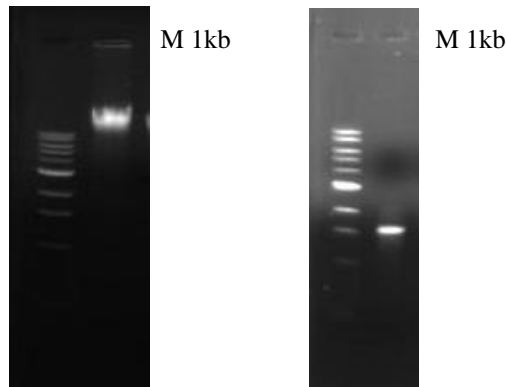


Fig. 3. DNA extraction (a) and amplification of 16S r RNA (b)

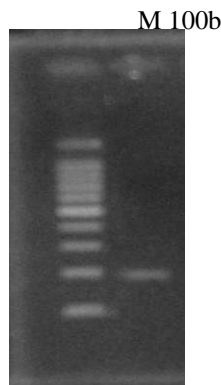


Fig. 4. Amplification of sakacin P structural gene in *Lactobacillus sakei* T 54

Lactobacillus sakei T54 is a producer of bacteriocin which is most probably sakacin P. This bacteriocin has strong antilisterial activity. The strain could be used as natural bioconservant in food industry.

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İRAN PENDİRİNDƏN İZOLYA OLUNMUŞ LACTOBACILLUS SAKEI T54 ŞTAMINDA SAKASIN P GENİNİN AMPLİFİKASİYASI

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XÜLASƏ

Tədqiqatda istifadə olunmuş ştam İranın Təbriz şəhərində ev şəraitində istehsal olunmuş pendirdən alınmışdır. Fenotipik identifikasiya Gram test, katalaza testi və hüceyrə morfoloqiyasının mikroskopik tədqiqatı vasitəsilə aparılmışdır. 16S rRNT fraqmentinin sekvensiyası ştamın *Lactobacillus sakei* kimi identifikasiyası ilə nəticələndi. Ştam antilisterial aktivlik nümayiş etdirdi. Antimikrob aktivlik proteolitik fermentlərin təsiri nəticəsində tamamilə itmişdir. Bu aktiv substansiyanın zülal təbiətli olduğuna dəlalət edir. *Lactobacillus sakei* növünə aid olan bakteriosinlərin praymerləri vasitəsilə tədqiq edilən ştamda bakteriosin geninin skrininqi aparılmışdır. PCR amplifikasiya nəticəsində *Lactobacillus sakei* T54 ştamının genomunda sakasin P geni müəyyən edilmişdir. *Lactobacillus sakei* T54 bakteriosin produsentidir və qida sənayesində təbii biokonservant olaraq istifadə oluna bilər.

Açar sözlər: süd turşusu bakteriyaları, antilisterial aktivlik, İran pendiri, PZR.

АМПЛИФИКАЦИЯ САКАЦИН Р КОДИРУЮЩЕГО ГЕНА У ШТАММА *Lactobacillus SAKEI T54* ИЗОЛИРОВАННОГО ИЗ ИРАНСКОГО СЫРА

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РЕЗЮМЕ

Штам, использованный в данном исследовании, был изолирован из сыра, произведенного в домашних условиях в городе Тебриз. Фенотипическая идентификация была проведена при помощи Грамм окрашивания, теста на продукцию каталазы и микроскопического исследования клеточной морфологии. В результате секвенирования фрагмента 16S рРНК было определено, что штамм относится к виду *Lactobacillus sakei*. Данный штамм проявил антилистериальную активность, которая полностью исчезла при действии протеолитических ферментов. Это указывает на белковую природу активного компонента. Ряд праймеров, специфичных для бактериоцинов вида *Lactobacillus sakei*, были использованы для идентификации типа бактериоцина, продуцируемого исследуемым штаммом. В результате ПЦР амплификации в геноме штамма *Lactobacillus sakei T54* был идентифицирован структурный ген сакацина Р. *Lactobacillus sakei T54* является продуцентом бактериоцина и может быть использован в качестве натурального биоконсерванта в пищевой промышленности.

Ключевые слова: молочнокислые бактерии, антилистерическая активность, иранский сыр, ПЦР.

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