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MOLECULAR DIAGNOSTIC OF *Streptococcus thermophilus*

DIAGNOSTYKA MOLEKULARNA *Streptococcus thermophilus*

Abstract: *Streptococcus thermophilus* is one of the most important lactic acid bacteria in the dairy industry. Despite the wide use of *Streptococcus thermophilus* in the industry, data on the phenotypic and genetic strain variations within the species are still limited. Genetic techniques are very useful for molecular discrimination of complex mixtures of starter and probiotic cultures in research laboratories. Detection and identification of various lactic acid bacteria species with rapid methods is often important for quality control of dairy products. This work deals with characterization and differentiation of strains *Streptococcus thermophilus* by PCR, RAPD and SDS-PAGE techniques. Fifteen strains of *Streptococcus thermophilus* from Czech Collection of Dairy Microorganisms (CCDM) and a strain of *Streptococcus thermophilus* from Czech Collection of Microorganisms (CCM) were used. Particular strains were confirmed with primer set THI/THII by PCR method. Consequently, their identities were examined by RAPD and SDS-PAGE techniques. Whereas, primers OPP-7 and RAPD-4, RAPD were used. It can be claimed that mentioned methods are good means for identification and characterization of streptococci.

Keywords: *Streptococcus thermophilus*, PCR, RAPD, SDS-PAGE

Streptococcus salivarius subsp. *thermophilus*, commonly named as *Streptococcus thermophilus* in food industry and throughout this paper, is one of significant species among the diverse group of lactic acid bacteria. *Streptococcus thermophilus* is widely used as a component of starters in milk fermentation processes, especially in yoghurt and cheese production and also in probiotic preparations [1–6].

Nowadays, the identification and classification of microorganisms are practised by a large variety of genotypic and phenotypic methods. The traditional time-consuming methods exploited for the identification and enumeration of bacteria are quickly replaced by methods of molecular biology, such as PCR, RAPD and SDS-PAGE. These

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genetic techniques are very useful for molecular discrimination of complex mixtures of starter and probiotic cultures in research laboratories. In addition, mentioned rapid methods are often important for quality control of dairy products [7–10]. PCR is highly sensitive technique, which is largely used as detecting and identifying tool for bacteria in different environments. Restriction analysis of genes amplified by PCR also contributes to strains distinction at the species, subspecies or other taxonomic levels [2, 11]. RAPD-PCR is one of the most popular genotypic typing techniques. It discriminates particular bacterial species of one strain and also serves as means for identification of DNA polymorphisms in the genome of selected strains. RAPD has been used for classification of a variety of food-borne microorganisms. For example, it allowed differentiation of both species and strains of lactic acid bacteria isolated from various collections and dairy products [6, 8, 12]. SDS-PAGE is widely applied method for identification of lactic acid bacteria [10].

Brigidi et al [2] tried to develop a rapid and easy-to-use PCR protocol for directly detecting and enumerating *Str. thermophilus* in their work and they affirmed that culture-independent or colony-based PCR method is available for detection of *Streptococcus thermophilus* species. Further, Langa et al [9] found that PCR is technically and economically affordable for laboratories, which do not have easy access to more sophisticated or expensive procedures. Delorme [13] used technique RAPD-PCR for investigation of new *Str. thermophilus* strains and their differentiation from *Str. salivarius* and *Enterococcus* spp. Soomro and Masud [10] analysed cell-free extracts of lactic acid bacteria by SDS-PAGE and they concluded that this method is reliable for molecular characterization of these microorganisms. Despite the wide-spread application of *Str. thermophilus* in the dairy industry, data on the phenotypic and genetic strain diversity within the species are still limited [12].

So, this work is focused on molecular diagnostic of *Streptococcus thermophilus* strains by mentioned genetic methods. Another goal of this study was to confirm applicability of given techniques for *Str. thermophilus* detection.

Materials and methods

In this study fifteen strains of *Streptococcus thermophilus* from Czech Collection of Dairy Microorganisms (CCDM) and a strain of *Streptococcus thermophilus* from Czech Collection of Microorganisms (CCM) were used. These microorganisms were grown in M17 broth for 48 hours at 37 ^{o}C.

Chromosomal DNA was isolated by modified method of Graves and Swaminathan [14]. PCR was accomplished in a DNA Engine® Peltier Thermal Cycler PTC-200 (BioRad). Amplification of *Str. thermophilus* strains was achieved using the 16S-23S rDNA primer set ThI/ThII (ThI: 5′-ACGGAATGTACTTGAGTTTC-3′; ThII: 5′-TGGCCTTTCGACCTAAC-3′) [7]. Composition of PCR reaction mixture (25 mm^3) was 15 mm^3 of distilled water, 0.5 mM dNTP mix, 0.8 μM each primer, 0.6 mM $MgCl_2$, 2.5 mm^3 of reaction buffer, 1 U of Taq polymerase and 2 mm^3 of chromosomal DNA. After optimization the thermocycle program comprise successive steps: 1 cycle of 95 ^{o}C for 5 min, followed by 40 cycles of 95 ^{o}C for 1 min, 52 ^{o}C for 30 s and 72 ^{o}C for 1 min,

then 1 cycle of 72 °C for 5 min. Amplified products were subjected to gel electrophoresis in 1.5 % gel and were visualized by ethidium bromide staining.

DNA polymorphisms were defined by RAPD-PCR using the same isolated chromosomal DNA as this for PCR. Amplification was performed on a DNA Engine® Peltier Thermal Cycler PTC-200 (BioRad) in a 25 mm^3 reaction mixture consisting of 15 mm^3 of distilled water, 0.5 mM dNTP mix, 8 μM each primer, 0.6 mM $MgCl_2$, 2.5 mm^3 of reaction buffer, 1 U of Taq polymerase and 2 mm^3 of chromosomal DNA. In this work, primers RAPD-4 (5′-AAGAGCCCGT-3′) and OPP-7 (5′-GTCCATGCCA-3′) were applied [6]. The PCR program was as follows: 1 cycle of 2 min at 94 °C, 45 cycles of 1 min at 94 °C, 1 min at 34 °C, 2 min at 72 °C and 1 cycle of 7 min at 72 °C. Amplified products from RAPD method were subjected to gel electrophoresis in 1.5 % gel and were visualized by ethidium bromide staining as well as PCR products.

Preparation of bacterial samples for SDS-PAGE was performed in this way: broth with accrued cells was centrifuged. The pellet was washed twice with distilled water and then resuspended in distilled water again. Then 3 mm^3 of lysosyme (3 $mg \cdot cm^3$) were added to 100 mm^3 of resuspended cells and this mixture was incubated at 37 °C for 3 hours. After that, 25 mm^3 of 20 % SDS and sample buffer were added so that total concentration of proteins was 100 $\mu g \cdot cm^3$ in sample and total volume of sample was 250 mm^3. Sample was immediately boiled for 10 min. In this manner prepared sample was used for analysis by SDS-PAGE. Protein Marker, Broad Range (212 to 2.3 kDa; BioLabs) was used as standard. Separating gel with concentration 12 % and 5 % resolving gel were chosen for proteins separation. Amount of 20 mm^3 of each sample was loaded on gel. After electrophoresis, gel was fixed for 20 min and then stained in silver nitrate solution according to Kirkeby et al [15].

Gels from all methods were analysed and molecular weights of DNA bands and protein fractions were calculated by using program UltraQuant (Ultra.Lum, USA). Surveyed gels from SDS-PAGE were statistically evaluated by cluster analysis using program Unistat 5.5, hence dendrograms were projected.

Results and discussion

Given strains were confirmed by technique PCR using primer set ThI/ThII. First of all, it was necessary to make gradient PCR to determine optimal annealing temperature. From obtained results temperature 52 °C was chosen. Another problem was purity of isolated DNA, so PCR reaction mixture was fortified with 0.4, 0.6, 0.8 and 1.0 mM $MgCl_2$. This study revealed that concentration 0.6 mM $MgCl_2$ is sufficient (Fig. 1). After these two optimizations, PCR with the others *Str. thermophilus* strains were performed. It was observed that applied primer set ThI/ThII is suitable for detection of the target species. Mentioned primers provided a PCR product with the expected size of 250 bp (Fig. 3). Similarly, Brigidi et al [2] described this size of PCR product in their study of primer set ThI/ThII specificity to *Streptococcus thermophilus* strains.

DNA polymorphism was examined by RAPD-PCR. After evaluation of gels by program UltraQuant (Ultra.Lum, USA), it can be claimed that obtained results of primer RAPD-4 were distinct from results of primer OPP-7. Regarding primer RAPD-4 the

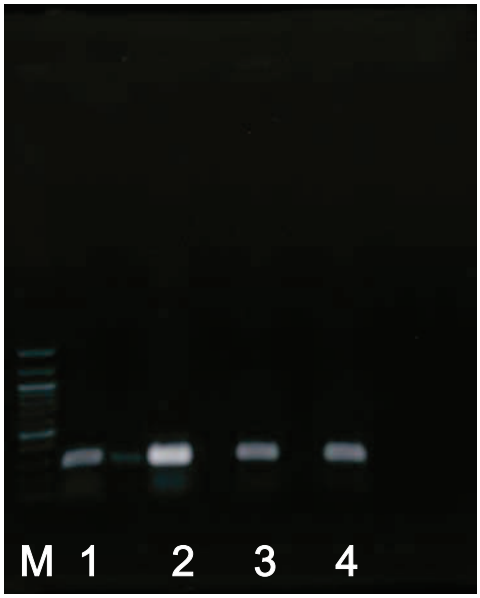

Fig. 1. Optimalization of PCR for strain CCDM 128 by addition of 10 mM $MgCl_2$ to reaction mixture. M – molecular weight marker 100-bp DNA ladder, lane 1 – 0.4 mM, lane 2 – 0.6 mM, lane 3 – 0.8 mM, lane 4 – 1.0 mM $MgCl_2$

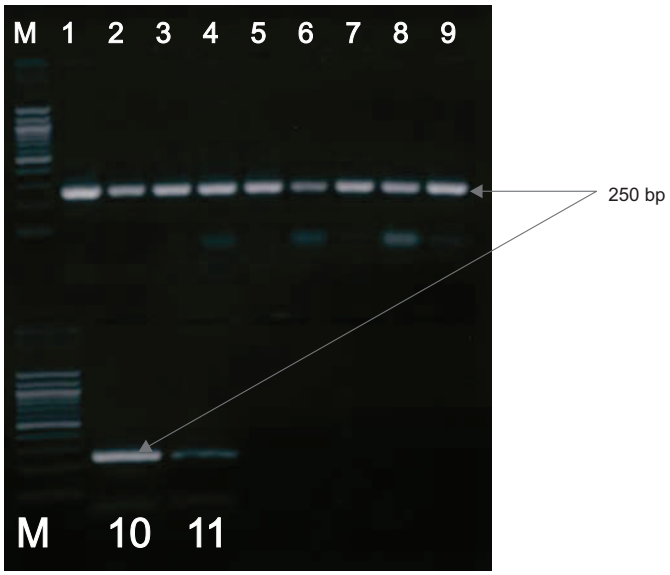

Fig. 2. PCR products of amplified chromosomal DNA of *Str. thermophilus* strains. M – molecular weight marker 100-bp DNA ladder, lane 1 – CCDM 69, lane 2 – CCDM 70, lane 3 – CCDM 126, lane 4 – CCDM 128, lane 5 – CCDM 129, lane 6 – CCDM 130, lane 7 – CCDM 131, lane 8 – CCDM 224, lane 9 – CCDM 437, lane 10 – CCDM 438, lane 11 – CCM 4757

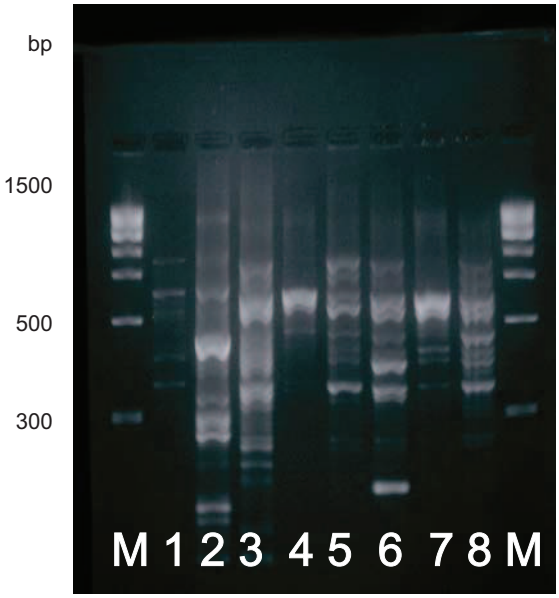

Fig. 3. RAPD products using chromosomal DNA of *Str. thermophilus* strains and RAPD primer. M – molecular weight marker 100-bp DNA ladder, lane 1 – CCDM 7, lane 2 – CCDM 33, lane 3 – CCDM 45, lane 4 – CCDM 55, lane 5 – CCDM 69, lane 6 – CCDM 70, lane 7 – CCDM 126, lane 8 – CCDM 129

DNA sizes ranged from 1.5 kb to 106 bp, biggest ones were 1.5 and 1.4 kb for strains CCDM 33 and 45, respectively. The third one was strain CCDM 131 with DNA size 0.9 kb. The lowest DNA size was observed in strain CCDM 224. The biggest DNA sizes of strains treated with primer OPP-7 were similar to that noticed in primer RAPD-4, thus 1.4 and 0.9 kb, but in contrast to primer RAPD-4 these sizes were determined in strains 128 and 70, respectively. DNA sizes for primer OPP-7 moved between 1.4 kb and 37 bp. The lowest DNA size belonged to mentioned strain 131, which is another discrepancy between these two primers. Mostly DNA sizes from 700 to 200 bp occurred in primer RAPD-4 and between 750 and 350 bp in primer OPP-7. The results are similar to that described by Urshev et al in their study [15].

Streptococcus thermophilus strains were compared by method SDS-PAGE. Figure 4 demonstrates protein profile of given strains. After normalization of gels by molecular weight standard Protein Marker, Broad range, sizes of particular proteins were obtained due to program UltraQuant (Ultra.Lum, USA). Protein sizes within the limits 10–160 kDa were determined that can be seen in Table 1, where number of proteins in mentioned molecular weight range were recorded in individual examined *Streptococcus thermophilus* strains. Salzano et al studied compute genom of *Streptococcus thermophilus* and concluded that it is possible to detect protein sizes from 10 to 210 kDa in this bacterium [16]. Mostly, proteins with molecular weights between 45–90 kDa were represented. It can be compared with results from Soomro and Masud [10] research of *Str. thermophilus*, in which they established four major proteins of about 100, 49, 47

Table 1

Amount of visualizated proteins in particular strains

Molecular mass [kDa]	CCDM 7	CCDM 33	CCDM 45	CCDM 55	CCDM 69	CCDM 70	CCDM 126	CCDM 128	CCDM 129	CCDM 130	CCDM 131	CCDM 133	CCDM 224	CCDM 437	CCDM 438	CCDM 4757
150–160	—	—	—	1	—	2	1	—	3	—	1	—	—	1	—	3
140–150	1	2	1	3	1	2	3	—	3	1	2	1	1	—	—	2
130–140	1	3	1	4	1	3	4	1	3	1	5	—	1	1	1	4
120–130	3	2	2	3	3	4	3	—	4	2	3	1	3	3	—	4
110–120	4	2	5	1	4	1	1	2	4	4	1	2	5	7	1	3
100–110	4	2	4	4	4	3	4	3	3	3	4	4	3	4	4	3
90–100	3	3	2	4	3	3	4	3	4	3	4	3	3	2	2	4
80–90	2	2	1	4	1	3	4	1	4	1	3	2	1	1	1	4
70–80	3	2	3	4	4	5	3	2	3	4	4	2	3	3	2	3
60–70	4	3	3	4	4	3	4	2	4	4	4	3	4	4	2	1
50–60	3	2	4	3	3	3	2	3	2	2	2	4	3	4	3	3
40–50	3	4	2	3	3	4	4	3	3	3	3	4	3	3	5	3
30–40	4	3	2	3	4	2	3	4	3	2	3	3	2	2	3	3
20–30	3	2	3	2	2	3	2	3	2	3	3	3	3	3	3	2
10–20	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2
Total number of proteins	40	34	35	45	39	43	44	29	47	35	44	34	37	40	29	44

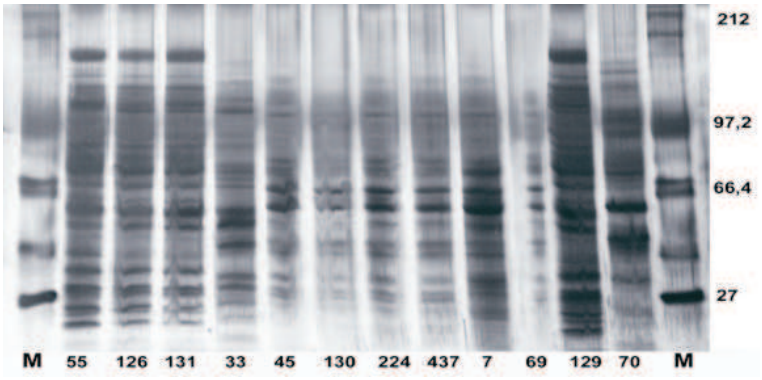

Fig. 4. Protein profile of studied *Str. thermophilus* strains obtained by SDS-PAGE method (12 % gel)

and 41 kDa. Table 1 shows number of proteins in molecular weight range of 10–160 kDa in individual examined *Streptococcus thermophilus* strains. Varcamonti et al [17] found that *Streptococcus thermophilus* protein profile also depends on temperature of cultivation, especiallly their production of heat shock proteins (Hsp proteins). With

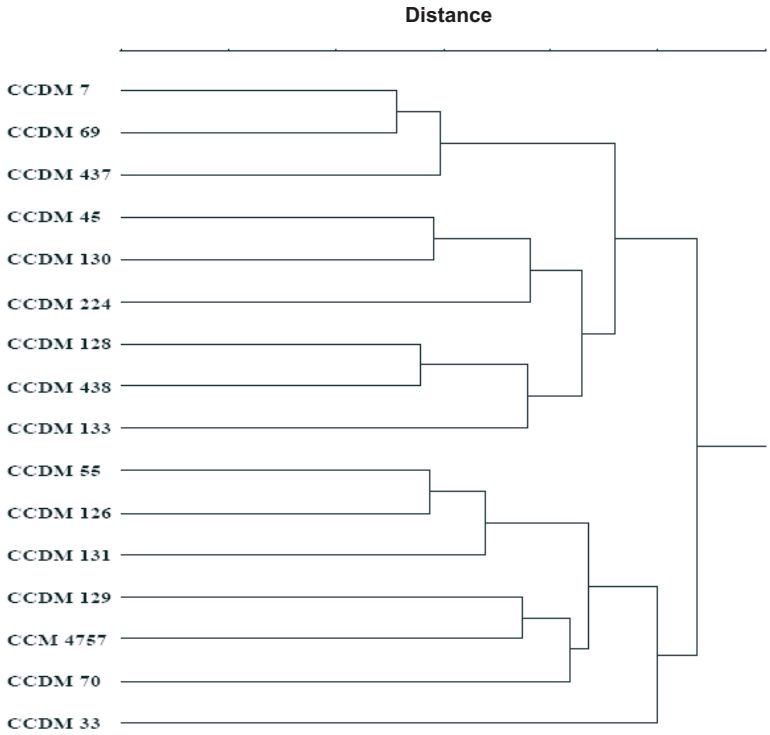

Fig. 5. Dendrogram made up of streptoccoci protein profile

respect to examined strains those were cultured at 37 ^{o}C, it could be interesting to observe influence of temperature on bacterial protein profile. Cluster analysis revealed two main groups (Fig. 5). The first group included 9 strains and it was divided into two subgroups, the first of them was consisted of 3 strains (CCDM 7, 69, 437) and the second one of 6 strains (CCDM 45, 128, 130, 133, 224, 438). The second main group was formed from two subgroups of 7 strains. The first subgroup was only one strain (CCDM 33), the second subgroup was composed of 6 strains (CCDM 55, 70, 126, 129, 131 and CCM 4757). Strain CCM 4757 resembled strain CCDM 129 the most because of their greater amount of proteins with high molecular weight.

Conclusions

In this study, expected size 250 bp of PCR products of amplified chromosomal DNA of *Streptococcus thermophilus* strains with primer set ThI/ThII were obtained. Further, technique RAPD provided similar DNA sizes in both used primers, but these sizes were observed in strange strains. Generally, DNA sizes 700 – 200 bp were detected in primer RAPD-4 and 750–350 bp in primer OPP-7. Analysis of protein profile by SDS-PAGE method revealed that proteins with middle molecular weight were mainly present. Proteins with high molecular weight were supplied only scanty and some strains lacked these proteins. From results of cluster analysis, it was established that strain CCM 4757 was mostly similar to strain CCDM 129. It could be declared that applied methods are useful means for *Streptococcus thermophilus* strains identification and detection.

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DIAGNOSTYKA MOLEKULARNA *Streptococcus thermophilus*

Abstrakt: *Streptococcus thermofilus* jest jednym z najważniejszych przedstawicieli bakterii kwasu mlekowego. Pomimo powszechnego zastosowania tego gatunku w przemyśle mleczarskim nadal nieliczne są dane na temat jego zróżnicowania fenotypowego i genetycznego *Streptococcus thermofilus*. Szybka identyfikacja różnych gatunków bakterii kwasu mlekowego ma duże znaczenie dla kontroli jakości produktów mleczarskich. Niniejsza praca dotyczy charakterystyk i różnic występujących między różnymi liniami *Streptococcus thermofilus*. Badania zostały przeprowadzone przy użyciu technik PCR, RAPD i SDS-PAGE. Do badań użyto 15 linii *Streptococcus thermofilus* z Czeskiego Zbioru Mikroorganizmów Mleczarskich oraz jednej linii *Streptococcus thermofilus* z Czeskiego Zbioru Mikroorganizmów. Dokonano porównania primerów THI/THII między poszczególnymi liniami bakterii za pomocą techniki PCR. Następnie próbki były badane i identyfikowane przy użyciu technik RAPD i SDS-PAGE. Natomiast primery OPP-7 i RAPD-4 zbadano techniką RAPD. Badania wykazały, że zastosowane techniki mogą być skutecznie wykorzystywane do identyfikacji i charakterystyki bakterii z rodzaju *Streptococcus.*

Słowa kluczowe: *Streptococcus thermophilus*, PCR, RAPD, SDS-PAGE