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An Efficient DNA Extraction Method for *Lactobacillus casei*, a Difficult-to-Lyse Bacterium

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Abstract

Background: There are several protocols to extract DNA from *Lactobacillus* spp. In the case of *L. casei* it is harder because of its especial and thick cell wall.

Objectives: In this study, nine DNA extraction protocols (by lysozyme treatment) were evaluated and compared in two categories (traditional and kit-based protocols) and an improved method was presented.

Materials and Methods: DNA quantity and quality was determined by spectrophotometry, agarose gel electrophoresis and polymerase chain reaction (PCR).

Results: The results revealed that the yield of extracted DNA differed by each protocol (5.8 - 17.1 μg/100 μL), but provided appropriate DNA for PCR amplification. The modified protocol offered the best total DNA extraction method when both quality (DNA purity; 1.54 μg) and quantity (DNA yield; 17.1 μg) were considered.

Conclusions: We suggest this protocol for effective and inexpensive DNA isolation from *L. casei* for downstream biological processes such as PCR.

Keywords: DNA, Extraction, Modified Protocols, *Lactobacillus casei*

1. Background

Cell wall of Gram-positive bacteria has several distinctive structures and protects the protoplast from mechanical damage and osmotic rupture or lysis. The murein layer (a thick peptidoglycan layer) is the ubiquitous component of the Gram-positive cell wall which provides shape, stability and viability. This layer contains almost equal amounts of polysaccharides and peptides (1, 2) and is composed of a polymer of disaccharide (glycan) chains of repeating N-acetylglucosamine and N-acetylmuramic acid residues (linked $\beta 1 \rightarrow 4$) and is cross-linked by short chains of amino acids (peptide) (1).

Undermining progress in *Lactobacillus* genetics has been the difficulty in achieving cell lysis with lysozyme (3, 4) and developing reliable procedures for DNA isolation (5). *L. casei* is a rod-shaped, Gram positive and highly lysis-resistant bacterium (6, 7). In this species, because of its special cell wall structure, genetic studies have several difficulties (8). Polysaccharide and peptidoglycan moieties are the major surface components of this bacterium (5, 7). The primary structure of its peptidoglycan has a common monomer GlcNAc–MurNAc–L-Ala–g-DGlu–L-Lys–D-Ala with an asparagine attached to the ω-amino group of lysine (9). The peptide side chains are then cross-linked by a transpeptidase (1).

Abed evaluated five methods for the extraction and purification of DNA from cultured Lactobacillus colonies isolated from dairy products. The results obtained in that study confirmed that wizard genomic DNA purification kit with modifications was superior to other methods because it produced a higher DNA yield with the highest purity (10). Scornec et al. set up a rapid 96-well plate DNA extraction protocol for *L. casei*. They optimized the DNA extraction procedure based on silica membranes in 96-column format to obtain genomic DNA from a large number of mutants (8). De et al. presented a simple, inexpensive and effective genomic DNA isolation procedure for *Lactobacillus* isolates. They verified the quality of the isolated genomic DNA by restriction digestion and polymerase chain reaction (PCR) (11).

In recent years, various molecular techniques have been used for the lysis of *L. casei* by chemical and mechanical protocols (12). In chemical methods, the peptidoglycan can be lysed by cell wall hydrolase enzymes (e.g. lysozyme or mutanolysin). Mutanolysin is costly, not gener-

ally available, and thus unsuitable for routine use in laboratories. High resistance to lysozyme is also observed in several lactobacilli species (1).

2. Objectives

In the current study, we evaluated several different DNA extraction protocols using lysozyme treatment and compared them with a new modified method.

3. Materials and Methods

3.1. Bacterial Strains and Growth Conditions

For the analysis of both plasmid and genomic DNA, a recombinant *L. casei* was constructed and used in this study. Briefly, beta toxin gene of *Clostridium perfringens* was synthesized by Generay biotechnology company (China) and cloned in NaeI and BamHI restriction sites of pT1NX vector obtained from BCCM/LMBP plasmid collection of university of Ghent, Belgium (http://bccm.belspo.be/about/lmbp.php). The modified vector was transformed to *L. casei* ATCC: 393 by a Gene PulserTM apparatus (Bio-Rad laboratories, Richmond, CA). This strain was grown statically at 37°C for 24 hours in *Lactobacillus* de Man, Rogosa and Sharpe (MRS) broth (Himedia, India) supplemented with erythromycin (7.5 μg/mL), anaerobically. Wild-type *L. casei* was grown in MRS broth without erythromycin, too.

3.2. DNA Extraction

All the DNA manipulations were performed according to standard procedures (13). In this study, eight DNA extraction protocols in two categories (traditional and kit-based protocols) and an improved method were tested. After 24 hours of incubation at 37°C, cultures of *L. casei* (3 mL) in the exponential phase of growth (approximately 1.6 unit of OD600 nm) were centrifuged for three minutes at 12000 rpm. These bacterial pellets were used for total DNA (genomic and plasmid) extraction.

3.3. Traditional Protocols

In this category, four protocols were used as follows.

3.3.1. P1 Protocol

This method, popularly known as boiling, was based on Abdulla (14) with no relevant modification. One milliliter of dH_2O was added to the pellet. After vortexing, the sample was boiled at 100°C for 15 minutes by placing in water bath. The suspension was cooled immediately to –20°C for 20 minutes and centrifuged at 13000 rpm for five minutes and the supernatant was kept frozen until used (14).

3.3.2. P2 Protocol

In this protocol, the cell pellet was suspended in 750 μL of 50 mM EDTA. Then, a volume of 100 μL of lysozyme solution (50 mg/mL) was added to the cell suspension and incubated overnight at 37°C. Subsequently 50 μL of proteinase K (20 mg/mL) was added and the tube was incubated for 30 minutes at 55°C. The suspension was centrifuged at 12000 rpm at room temperature for five minutes and the pellet was gently resuspended in 950 μL of lysis solution (10 mM Tris-HCl, PH 8.0, 1 mM EDTA, 0.1% (w/v) sodium dodecyl (SDS)). Then, 15 μL of RNase A (20 mg/mL) was added to the lysate and incubated for 45 minutes at 37°C with gentle inversion. For protein precipitation, 300 μL of protein precipitation solution (6 mL of 5 M potassium acetate, 1.15 mL of glacial acetic acid and 2.85 mL of distilled water) was added to the lysate mixture and vortexed at medium speed for 20 seconds. The lysate was centrifuged at 12000 rpm for 20 minutes and the supernatant was transferred to a clean 1.5 mL microtube. One additional centrifugation step at 12000 rpm for 10 minutes was performed to remove any residual protein. To precipitate DNA, 600 μL of cold isopropanol was added and the sample was centrifuged at 12000 rpm for 20 minutes; then, the pellet was washed with 70% ethanol before air drying for 15 minutes. Finally, the pellet was resuspended in 100 μL of Tris-EDTA buffer (10 mM Tris Hcl, 1 mM EDTA, pH = 8.0) and kept at 65°C for 15 minutes and stored at –20°C till further analysis (14).

3.3.3. P3 Protocol

In the third protocol, the pellet was washed thrice with 2 mL of NaCl-EDTA (30 mM NaCl, 2 mM EDTA, pH = 8.0) and resuspended in 100 μL of this buffer and then 100 μL of freshly prepared lysozyme solution (10 mg/mL in NaCl-EDTA) was added and mixed. This mixture was incubated at 37°C for one hour with periodic shaking. To remove RNA, 1 μL of RNase A solution (20 mg/mL) was also added to the mixture before incubation. The volume of the mixture was then made up to 500 μL with additional NaCl-EDTA, 50 μL of a 10% SDS solution and 10 μL of proteinase K solution (20 mg/mL). The contents were mixed thoroughly and incubated at 55°C for one hour. After incubation, 200 μL protein precipitation solution (same as the P2 protocol) was added and vortexed at medium speed for 20 seconds and kept on ice for five minutes. The lysate was centrifuged at 12000 rpm for three minutes and the supernatant was transferred to a clean 1.5 mL tube. DNA in the supernatant was precipitated with 600 μL of cold isopropanol and pelleted by centrifugation at 12000 rpm at room temperature for three minutes. The supernatant was discarded and the DNA pellet was washed once with freshly prepared 70% ethanol and air-dried. The final pellet obtained was dissolved in 100 μL TE buffer (10 mM Tris HCl, 1 mM EDTA, pH = 8.0) and kept at 65°C for 15 minutes and stored frozen at –20°C till further analysis (11).

3.3.4. P4 Protocol

In this protocol, firstly, the bacterial pellet was resuspended in 480 μL of 50 mM EDTA and gently vortexed. Then, a volume of 120 μL lysozyme (20 mg/mL) was added to the cell suspension and incubated at 37°C for two hours

with periodic mixing. The suspension was centrifuged at 12000 rpm for three minutes in room temperature and the supernatant was removed. The pellet was gently resuspended in 600 µL genomic lysis buffer (10 mM Tris-HCl, pH = 8.0, 1 mM EDTA, 0.1% (w/v) SDS) containing 6 µL proteinase K solution (20 mg/mL). The sample was incubated at 60°C for one hour and after the incubation, 200 µL protein precipitation solution (same as P2 protocol) was added and kept on ice for five minutes. After this step, the protocol continued as same as P3 protocol.

3.4. Kit-Based Protocols

In this category, five kit-based protocols were performed as described below.

3.4.1. P5 Protocol

This protocol was performed exactly based on DNA extraction kit (DN8115C, SinaClon, Iran) according to the manufacturer's instruction.

3.4.2. P6 and P7 Protocols

In these protocols, the pellets were resuspended in 180 µL of lysis buffer composed of TE (Tris-HCl 20 mM, EDTA 2 mM, pH = 8.0), lysozyme (20 mg/mL) and triton X-100 (1% v/v). After this step, in P6 protocol we used kit and performed the protocol according to the manufacturer's instruction, but in P7 protocol we incubated the mixture for two hours at 37°C and after the incubation, we followed the kit protocol from the lysis step.

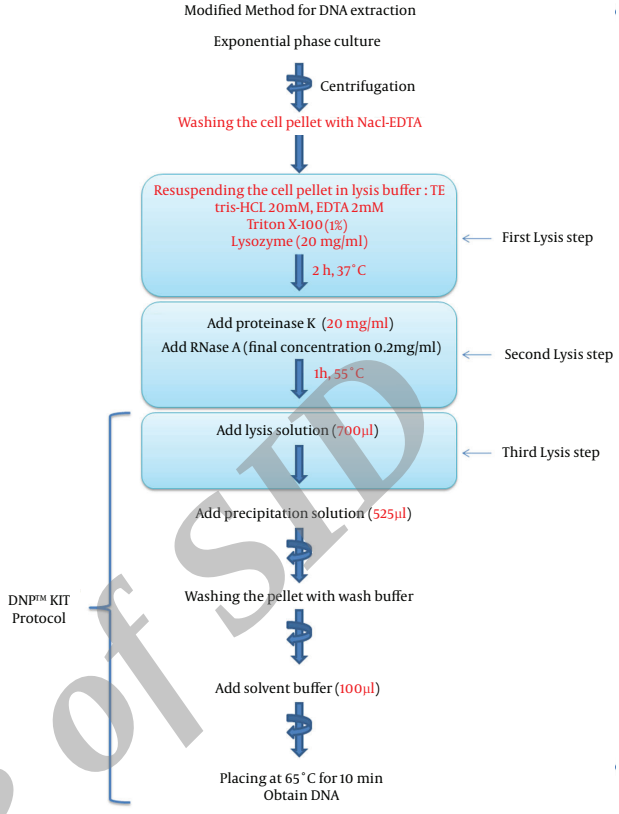
3.4.3. P8 Protocol

In this procedure, the pellet was washed once with 500 µL NaCl-EDTA (30 mM NaCl, 2 mM EDTA, pH = 8.0) and resuspended in 100 µL NaCl-EDTA buffer and 100 µL of freshly prepared lysozyme solution (25 mg/mL). To remove RNA, 1 µL of RNase A solution (20 mg/mL) was also added and the mixture was incubated at 37°C for two hours with periodic shaking. The lysate was centrifuged at 13000 rpm for two minutes and the supernatant was poured off. After this step, we used kit and performed the protocol from the lysis step according to the manufacturer's protocol.

3.4.4. P9 Protocol (Modified Method)

In this new modified protocol, several modifications were made. Three lysis steps were used in this protocol (Figure 1). The pellet was washed once with 500 µL NaCl-EDTA (30 mM NaCl, 2 mM EDTA, pH = 8.0) and resuspended in 180 µL of lysis buffer composed of TE buffer (Tris-HCl 20 mM, EDTA 2 mM, pH = 8.0), lysozyme (20 mg/mL) and triton X-100 (1% v/v). The mixture was incubated for two hours at 37°C. Then, proteinase K (20 mg/mL) and RNase A (final concentration: 0.2 mg/mL) were added to the mixture and incubated for one hour at 55°C. After the incubation, we continued according to the kit protocol, except for the buffer volumes which are indicated in Figure 1.

Figure 1. Schematic Representation of Modified DNA Extraction from *Lactobacillus casei*



The main protocol modifications are in red letters.

3.5. Evaluation of Quantity and Quality of Extracted DNA

The presence and integrity of the extracted DNA from different protocols were evaluated by agarose gel (0.7%) electrophoresis using horizontal electrophoresis system (Bio-Rad). The type of band pattern indicated the quality of DNA.

The extracted DNA from each protocol was also quantified by spectrophotometry using a BioPhotometer Plus (Eppendorf, Germany) at 260 nm and 280 nm. The quality of DNA was determined by A260/A280 ratio value. The system software provides the DNA concentration (ng/µL) and automatically calculates the absorption ratio of 260/280 (A260/A280). The total DNA yield (in 100 µL of samples) was calculated as described by Sambrook (13):

DNA yield (µg) = DNA concentration (ng/µL) × total sample volume (µL)

3.6. Polymerase Chain Reaction Using Extracted DNA

To check the efficiency and applicability of the extracted genomic and plasmid DNA, each DNA was tested by PCR. The extracted total DNA samples were used as template for selective amplification of DNA from the 16S rRNA gene of *L. casei* and cloned beta toxin gene (cpb) of *C. perfringens*. The primers used for different PCRs are listed in Table 1.

PCR reaction was performed using 5 µL of the extracted DNA with 25 µL of ready-to-use PCR master mix 2x (PR901638, SinaClon, Iran), 2.5 µL (20 pmol/µL) of each primer and dH_2O till 50 µL volume was reached. Amplification of DNA from the 16S rRNA gene of *L. casei* was performed as described previously (15). Amplicons of cpb were obtained with 35 cycles following an initial denaturation step at 95°C for 10 minutes. Each cycle involved denaturation at 94°C for one minute, annealing at 52°C for one minute, synthesis at 72°C for one minute, and a final extension step at 72°C for 10 minutes. The PCR products were then examined for clarity and intensity. The amplified products were electrophoresed in 1.7% agarose gel and observed with gel documentation system.

4. Results

4.1. Quantity and Quality of Extracted DNA

This study evaluated different DNA extraction methods of *L. casei*. In the nine protocols described in this work, total DNA was isolated from *L. casei* by the lysozyme treatment method and was estimated spectrophotometrically. The results revealed that DNA extraction with modified protocol produced acceptable DNA purity (1.54) and highest DNA yield (17.1 µg) when compared with other protocols (Table 2). The DNA yield varied significantly depending on the category of DNA extraction used (traditional and kit-based protocols). The DNA yields were lower with traditional protocols when compared to the kit-based protocols. In almost all DNA extraction protocols it was possible to visualize the DNA. Agarose gel electrophoresis showed better results for kit-based protocols (Figure 2).

4.2. Polymerase Chain Reaction Amplification of DNA

All nine protocols provided effective DNA for PCR amplification with the pairs of primers used. In all the samples, a single band of 196 bp of target cloned beta toxin gene was amplified and visualized on agarose gel (Figure 3). In addition, in all the protocols, the 16S rRNA gene was amplified (Figure 4). These results indicated that there was no difference for PCR amplification of the target genes between different protocols.

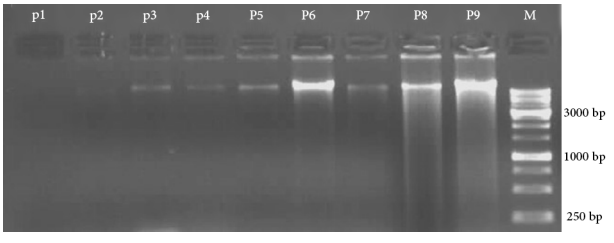
Table 1. Primers to Detect 16S rRNA and Clone Beta Toxin Genes

Gene	Primer	Primer Sequence (5' – 3')	Amplicon Size, bp	Reference
16SrRNA			290	(15)
	Casei-F	CCCACTGCTGCCTCCCGTAGGAGT		
	Casei-R	TGCACTGAGATTCGACTTAA		
cpb			196	(16)
	CPB-F	GCGAATATGCTGAATCATCTA		
	CPB-R	GCAGGAACATTAGTATATCTTC		

Table 2. Yield and Quality of DNA Extracted From *Lactobacillus casei* by Different Protocols

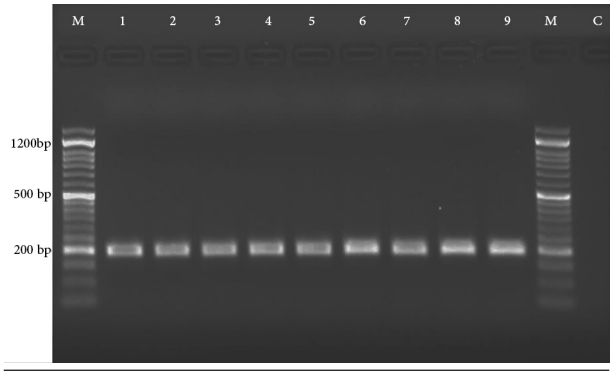
Protocols	Traditional Protocols				Kit-Based Protocols				
	P1	P2	P3	P4	P5	P6	P7	P8	P9
DNA Concentration, ng/µL	62	58	112	108	120	158	90	145	171
Total Yield of DNA, µg/100 µL	6.2	5.8	11.2	10.8	12.0	15.8	9.0	14.5	17.1
Quality of DNA (A260/A280 ratio)	1.23	1.81	1.92	1.56	1.50	1.48	1.44	1.45	1.54

Figure 2. Agarose Gel Electrophoresis Pattern of Extracted Total DNA From *Lactobacillus casei*



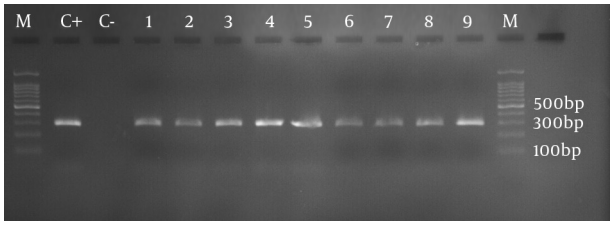
Ten microliters of DNA samples were run in each lane of a 0.7% agarose gel. Lanes P1 - P9, Nine DNA extraction protocols performed in this study. Lane M: 1 kb DNA marker (Fermentas).

Figure 3. Amplified Polymerase Chain Reaction Products From *Lactobacillus casei* With the Primer Set of Beta Toxin Gene



Lanes 1 - 9, Amplified PCR products (196 bp) from nine extracted DNA protocols, respectively; Lanes M, 50 bp DNA markers (Fermentas); Lane C, negative control (wild-type *L. casei*).

Figure 4. Results of 16s rRNA Polymerase Chain Reaction Amplification for the Identification of *Lactobacillus casei*



Lanes 1 - 9, PCR products amplicons (290 bp) from nine extracted DNA protocols, respectively; Lanes M: 100 bp DNA markers (Fermentas); Lane C+, Positive control (wild-type *L. casei*); Lane C–, Negative control (dH_2O).

5. Discussion

The use of reproducible and efficient strategies for DNA extraction is essential for most protocols in molecular biology analyses (10, 17). In this study, we tried to evaluate different methods to find the most efficient, economic and performable way associated with the acceptable purity of the extracted DNA from *L. casei*. Our findings indicated that the use of the modified protocol for the extraction of genomic and plasmid DNA from recombinant *L. casei* resulted in superior performance when compared to the other methods applied under similar conditions.

Previous studies have been performed to evaluate different DNA extraction methods in *L. casei*, but an efficient, suitable and economic method for *L. casei* extraction is still required (8, 10, 11, 14). In our study, we sequentially tested several traditional and kit-based methods to extract DNA from *L. casei* and an improved method was designed for this purpose. In the traditional category, only four protocols were evaluated, as described. We did not perform other old protocols such as phenol-chloroform DNA extraction. The disadvantages of this method are the toxicity of phenol/chloroform, troubles of leftovers with enzymes (PCR digestion, etc.) and being time-consuming. In kit-based protocols, DNA is extracted much faster, cheaper and easier than traditional methods.

Several methods are used to isolate DNA from bacteria, but they often involve multiple time-consuming steps (10). These methods can vary due to the efficiency of physical and chemical characteristics of samples (17). In the current work, we used one type of bacterium in the same cultivating condition and time. With this policy, the effect of physical and chemical characteristics of culture medium was eliminated.

The failure of complete lysis of *L. casei* is due to the inherent nature and specific cell wall which contains a high concentration of peptidoglycan (11). Cleavage of the covalent cross-links in the peptidoglycan by enzymes can help to disrupt the cell wall. Various enzymes such as lysozyme, mutanolysin and labiase have been discovered over the years and utilized with varying success rates by different researchers (11). Mutanolysin and labiase are costly, not generally available and thus unsuitable for routine use in laboratories. Lysozyme is the best known among hydrolases as binds the bacterial surface and attacks peptidoglycans (18). For this reason, this suitable and economical hydrolase was used in our protocols. The use of lysozyme alone is insufficient for the lysis of *L. casei* and results in lower yield of DNA (11). For this reason, we tested and analyzed lysozyme treatment at various time, temperature and chemical conditions to obtain a higher yield of pure DNA. Lysozyme is especially effective in disrupting bacterial cells when used in combination with EDTA (10, 19), which was also confirmed in our experience. As our results showed, the complete lysis of *L. casei* was achieved in concurrent use of lysozyme, EDTA and Triton X-100.

The yield of DNA was significantly higher in the kit-based protocols (ranged: 90 - 171 ng/μL) in comparison to traditional procedures (ranged: 58 - 112 ng/μL). The highest yield of DNA was extracted from the modified protocol (17.1 μg). This was due to concurrent use of lysozyme, EDTA, Triton X-100 and proteinase K in multiple lysis steps of the protocol.

Another key issue in the sensitivity and usefulness of biological analyses such as PCR is the quality of extracted DNA from bacterial isolates (10). In the present study, the purity of extracted DNA varied between 1.23 - 1.92 in different protocols. In the boiling method (P1 protocol), the lowest quality product was obtained (A260/A280 = 1.23). This ratio was due to the high protein contamination and can lead to an overestimation of the real concentration of DNA (20). P2 and P3 protocols had high-purity products (A260/A280 1.81 and 1.92, respectively). This may be due to the use of high concentration of lysozyme (50 mg/mL) for P2 protocol and the additional step of protein precipitation and RNase (20 mg/mL) for P3 protocol, which may have resulted in the removal of contaminants and increased the purity, similar to the previously described investigations (10, 21). The purity of DNA in all kit-based protocols was ~1.50 which was lower than that of the traditional protocols. This may be due to using a single tube during protein precipitation and purification steps.

The time taken for the isolation of DNA by the modified protocol was slightly longer than the other protocols, due to the incubation times required for multiple lysis steps. However, considering the yield, purity and economy of the presented method, it made it ideal. Hence, this method can be an economical and efficient method for the isolation of DNA from the difficult-to-lyse bacteria: *Lactobacillus* (11).

In conclusion, the comparison of nine DNA extraction protocols from recombinant *L. casei* showed that the modified protocol can be the best method for total DNA extraction from this difficult-to-lyse bacterial cell. Therefore, we offer it for many purposes such as screening of *L. casei* colonies after transformation. Overall, this universal protocol is an inexpensive, safe and effective DNA isolation procedure with acceptable quality and quantity.

Footnotes

Authors' Contribution: Mehdi Golchin designed the project and supervised the entire experiments and manuscript writing. Mojtaba Alimolaei performed the experiments and data analysis and wrote the manuscript.

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