

## A Simple and Inexpensive Procedure for Chromosomal DNA Extraction from *Streptococcus thermophilus* Strains

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**Abstract:** A simple and rapid procedure to extract chromosomal DNA to be used as template for polymerase chain reaction (PCR) fingerprinting experiments from *Streptococcus thermophilus* (*St.thermophilus*) is proposed. Different procedures for rapid extraction of chromosomal DNA from lactic acid bacteria were also compared to the proposed procedure. Seven *St. thermophilus* strains were tested for their quantitative as well as qualitative yields of chromosomal DNA. Results showed that, the physical treatment, use of a detergent and enzyme for lysis, treatment with proteinase K, Lysozym and Mutanolysin were essential steps for extraction of chromosomal DNA from *St.thermophilus*. The protocol allowed obtaining chromosomal DNA sample patterns comparable with those obtained by classical DNA extraction. The quality of isolated chromosomal DNA was high and the DNA concentrations of the samples was 50 to 90 ng/  $\mu$ l and the size of the DNA obtained was approximately 22 to 23 kb without extensive shearing. The proposed protocol was satisfactory because they yielded high quality and quantity DNA and were able to remove the inhibitors of DNA amplification compared to the traditional methods. Since this protocol provided high quality genomic DNA for PCR amplification, restriction enzyme digestion and presumably some other related molecular biology studies. Also it may be used for a range of techniques that require high quality genomic DNA. This protocol was very attractive for the large-scale isolation of chromosomal DNA. It could be concluded that the convenience of the technique makes this rapid mini-prep procedure suitable for routine DNA isolation from *St.thermophilus*.

**Key words:** *St. thermophilus* • Chromosomal DNA Extraction • PCR

### INTRODUCTION

Lactic acid bacteria (LAB) are a group of related bacteria that produce lactic acid as a result of carbohydrate fermentation. These microbes are broadly used by us in the production of fermented food products. Maria *et al.* [1] reported that LAB are attractive approach for generation of functional foods enriched in bioactive peptides given the low cost and positive nutritional image associated with fermented milk drinks and yoghurt.

*St.thermophilus* is an important microorganism with essential catalytic functions in dairy food fermentations. Along with the metabolic activities of these bacteria commonly used as starters, they are required to be resistant to antibiotics and phages [2]. Studies on *St. thermophilus* strains from commercial sources indicated that up to 80% of isolates of this species currently use as starter culture are plasmid free [3,4]. When plasmids were

present in these bacteria, they are found in small sizes (2-8 Kb). The role of many plasmids and chromosomal DNA in *St. thermophilus* is unclear. For this reason, it is interest to examine the sequence of native *St. thermophilus* DNA with a view to explore their application as food-grade vectors and determine the biological role of *St. thermophilus* plasmid [5]. Genomic DNA/RNA isolation is the first and the most important requirement in carrying out molecular biology techniques such as PCR, restriction enzyme analysis, Southern hybridization, genomic DNA library construction [6], mutation detection or linkage analysis [3,7], as well as DNA microarray gene expression profiling [8]. All these techniques require a reasonable amount of DNA with good enough quality, fidelity and concentration [9].

Several authors have described methods for isolating from small cultures genomic DNA suitable for PCR amplification, DNA sequencing, mapping purposes and

endonuclease digestion [4,10]. Because each method has its advantages and disadvantages, the appropriate extraction method should be chosen according to the purpose in mind. Therefore, an efficient procedure for DNA extraction is required, combining simplicity with high yields of pure DNA suitable for molecular biology studies. Moreover, when a large number of samples must be screened, simple and rapid methods are needed to eliminate time-consuming steps, multiple pipettings, extractions with organic solvents and changes of microfuge tubes. Most of the routinely used protocols for preparation of bacterial chromosomal DNA are primarily based on two strategies including either cetyl trimethyl ammonium bromide (CTAB) or lysozyme/detergent based method followed by improvement of the procedure by removal of protein and RNA contamination [11,12]. However, many gram positive bacteria, such as *St.thermophilus* are resistant or weakly susceptible to lysozyme due to their cell wall structure [6]. Some problems such as poor yields and interference with restriction enzymes digestion have been reported to be associated with some standard DNA extraction protocols [13,14]. The basis for genetic manipulation of these industrially significant bacteria begins finds a technique to facilitate the rapid purification of genomic DNA that can be used directly for molecular analysis. A modified protocol is described to isolate chromosomal DNA from various Gram positive bacteria without the need more for phenol:chloroform extractions and caesium chloride gradients [15,16].

In this study, we describe a very simple, economical and rapid method for extraction of very pure genomic DNA. Both plasmid and chromosomal DNA from colonies or pellets of *St.thermophilus* strains that is suitable for subsequent PCR. The method involves only chloroform as the cell disrupting and extracting agent. Also, it consists of only three steps prior to DNA analysis.

## MATERIALS AND METHODS

**Bacterial Strains and Culturing Condition:** Seven *St. thermophilus* strains representing different types of *subsp* (differing in cell wall structure and extracellular material) were obtained from the BafM, Kiel, Germany.

Strains were grown in LTM17 broth medium. The inoculated medium was incubated at 40°C for 48 hrs. The bacteria were identified as *St. thermophilus* based on their characteristics as described in Bergey's Manual [17] and by using molecular technique. The bacterial strains were stored in 70% glycerol at -80°C to supply stable

inoculums for this study. Chromosomal DNA was extracted from *St. thermophilus* grown in LTM17 medium containing 20 mM DL-threonine.

**Chromosome DNA Isolation from Bacteria (Proposed Protocol):** Chromosomal DNA from Gram-positive bacteria was isolated as follows: Cells from an overnight culture (10 ml) were collected by centrifugation at 10000 rpm/ 3min. Resuspend cell pellets with 200  $\mu$ l lysis buffer. Immediately add (4 $\mu$ l each from 10 mg ml<sup>-1</sup> and 100 mg ml<sup>-1</sup> stock solutions respectively) to the suspension, which was then incubated at 37°C for 30 min subsequently, 10  $\mu$ l Proteinase K (10 mg/ml). Vortexes to make sure no chunks. 7  $\mu$ l 20 % SDS were added and the suspension was incubated at 60°C for 1 hr with inverting every 15 min.

The suspension was then extracted once with phenol and chloroform/isoamyl alcohol (25:24:1), mix thoroughly by inverting for 5 min, spin at 10000 rpm for 5min. The supernatant was then transferred to a new tube. A cotton-like genomic DNA was seen at this stage. DNA was ethanol-precipitated from the water phase, the DNA was resuspended in 1/10 volume of 3M sodium acetate and 2 volume of ice cold ethanol, mix gently and resuspended in 50  $\mu$ l TE buffer. DNA yields from standard 10 ml cultures ranged between 30-50  $\mu$ g.

Presence of DNA can be confirmed by electrophoresing on an agarose gel containing ethidium bromide, or another fluorescent dye that reacts with the DNA and checking under UV light. The DNA might be treated with restriction endonucleases for a Southern transfer and hybridization.

The conditions to achieve a complete cell lysis have to be optimized: Although overnight cultures can be used for almost all streptococci, some strains might need to be processed in the exponential growth phase. For exopolisaccharide-producing strains, the use of the low-salt-medium is recommended.

**Solutions and Buffers:** Lysis buffer (6.7% sucrose; 50mM Tris/HCl, pH7.0; 1mM EDTA); TE buffer (10mM Tris/HCl,pH7.5;0.5mM EDTA); Proteinase K (10 mg/ml); Lysozym 2 mg/ml; Mutanolysin 150 U/ml; 3M sodium acetate (pH5.2).

**Evaluation of Quantity and Purity of Extracted DNA:** The extracted DNA was quantified using a NanoDrop 2000 Spectrophotometer (Thermo Scientific, Germany). The 260/280 nm absorbance ratio was used to determine undesired contaminations as described by

Sambrook *et al.* [18]. To evaluate the quality and intactness of the extracted DNA, gel electrophoresis was used in the presence of DNA mass ladders.

**PCR Fingerprinting and Amplification:** Analysis of PCR was carried out in order to test the intactness/fidelity of the genomic DNA and to determine the possible inhibitory materials which may interfere with the reaction. PCR reaction was performed in a 20 µl volume, consisting of: 10 µl PCR Master Kit (2×) (Promega, USA), 0.4 µM; gene specific primer set as St1: TTATTTAGGGGCAATTGC, St2:GTGAACCTTCCACTCTCACAC[19], (Eurofins, mwg/operon, Germany) and 50 ng/µl genomic DNA. PCR were done in a (My Cycler thermal cycler v.1065, Bio-Rad, Laboratories, Inc.USA).The amplification program consisted of one initial denaturation at 94°C for 4 min followed by 30 cycles of 60 s at 94°C for denaturation, 1 min at 40°C for primer annealing, 1 min at 72°C for extension and DNA synthesis and final extension at 70°C for 10 min. The products were separated on 1.5% agarose gel containing ethidium bromide (1 µg/ml), then images were taken using a G: Box™ gel documentation system (Syngene, Cambridge, United Kingdom).

**Digestion with Restriction Enzymes:** The restriction enzyme digestion methodology was used to assess the purity, quality and intactness of the extracted DNA. Each reaction was carried out in a total volume of 20 µl containing the following: 2 µg DNA and restriction enzyme *Eco*R1 (10 u/µl) (Fermentas, GmbH, Germany) incubated at 37°C for 2 h.

## RESULTS

**Chromosomal DNA Isolation from Bacteria:** Gram-positive species are much more resistant to cellular lysis resulting from the extensive concentration of peptidoglycan within the cell wall. Many protocols have been developed for extraction of bacterial genomic DNA. However, only few of them provide for optimal DNA isolation from widespread types of bacteria. This study aimed to present such protocols to achieve an easy to handle and highly efficient extraction system of chromosomal DNA. The method is based on a modification of procedure described by Miller *et al.* [20] and Zhang [21] and consists of only three steps prior to DNA analysis: resuspension, lysis and purification by precipitation of the proteins with high salt concentrations. It is simple, rapid and cheap and it requires neither

specialized equipment nor complicated extraction agents. The quality of the DNA obtained by this procedure is similar to that obtained by other common laboratory and commercial techniques and is suitable for both PCR amplification and digestion. This new procedure requires a single step for lysing the bacterial cells, which is then followed by straight forward isolation of DNA with chloroform-isoamyl alcohol and ethanol for precipitation and purification of the genomic DNA. In order to eliminate RNA from extracts, RNAase was added in the lysing buffer or incubated with the extracted DNA at the end of the extraction. Addition of DL-threonine to the growth medium improved the culture lysis. Using mix from lysozyme and mutanolysin (2 mg/ml lysozym and 150 U/ml Mutanolysin) for the lysis buffer gave highest recovery of chromosomal DNA from all strains tested. Ethanol precipitates the DNA and RNA while isopropanol selectively precipitates DNA leaving RNA and polysaccharides in the solution. No doubt ethanol plays a vital role in DNA precipitation, but special care has to be paid to the removal of ethanol after precipitation. It has been reported that any residual ethanol may hinder the PCR as an inhibitor. Thus, we suggest that use of both physical and chemical steps for cell lysis. Use of proteinase K and phenol:chloroform for DNA purification and ethanol precipitation, are essential steps for extraction of chromosomal DNA from *St.thermophilus* strains. The lysate can be treated with proteinase K (10 mg/ml) and incubated for 15 min at 37 C, this treatment is essential for good extraction of chromosomal DNA. An incubation step at 60°C for 60 min followed by cooling to room temperature for 5 min is included to improve cell lysis and DNA yields and also to inactivate any lytic enzymes (lysozyme-mutanolysin or protease) that may be present. The extracted chromosomal DNA from seven *St.thermophilus* strains was found to be intact by agarose gelelectrophoresis (1%) even though it was exposed to several steps of extraction/purification (Fig. 1).

**Evaluation of Quantity and Purity of Extracted DNA:** The spectrophotometric analysis for (OD.260/280 nm) resulted in average between 1.6-2.09. This indicates that the extracted DNA was free from protein a contamination (Table 1). This procedure may yield between 50 to 90 ng/µl from 10 ml of a stationary phase bacterial culture.

The DNA concentrations of the samples are 50 to 90 ng/µl and the size of the DNA obtained is approximately 22 to 23 kb (Fig. 1). The typical yield from the protocol is attributed to only 10 ml starting culture ( $10^6$  to  $10^7$  cells/ml). Time considerations: the protocol takes 2 hr,

Table 1: Determination the quality of isolated chromosomal DNA of *St.thermophilus* strains by measure the absorbance ratio by NanoDrop 2000 spectrophotometer (260/280nm) and species yield of chromosomal DNA (ng/μl).

St.thermophilus strains	Yield of chromosomal DNA (ng/μl)	Purity of chromosomal DNA (260/280 nm)
ER1	64.5	1.74
S4	92.1	2.09
S0	75.7	1.88
S8	51.0	1.62
S11	66.3	1.80
ER1-1	69.0	1.77
S106	70.2	1.91

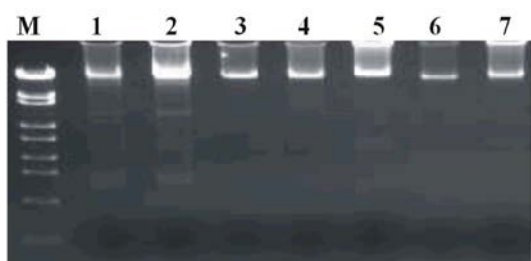


Fig. 1: Agarose gel electrophoresis showing the chromosomal DNA obtained from seven *St.thermophilus* strains by using our modified protocol. Lanes 1-7 were chromosomal DNA of *St.thermophilus* strains, the size of chromosomal DNA ranged from 22 to 23kb. Lane M, is DNA marker was used as a reference standard (BioLabs, Inc, New England). (Fragment length: 23.130, 9.416, 6.557, 4.361, 2.322, 2.027, 564 bp).

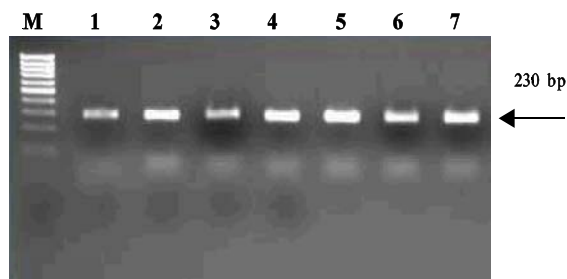


Fig. 2: PCR amplification of DNA extracted from seven of *St.thermophilus* strains using specific primer (St1, St2). Lanes 1-7 were amplified PCR product of *St.thermophilus* strains. Amplified PCR products were electrophoresed on an agarose gel (1.5%). Lane M, is PCR marker was used as a reference for gel electrophoresis (Qiagen, GelPilot, Inc, Germany).

including the incubation time. Other large-scale prep takes slightly longer to reach the point of the isolation of the chromosomal DNA.

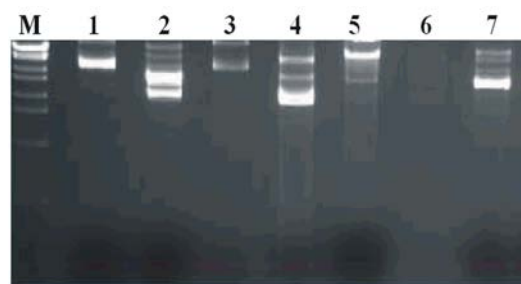


Fig. 3: Agarose gel electrophoretic patterns of *EcoRI* restriction digestion of *St.thermophilus* strains (lane1, ER1; lane 2, S4; lane 3, S0; lane 4, S8; lane 5, S11; lane 6, ER1-1(have a fine two fragments); lane 7, S106). Lane M, is DNA marker was used as a reference standard (Qiagen, GelPilot, Inc, Germany).

Generally, the present protocol could serve as a universal competent method for isolation of genomic DNA from a variety of samples.

#### PCR Fingerprinting Amplification and Digestion with Restriction Enzymes:

To test the quality of the DNA and reproducibility of the method, molecular typing methods were used to identify these bacteria, including 16S/23S ribosomal DNA (rDNA) and random amplification polymorphism DNA (RAPD) analysis (Fig. 2). Primers St1and St1[19]was used to amplify a DNA fragment of 230 bp with different substrates (Fig 2).The 230-bp fragment was amplified in all cases, indicating that the protocol provides DNA suitable for PCR analysis. Extracted DNA obtained using this protocol was directly used for quality assessment with restriction digestion and the result for digestion with *EcoRI* showed that the isolated DNA was very pure with high quality for enzymatic reaction, in which a minimal inhibitory effect of the extraction process was observed (Fig. 3). *EcoRI* was found to possess a single site on this *St.thermophilus* isolated DNA, while some strains was the result of digestion obtained two fragments. The sizes of the fragments were calculated by comparing to the marker (lambda DNA digested with *Hind III*) which has been used as marker. The *EcoRI* fragment from *St. thermophilus* is approximately ranged from 0.9 to 1.5 kb. *PamH1*, *EcoRII* and *PstI* restriction enzymes (Fermentase, Thermofisher, INC) were also used (data not shown). DNA was completely digested with restriction enzymes and there was no evidence of the presence of nuclease in any sample.

## DISCUSSION

*St.thermophilus* represents a major genus of the LAB that has widespread use in fermented milk production. Several protocols have been described for chromosomal DNA isolation of this genus. The isolation mainly was based on two strategies including either CTAB or lysozyme/detergent based method followed by improvement of the procedure by removal of protein and RNA contamination [11]. More improvement of bacterial cell lysis could be achieved by use of suitable chemical and enzymatic digestion of bacterial cell wall. Lysozyme is a frequently used enzyme in the lysis buffer of various reported methods for the complete lysis of bacterial cell wall. Use of lysozyme in mix with mutanolysin or protease with also recommended. Use of proteinase K was found to be helpful in removing DNA bound proteins resulting in improvement in quality of template DNA. Extraction of DNA using phenol:chloroform:iso-amyl alcohol improved the quantity as well as the quality of target DNA. Querol *et al.* [22] achieved 97% PCR positivity by using phenol: chloroform: isoamyl alcohol extraction followed by isopropanol precipitation of DNA. Sina *et al.* [23] reported that the use of phenol and chloroform for extraction and ethanol and/or isopropanol for precipitation of DNA surely improves the yield of the purified target DNA, which finally results in an increased sensitivity of PCR. However, Brisson-Noel *et al.* [24] estimated that 5% of clinical specimens contain some inhibiting component(s), which were not removed by phenol extraction of DNA. Ethanol precipitates the DNA and RNA while isopropanol selectively precipitates DNA leaving RNA and polysaccharides in the solution. No doubt ethanol plays a vital role in DNA precipitation, but special care has to be paid to the removal of ethanol after precipitation. Any residual ethanol may hinder the PCR. Thus, we suggest that use of both physical and chemical steps for cell lysis, use of proteinase K and phenol:chloroform for DNA purification and ethanol precipitation, are essential steps for extraction of chromosomal DNA from *St.thermophilus* strains. Quantitation and purity of chromosomal DNA was done spectrophotometrically by measuring absorbance at 260/280 nm for comparative quantitative analysis of DNA [18]. Serial dilutions of DNA extracted from all protocols were prepared ranging from 50 to 90 ng/μl. All of these dilutions were amplified to compare the quality and the minimum amplifiable quantity of DNA.

In order to check the efficiency and reliability of the method, the PCR technique was applied and the PCR product yielded a clear band pattern and an adequate intensity (Fig. 2). Primers St1and St1 [19] were used to amplify a DNA fragment of 230 bp with different substrates (Fig 2).The 230-bp fragment was amplified in all cases, indicating that the protocol provides DNA suitable for PCR analysis. Although many of the PCR based techniques do not necessarily require high quality DNA, pure DNA is recommended for PCR based assays such as RAPD, which is susceptible to artifactual polymorphisms [15, 25].

Extracted DNA obtained using this protocol was directly used for quality assessment with restriction digestion. The digestion with *EcoRI* showed that the isolated DNA was very pure with high quality for enzymatic reaction, in which a minimal inhibitory effect of the extraction process was observed. This new chromosomal DNA procedure provides higher quality genomic DNA for PCR amplification, restriction enzyme digestion and presumably some other related molecular biology studies.

Generally, the application of this protocol for *St.thermophilus* was time efficient and adequate for PCR amplification and other library analysis. This protocol produced chromosomal DNA free of contamination and similar to which isolate by using the specific kits (data not shown). It is simple, rapid and universally competent method for isolation of genomic DNA from a variety of samples.

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