Properties of exopolysaccharide producer *Streptococcus thermophilus* ST8.01 isolated from homemade yoghurt

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Summary

A total of 38 presumptive *Streptococcus thermophilus* strains were isolated from homemade yoghurts from Turkey. Isolated strains were analysed for exopolysaccharide (EPS) production by evaluating the colour of the colonies grown in ruthenium red milk agar. Only one strain, ST8.01, produced EPS and was selected for further analyses. As a result of the identification tests, EPS producer strain was identified as *Strep. salivarus* subsp. *thermophilus* ST8.01. The presence of *epsCD* genes in *Strep. thermophilus* ST8.01 was determined and *epsCD* fragments were sequenced. The *epsCD* fragment showed from 89% to 94% similarity with EPS synthesis gene clusters of different *Strep. thermophilus* strains. The amount of EPS produced in M17 broth at 42 °C for 24 h was found to be (132 ± 3.0) mg·l^{-1}. The viscosity of the culture of strain ST8.01 in skim milk was determined to be (142 ± 5.0) mPa.s. The acid production experiment showed that ST8.01 strain was a fast acidifying strain. The proteolytic activity of ST8.01 strain was determined to be (0.15 ± 0.01) mg·of tyrosine per 1 ml. Acetaldehyde was detected as a major flavour compound produced by *Strep. thermophilus* ST8.01. The results of this study suggest that *Strep. thermophilus* ST8.01 strain may be used as commercial starter culture to manufacturing dairy products.

Keywords

Streptococcus thermophilus; exopolysaccharide; technological properties; flavour compounds; yoghurt

Streptococcus thermophilus is a homofermentative thermophilic lactic acid bacterium (LAB) used world-wide as a starter for the preparation of various dairy products such as fermented milks, yoghurt and several varieties of hard-cooked cheeses (e.g. Italian and Swiss varieties). *Strep. thermophilus* is regarded as the second most important starter culture in dairy industry after *Lactococcus lactis* [1–3]. *Strep. thermophilus* strains used in commercial starter cultures possess several important technological properties such as production of lactic acid, proteolytic activity, synthesis of flavour compounds and exopolysaccharide (EPS) production. These metabolic traits are strain-dependent [2–6]. The major function of *Strep. thermophilus* in milk fermentation is to produce lactic acid from lactose, causing a rapid decrease in pH [2] and also the production of certain levels of flavour compounds such as acetaldehyde, diacetyl, acetoin, ethanol, acetone and butanone-2 [3, 7].

Many *Strep. thermophilus* strains synthesize EPS and these are commonly used, particularly in the dairy industry, for the manufacture of fermented dairy products, especially yoghurt and cheeses [4, 8, 9]. EPS plays a major role in the rheological behaviour, mouth-feel and texture of fermented products without the use of additives [4]. The *eps* genes share a high degree of similarity in various lactic acid bacteria. The *eps* genes are located in an operon structured as follows: regulation of EPS production (*epsA, epsB*), determination of chain length and export (*epsC, epsD*), biosynthesis of the repeating units for synthesizing exopolysaccharide (*epsE, epsF, epsG, epsH, epsI*), and polymerization and export of the polysaccharide molecule (*epsK*, *epsL*, *epsM*) [10–13].

The purpose of the present investigation was to determine the properties of an exopolysaccharide producer *Strep. thermophilus* ST 8.01 strain isolated from homemade yoghurt, which would be important for its use as a commercial starter.

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MATERIAL AND METHODS

Isolation and growth conditions

Homemade yoghurt samples were obtained from local bazaars of Isparta province located in West Mediterranean region of Turkey. Yoghurt samples were diluted and inoculated onto Neutral Red Chalk Lactose Agar (NRCLA) [14] plates incubated at 37 °C for 24 h for selecting the presumptive *Strep. thermophilus* strains. The randomly selected presumptive *Strep. thermophilus* colonies from NRCLA plates were transferred into M17 broth (Merck, Darmstadt, Germany). Stock cultures were maintained at –20 °C in M17 broth supplemented with 15% glycerol. The isolates were characterized based on Gram staining, catalase reaction and cell morphology.

Screening of exopolysaccharide production

Exopolysaccharide production was examined on ruthenium red milk (RRM) plates containing 5 g yeast extract, 100 g skim milk powder, 10 g saccharose, 15 g agar and 0.08 g of ruthenium red per liter. After the incubation, colonies of non-ropy strains develop red colour and colonies of ropy strains appear white on RRM plates [10].

Identification of the exopolysaccharide-producing strain

Exopolysaccharide-producing strain ST8.01 was identified phenotypically based on production of acids from saccharides and related compounds, and genotypically by 16S rDNA homology. The production of acids from saccharides and related compounds was investigated using API 50 CH strips and API 50 CHL medium (BioMérieux, Marcy l'Étoile, France) according to manufacturer's instructions. Strain ST8.01 was characterized genotypically by 16S rDNA homology using PCR with universal primers pA 5'-AGA GTT TGA TCC TGG CTC AG-3' and pE' 5'-CCG TCA ATT CCT TTG AGT TT-3' [15]. PCR amplification was performed in a total reaction volume of 50 μl containing 3 μl of bacterial DNA solution obtained from overnight culture of ST8.01 strain by the method of Cancilla et al. [16]. A total of 30 amplification cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 60 s and extension at 72 °C for 90 s were performed using a thermocycler Genius (Techne, Cambridge, United Kingdom). After the amplification, 10 μl of products were electrophoresed at 6 $V \cdot cm^{-1}$ (1% w/v agarose gel, 0.2 $\mu g \cdot ml^{-1}$ of ethidium bromide) in Tris-acetate-EDTA buffer using the O'GeneRuler 100 bp DNA ladder (Fermentas, Vilnius, Lithuania). Sequencing of 16S rDNA was performed in RefGen (Ankara, Turkey) by using an automated gene sequencer ABI PRISM 3730XL (Perkin Elmer, Foster City, California, USA). For detection of the closest relatives, the sequence of the PCR product was compared to the 16S rDNA gene sequences of Genbank using the BLAST programme [17].

Analysis of the presence of *epsCD* genes

The presence of *epsC* and *epsD* genes in *Strep. thermophilus* ST8.01 strain was analysed by PCR. Genes *epsCD* genes were amplified using epsCF 5'-AGT GAT GAA ATC GAC GTA CT-3' and epsDR 5'-CCA ACC GAC TTT TCT ACG AC-3' primer set proposed by Stingele et al. [10]. PCR was performed in 50 μl of total reaction volume containing 3 μl of the bacterial DNA solution. The reaction involved 35 cycles of denaturation at 94 °C for 45 s, annealing at 55 °C for 35 s and extension at 72 °C for 60 s. The PCR products were electrophoresed and visualized as described above. Sequencing of the *epsCD* genes was performed in RefGen and the sequence was compared to the *epsCD* genes sequence of Genbank using the BLAST programme. The *Strep. thermophilus* ST6.05 strain was used as a negative control in amplification experiments.

Isolation and quantification of the exopolysaccharide

Exopolysaccharide from *Strep. thermophilus* ST8.01 strain was purified as previously described by Frengova et al. [18]. Briefly, ST8.01 culture was heated, precipitated with trichloroacetic acid and then chilled in absolute ethanol. Purified EPS was submitted to preliminary determination of the total saccharide concentration using the phenol-sulphuric method of Dubois et al. [19], glucose being used as the standard and the results being expressed as mg of glucose equivalents per litre. The experiment was performed in triplicate.

Viscosity measurement

The overnight culture of *Strep. thermophilus* ST8.01 was inoculated into sterile skim milk (10%, w/v; Fluka, Steinheim, Switzerland; cat. no. 70166) and incubated at 42 °C until pH reached 4.6 ± 0.1. The viscosity was evaluated with a Brookfield viscometer model LVDV-II+Pro (Brookfield Engineering Laboratories, Middleboro, Massachusetts, USA) and quantified at 25 °C for 2 min at 1.67 Hz. The viscosity measurement was carried out in triplicate.

Acid production ability

The acid production ability of strain ST8.01 was tested by inoculating (2%, v/v) the 18 h cul-

ture into sterile skim milk (10%, w/v) and incubating at 42 °C [20]. The pH was measured at the beginning and at the end of the incubation period of 5 h using a pH meter (WTW 3110 pH meter; Wissenschaftlich-Technische Werkstätten, Weilheim, Germany). The acidification was calculated as $\Delta pH = pH_{at\ time} - pH_{beginning\ time}$.

Proteolytic activity

The proteolytic activity of the strain ST8.01 grown in skim milk (10%, w/v) was measured by the method of Citti et al. [21]. The concentration of liberated free aromatic amino acids was measured spectrophotometrically at 650 nm in a Shimadzu UV-1601 spectrophotometer (Shimadzu, Kyoto, Japan). Proteolytic activity of the strain was expressed as mg of tyrosine per 1 ml. The acid production ability and proteolytic activity experiments were performed in triplicate.

Determination of flavour compounds

The flavour compounds acetaldehyde, diacetyl, ethanol and acetone were investigated by headspace analysis using slightly modified procedures of Beshkova et al. [7]. The skim milk (10%, w/v) was inoculated with 2% (v/v) of the overnight culture of *Strep. thermophilus* ST8.01 and incubated at 42 °C for milk coagulation until pH reached 4.6 ± 0.1. An amount of 2 g of anhydrous sodium sulfphate (Merck) was transferred into a vial followed by 2 g of the homogenous culture sample. The vial was sealed with a cap and then the solution was mixed on a vortex for 5 min. Gas chromatography analysis was performed by using a Perkin-Elmer Autosystem XL (Perkin-Elmer) equipped with a flame-ionization detector. The following conditions were used: capillary column, CP-WAX (50 m × 0.32 mm i.d. × film thickness); oven temperature program 35 °C for 2 min, raised to 240 °C at a rate of 5 °C·min^{-1} and then kept at 240 °C for 20 min; injector and detector temperatures, 180 °C and 200 °C, respectively. The carrier gas was helium (1.7×10^5 Pa). The experiment was done in duplicate.

RESULTS AND DISCUSSION

Strain isolation and screening of exopolysaccharide production

A total of 38 Gram-positive and catalase-negative cocci, presumptive *Strep. thermophilus* isolates, from NRCLA plates corresponding to 20 homemade yoghurt samples were selected for screening of exopolysaccharide production. All presumptive *Strep. thermophilus* strains were then tested for exopolysaccharide production on RRM medium. Only one strain generated light pink colonies and it was consequently identified as a ropy strain. This EPS producing strain was selected for further analyses. Ruthenium red stains the bacterial cell wall, so non-ropy strains generate red colonies on RRM medium. In ropy strains, EPS prevents this staining, and the colonies appear white [10, 22]. On the other hand, Mora et al. [6] reported that exopolysaccharide-producing *Strep. thermophilus* strains generated white or pink colonies on RRM medium, as confirmed in this study.

Identification of the exopolysaccharide-producing strain

The exopolysaccharide-producing strain ST8.01 was found to be Gram-positive and catalase negative cocci. The strain was identified phenotypically by the API system and genotypically by 16S rDNA homology. The saccharide fermentation analysis showed that the strain fermented only three saccharides (glucose, lactose and saccharose). Comparison of this saccharide fermentation profile to the API 50 CHL databank lead to the strain identification with 99.2% probability as *Strep. salivarius* subsp. *thermophilus*. Further confirmation was done by PCR amplifying a fragment of approximately 900 bp of 16S rDNA sequence

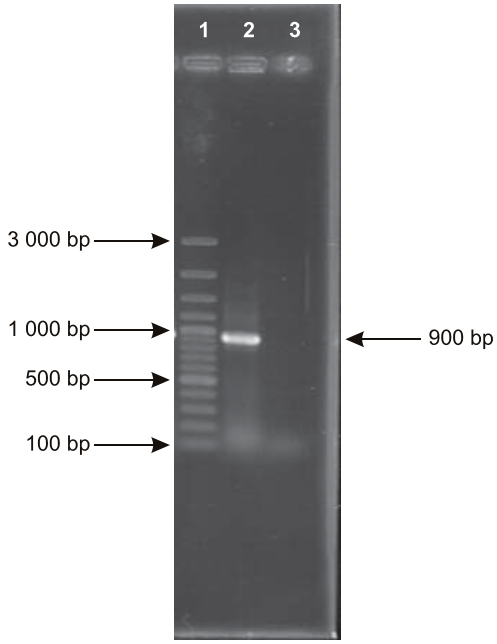

Fig. 1. PCR amplification of 16S rDNA fragment of genomic DNA of *Strep. thermophilus* ST8.01.

1 – molecular weight marker (O'GeneRuler 100 bp DNA ladder), 2 – *Strep. thermophilus* ST8.01 strain, 3 – negative control.

(Fig. 1, line 2) using a universal primer set proposed by EDWARDS et al. [15]. The PCR product was sequenced and the sequence pattern of 900 bp fragment showed 99% homology with *Strep. thermophilus* genome using the BLAST programme.

Analysis of the presence of *epsCD* genes

The appropriate primers were used for analysis the presence of *epsCD* genes in *Strep. thermophilus* ST8.01. The results showed that about 1435 bp fragment was amplified from the genomic DNA of *Strep. thermophilus* ST8.01 (Fig. 2, lines 1 and 3). No DNA fragment was amplified using genomic DNA of *Strep. thermophilus* ST6.05, used as a negative control (Fig. 2, lines 2 and 5). The PCR products of *epsCD* genes were sequenced and the sequence pattern of this fragment showed a high degree of similarity (89–94% identity) with exopolysaccharide synthesis gene clusters of different *Strep. thermophilus* strains. Previous studies on *eps* loci of *Strep. thermophilus* also showed that they are very polymorphic [6, 11, 13, 23, 24]. The sequence pattern of the amplified fragment showed a significant homology with capsular polysaccharide biosynthesis operon of *Strep. iniae* (67% identity), *Strep. pneumoniae* (66% identity), and *Strep. agalactiae* (63% identity). The *epsA*, *epsB*, *epsC*, *epsD* and *epsE* genes of *Strep thermophilus*, *cpsA*, *cpsB*, *cpsC* and *cpsD* genes of *Strep. agalactiae* and *cpsA*, *cpsB*, *cpsC, cpsD* and *cpsE* genes of *Strep. pneumoniae* are organized in the same form and share a very high homology [10].

Isolation and quantification of the exopolysaccharide

The total saccharide concentration of EPS was determined using the phenol-sulphate method. The amount of EPS produced by *Strep. thermophilus* ST8.01 in M17 broth at 42 °C for 24 h was found to be (132 ± 3.0) $mg \cdot l^{-1}$ (glucose equivalents; mean ± standard deviation). EPS produced by thermophilic streptococci was reported to range between 25 $mg \cdot l^{-1}$ and 150 $mg \cdot l^{-1}$ [25, 26]. Similarly, ASLIM et al. [27] indicated that the quantities of EPS produced by different *Strep. thermophilus* strains were 16 $mg \cdot l^{-1}$ to 114 $mg \cdot l^{-1}$ and 24 $mg \cdot l^{-1}$ to 140 $mg \cdot l^{-1}$ in M17 broth and skim milk, respectively. EPS produced by *Strep. thermophilus* strains isolated from traditionally made Indian fermented milk products were reported to range between 77 $mg \cdot l^{-1}$ and 211 $mg \cdot l^{-1}$ in deproteinized whey supplemented with 10 $g \cdot l^{-1}$ glucose [28]. The amount of EPS synthesized by *Strep. thermophilus* ST 8.01 was found to be similar to the reported values.

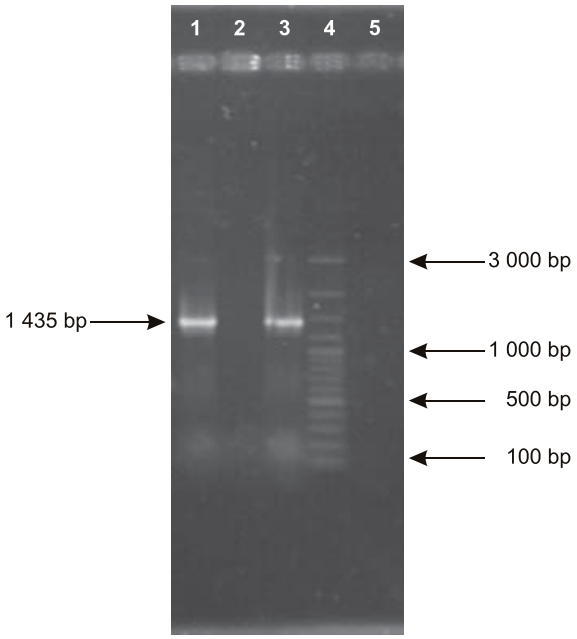

Fig. 2. Specific PCR amplification of *epsCD* genes using total DNA of the exopolysaccharide-producing strain *Strep. thermophilus* ST8.01 as a template.

1 and 3 – exopolysaccharide-producing strain ST8.01, 2 and 5 – negative control (exopolysaccharide non-producing strain *Strep. thermophilus* ST6.05), 4 – molecular weight marker (O'GeneRuler 100 bp DNA ladder).

Viscosity

The viscosity of the culture of strain ST8.01 in skim milk was determined to be (142 ± 5.0) mPa.s (mean ± standard deviation). In a previous study conducted by ASLIM et al. [27], the viscosity in skim milk of exopolysaccharide-producing *Strep. thermophilus* strains ranged between 15 mPa.s and 97 mPa.s. In addition, BEHARE et al. [28] indicated that viscosity in 19 exopolysaccharide-producing *Strep. thermophilus* strains ranged between 67 mPa.s and 179 mPa.s. The viscosity of *Strep. thermophilus* ST 8.01 in skim milk was found to be compatible with results of previous studies.

Acid production ability and proteolytic activity

After incubation in skim milk at 42 °C for 5 h, the ΔpH values of exopolysaccharide-producing *Strep. thermophilus* ST8.01 was determined to be 1.55 ± 0.05 (mean ± standard deviation). This result showed that *Strep. thermophilus* ST8.01 strain was a fast acidifying strain. One of the main important criteria for the selection of *Strep. thermophilus* strains that are intended to be used as starters in milk fermentation is rapid acidification ability. AYHAN et al. [29] reported that ΔpH levels of 28 domestic strains and 9 commercial *Strep. thermophilus* strains ranged between 0.30 and 1.50.

Strep. thermophilus ST 8.01 showed a higher ΔpH value than these strains. Proteolytic activity of strain ST8.01 was found to be as (0.15 ± 0.01) mg of tyrosine per 1 ml (mean ± standard deviation), after 24 h in skim milk at 37 °C. Proteolytic activity of strain ST8.01 was compatible with results of domestic and commercial *Strep. thermophilus* strains in the previous study by AYHAN et al. [29]. Values for these strains ranged between 0.24 mg and 0.30 mg of tyrosine per 2 ml.

Determination of flavour compounds

Production of flavour compounds acetaldehyde, diacetyl, ethanol and acetone by strain ST8.01 was investigated by headspace analysis. Acetaldehyde was detected as a major flavour compound produced by *Strep. thermophilus* ST8.01, being produced at 31.17 mg·kg^{-1} at 42 °C (Fig. 3). The other flavour compounds were found to be ethanol (6.34 mg·kg^{-1}) and acetone (0.99 mg·kg^{-1}). Diacetyl was not detected. According to headspace analyses results, ST8.01 strain produced a high level of acetaldehyde. Acetaldehyde, diacetyl, acetoin, ethanol, acetone and butanone-2 are the most important flavour compounds in yoghurt. However, among them, acetaldehyde is considered as the major flavour compound for the typical yoghurt aroma [5, 7, 30]. AYHAN et al. [29] reported that the most of the commercial and domestic *Strep. thermophilus* strains produced acetaldehyde at levels varying from 25.92 mg·kg^{-1} to 43.65 mg·kg^{-1} and from 25.00 mg·kg^{-1} to 55.00 mg·kg^{-1}, respectively. Our result was compatible with this study. On the other hand, ZOURARI et al. [31] noted that acetaldehyde amounts in *Strep. thermophilus* cultures in milk varied from 2.5 mg·kg^{-1} to 6.5 mg·kg^{-1}. XANTHOPOULOS et al. [5] indicated that a total of 74 *Strep. thermophilus* cultures produced acetaldehyde at levels varying from 0.0 mg·kg^{-1} to 7.4 mg·kg^{-1}, after incubation at 42 °C until culture pH reached 4.6 ± 0.1. In addition, BESHKOVA et al. [7] showed that acetaldehyde was a predominate (1.511 μg·g^{-1} and 0.996 μg·g^{-1}) flavour compound in *Strep. thermophilus* 13a and 15a cultures, followed by ethanol (0.8 μg·g^{-1} and 0.7 μg·g^{-1}), acetoin (0.78 μg·g^{-1} and 0.88 μg·g^{-1}), acetone (0.31 μg·g^{-1} and 0 μg·g^{-1}), diacetyl (0.21 μg·g^{-1} and 0 μg·g^{-1}) and butanone-2 (0.025 μg·g^{-1} and 0 μg·g^{-1}), after incubation at 42 °C for 4 h, respectively.

CONCLUSION

Rapid acidification ability, good proteolytic activity and good flavour compound production of the exopolysaccharide-producing strain *Strep. thermophilus* ST 8.01 suggest that this strain has a potential to become a commercial starter culture for the production of fermented dairy products, in particular yoghurt.

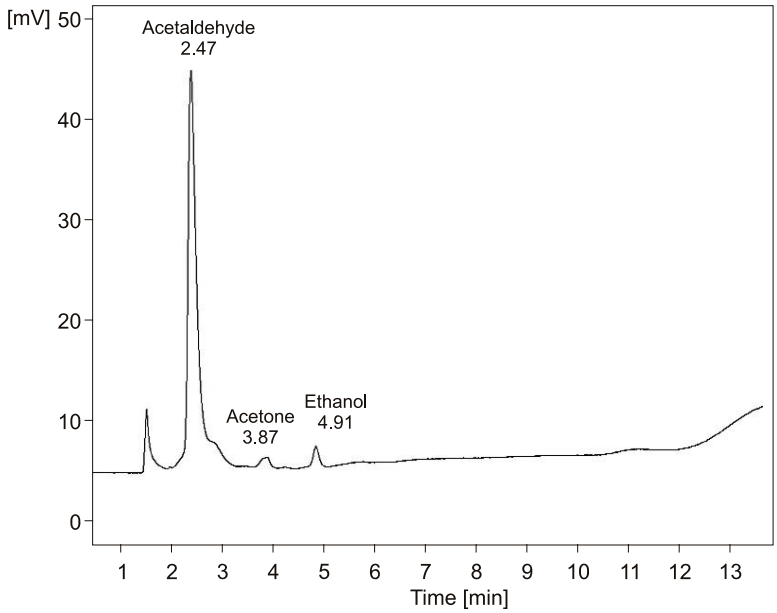

Fig. 3. Headspace gas chromatogram of flavour compounds produced by *Strep. thermophilus* ST8.01 in skim milk at 42 °C.

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