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Comparison of DNA Extraction Methods for Molecular Detection of Probiotic *Lactobacilli*, Lysis-resistant Bacteria

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Abstract

DNA extraction is a crucial step in all nucleic acid-based protocols to identify microorganisms. Lactic acid bacteria are a significant part of healthy microbiota in the human gastrointestinal tract. These gram-positive bacteria have several layers of peptidoglycan in the cell walls. These structures cause difficulties in the cell lysis and obtaining reliable protocols for DNA isolations. The purpose of this study was to assess the autoclave and lysozyme-based DNA purification approaches for achieving the high-quality genomic DNAs of *Lactobacillus acidophilus* bacteria. DNA concentrations and qualities were also compared with the commercial kit. The results showed that the proper DNA isolation methods were various, according to the downstream applications. Protocols that included lysozyme produced a higher amount of DNA than the autoclave method. Lysozyme treatment combined with silica -guanidinethiocyanate procedure was the efficient protocol with affordable cost for routine lysis of *L. acidophilus* bacteria. Appropriate DNA concentration and quality were obtained through this method comparable to those of the commercial kit. Inversely, autoclave treatment had little effect on the breakage of the cell walls indicating low concentrations of extracted DNAs. This method could not completely break down all the bacterial cell walls. However, the breakage of low numbers of cell walls was microscopically observed in the supernatant of the autoclaved cell suspension. The quality of this protocol was found to be adequate for performing direct polymerase chain reaction (PCR) assay on samples with large amounts of *lactobacilli*. These conclusions suggest attentively selecting the DNA extraction method based on the planned downstream analysis of PCR products.

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Keywords
Autoclave
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Lysozyme

Introduction

Lactic acid bacteria are a significant part of healthy microbiota in the human gastrointestinal tract. Also, in many traditional fermented foods, beneficial strains of these bacteria have been isolated and are well-known as probiotics (Angelescu *et al.*, 2019; Ehsanbakhsh *et al.*, 2017; Markowiak & Śliżewska, 2017). The study of genomic characterization of lactic acid bacteria (LABs) has significant impacts on the accurate identification of the

bacteria in the clinical, environmental, and food laboratories. However, achieving successful scientific results requires the high quality of the isolated nucleic acids (Ketchum *et al.*, 2018). Moreover, characterization of the microbial biodiversity using 16S rRNA gene sequencing is a common approach, including that related to fermented foods or human microbiota. A fundamental challenge in these assays is DNA extraction efficiency from all bacteria in the community (Ketchum *et al.*, 2018; Lim *et al.*, 2018; Yuan *et al.*, 2012).

In general, efficient disruption of cells is one of the crucial steps in nucleic acid purification. Breaking the bacterial cells can be performed by mechanical (high-pressure homogenizer, bead mill), and non-mechanical techniques (physical, chemical, and biological assays) (Ketchum *et al.*, 2018; Shehadul Islam *et al.*, 2017). Depending on the strains or sample types, different results were represented. Furthermore, there are lots of commercial kits that shown various yields. The global market for cell lysis is increasing. A few equipment and chemicals such as sonicators and enzymes are commercially available for cell lysis (Shehadul Islam *et al.*, 2017).

The gram-positive bacteria have thick cell walls and consist of several layers of peptidoglycan (Vermassen *et al.*, 2019). These structures cause difficulties in cell lysis of *lactobacilli* and obtaining reliable protocols for DNA isolations. De *et al.* (2010) reported that the combination of ampicillin and lysozyme treatment could produce high-quality pure genomic DNA from *lactobacillus* isolates (De *et al.*, 2010). In another study, lysozyme-based protocols were evaluated for DNA extraction from recombinant *Lactobacillus casei*. The modified protocol, including three lysis steps, was suggested as an effective DNA isolation method (Alimolaei & Golchin, 2016). Some investigators have used physical disruption methods such as bead beating for improving the cell lysis. Yuan *et al.* (2012) used several DNA extraction methods in which different mechanisms were employed for cell lysis of the human microbiome. They demonstrated that the bead beating and, or mutanolysin treatment could be a very effective for cell lysis in community analysis of the human microbiome (Yuan *et al.*, 2012). Quigley *et al.* (2012) assessed the efficiency of several protocols, including commercial kits and manual protocol for DNA purification from milk and cheese. Their results highlighted that the Power Food-Microbial DNA Isolation kit (MoBio Laboratories Inc.) was an excellent candidate for PCR-based identification assays (Quigley *et al.*, 2012). Ketchum *et al.* (2018) also showed that the bead-beating and lysozyme treatment more effectively improves the quality of the extracted DNA for the microbiome analysis of marine invertebrates (Ketchum *et al.*, 2018). In another work, Lim *et al.* (2018) compared 3 commercial DNA extraction kits with or without the bead-beating steps for profiling of the human gut microbiome. They emphasized that the mechanical disruption step resulted in higher degrees of microbial diversity (Lim *et al.*, 2018). In addition, Douglas *et al.* (2020) evaluated several approaches of bacterial DNA extraction for breast milk microbiota analysis. the substantial influence of the selected extraction methodology on the obtained data was highlighted (Douglas *et al.*, 2020). Since there is no consensus about the methods evaluated in these studies and extraction methods are advancing, it is essential to compare the new protocols with appointed procedures. On the other hand, rapid DNA extraction and direct PCR are time and cost-effective approaches for high-throughput applications. Many researchers have reported that universal direct PCR based on boiling the samples in water is an efficient method for many cells (Harrel & Holmes, 2022; Videvall *et al.*, 2017). However, using autoclave treatment for direct PCR-based determination of probiotic bacteria has not been reported. Therefore, our study aimed to compare the autoclave and lysozyme treatment for

extracting genomic DNA from the gram-positive bacterium *Lactobacillus acidophilus* for subsequent direct and indirect PCR-based identification.

Materials and methods

Bacterial strains

The lyophilized culture of *Lactobacillus acidophilus* (ATCC 4356) was used in the present study, which was obtained from the Iranian Research Organization for Science and Technology (Tehran, Iran) as a probiotic strain. The bacterial cultures were activated in deMan Rogosa Sharpe (MRS) broth (Merck-Darmstadt, Germany) at 37 °C. Overnight cultures of the bacteria (OD630nm= 0.7-0.8) were harvested by centrifugation at 6000 g for 5 min and stored at -20 ºC until DNA extraction.

DNA preparation methods

The primary genomic DNA extraction method was performed according to the silica-guanidinethiocyanate method, which is based on silica powder for nucleic acid purification (Boom *et al.*, 1990). Two protocols with lysozyme and autoclave treatments were used (methods 1 and 2). For comparison, the bacterial genomic DNA was also extracted by a commercial kit (Bioneer, Korea), (Method 3).

Method 1 (M1)

For methods including enzyme treatment, the cell pellets were treated with lysozyme and proteinase K. Briefly; the washed pellets were resuspended in 500 µL of TE (10 mM Tris–HCl pH 8.0, 1 mM EDTA) buffer with 15 mg/mL lysozyme and incubated for 1 h at 37 °C. Then 10 µL proteinase K (20 mg/mL) was added. Incubation was carried out at 50 °C for 1 h. In this method (M1), the remaining steps were performed based on the Guanidine Thiocyanate-Silica Gel method as described by Shakeri *et al.* (2014). Briefly, 500 µL of lysis buffer was added and incubated at 65 °C for 5 min. The solution was mixed with 25 µL of silica suspension. After centrifuging at 6000 rpm for 20 S, the pellet was washed with saline buffer and left to air dry. The DNA was dissolved in 50 µL of elution buffer. All reagents and buffers were prepared according to Boom *et al.* (1990).

Method 2 (M2A and M2AE)

Cell pellets from overnight cultures of bacteria were washed with 1 mL sterile water and suspended in 100 µL of TE (10 mM Tris–HCl pH 8.0, 1 mM EDTA) buffer in sterile microcentrifuge tubes. The tubes were autoclaved at 121 °C for 5 min and short exhaust. In this protocol, autoclaving (M2A) or combined autoclaving followed by enzyme treating (M2AE) was performed. In the M2AE method, after centrifugation of the autoclaved bacterial suspension at 14000 rpm for 5 min, the pellet was subjected to lysozyme and proteinase K. In both modes, after the treatments, DNAs were extracted by the Guanidine Thiocyanate-Silica Gel method as mentioned in section Method 1 (M1).

Method 3 (M3)

In this method (M3), lysozyme treatment was conducted before the AccuPrepTM Genomic DNA Extraction Kit (Bioneer, Korea) was used. DNA extraction was performed according to the manufacturer's instructions.

Direct PCR

Cell pellets were autoclaved in 150 µL of TE buffer with sterilization temperature of 121 °C for 5 min and short exhaust. The autoclaved samples were centrifuged at 14,000 rpm for 5 min, and 10 µL of the supernatant was used directly in the PCR amplification. This protocol compared with the boiling extraction method that is usual for direct PCR (Alimolaei & Golchin, 2016).

Microscopic analysis

The effect of lysozyme or autoclave treatment on the cell lysis was evaluated by light microscopy. The lysate expanded on the glass slide and heat-fixed. The smear was subjected to microscopic observation

(100× oil immersion objective lens) under an optical microscope Nikon YS100 (Olympus, Tokyo, Japan).

Qualitative and Quantitative measurement of DNA

The concentration and purity of extracted DNAs were obtained by a nanodrop spectrophotometer (Nanodrop Technologies, DE, USA). The ratios of optical density A260/A280 and A260/A230 were assessed as DNA quality indicators. Agarose gel electrophoresis was also used for the integrity evaluation of the extracted genomic DNAs.

PCR amplification and bacterial identification

The primer sequences and PCR conditions for amplifying a 227 bp-region in the 16S rRNA encoding genes of *L. acidophilus* bacteria were similar to the previous studies (Shakeri *et al.*, 2018). The primers were Acidfor (5′-AGCGAGCTG AACCAACAGAT-3′) and Acidrev (5′-AGGCCGTTACCCTACCAACT-3′). PCR was performed with the following parameters: 3 min at 94 °C, followed by 35 cycles; 30 s at 94 °C, 20 s at 60 °C, and 20 s at 72 °C, followed by a final extension step of 5 min at 72 °C. The expected size of PCR products was checked on 1.5% agarose gel containing ethidium bromide by visualization under UV light and photographed. The band intensities were measured with ImageJ 1.38X software (Bethesda, MD, USA). In all experimental tests, the reproducibility of the data was confirmed by repeating in three runs.

Results and discussion

DNA yield and quality

Various quantities and qualities were observed by different DNA extraction procedures. The concentration, DNA quality, DNA yield and, PCR quality are shown in Table (1). While DNA extracted with M1, M2AE and M3 methods met the A260/A280 absorbance ratio (about 1.8-2.0) recommended for PCR amplifications, the M2A method deviated from this range. These results indicated that the M2A method was less effective at removing protein and RNA contaminations. However, all methods provided sufficient amounts of DNA (i.e., ≥1ng) for PCR identifications, the M2A method had the lowest concentration and PCR band intensity. These results demonstrated that autoclave treatment could not break all bacterial cell walls. Still, lysozyme treatment was the most effective procedure for disruption of them as reported by Alimolaei & Golchin (2016) for *lactobacilli* strains. The results of the present study showed that using combination of physical (autoclave) and enzymatic treatments generated the very pure extracted genomic DNA with the greater yield, which was similar to the previous work by Ketchum *et al.* (2018). However, the used commercial kit (M3 method) produced the purest DNA. This kit employs glass fibers fixed in a column for extraction of genomic DNAs, which is expensive. However, using silica powder (M1 method) also provided equally effective DNAs, which is cheaper than commercial test kits. According to the findings of other studies (Boom *et al.*, 1990; Urbaniak *et al.*, 2019), the results proved that silicon-based extraction method was suitable for extracting good-quality DNAs from gram-positive probiotics.

Table 1. Concentration, quality, and yield of DNAs extracted with different methods and their PCR qualities

Extraction method	Concentration(ng/µl)	DNA quality (A260/A280 ratio)	Total yield (µg)	PCR quality (band intensity)
M1	251.10±11.00	1.98±0.05	17.58	200.78±4.88
M2A	27.60±4.48	1.56±0.03	1.93	49.73±4.78
M2AE	193.50±12.10	2.01±0.05	13.54	159.49±4.18
M3	759.07±98.38	1.84±0.06	37.95	248.16±4.69

Data±standard deviation, Total DNA yield = (elution volume×DNA quantity using the nanodrop spectrophotometer).

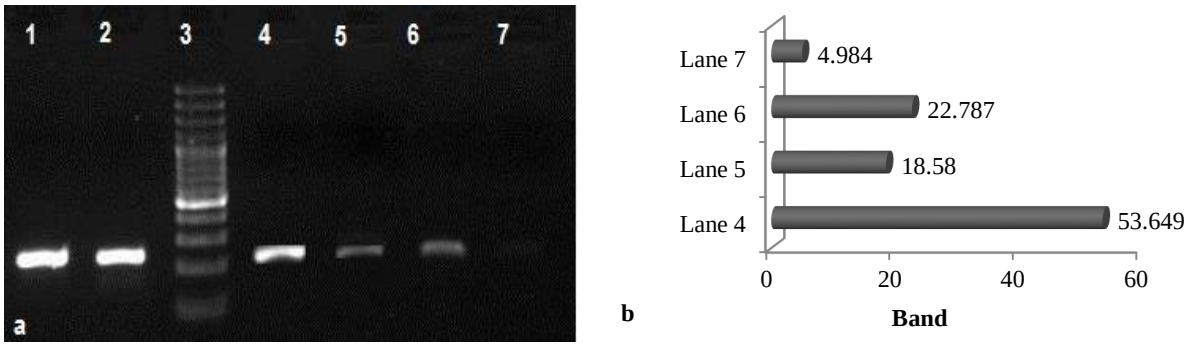

Fig 1. PCR amplification and electrophoresis in direct boiling and autoclaving PCR methods (a), Intensities of PCR bands for 10^4 and 10^3 CFU/mL of the bacteria (b). Lane 1- direct boiling PCR from ~10^8 CFU/mL of the bacteria; lane 2- direct autoclaving PCR from ~10^8 CFU/mL of the bacteria; lane 3- GeneRuler 100 bp Plus DNA Ladder (Thermo Scientific); lane 4-5- direct boiling PCR from ~10^4 and 10^3 CFU/mL of the bacteria; lane 6-7- direct autoclaving PCR from ~10^4 and 10^3 CFU/mL of the bacteria.

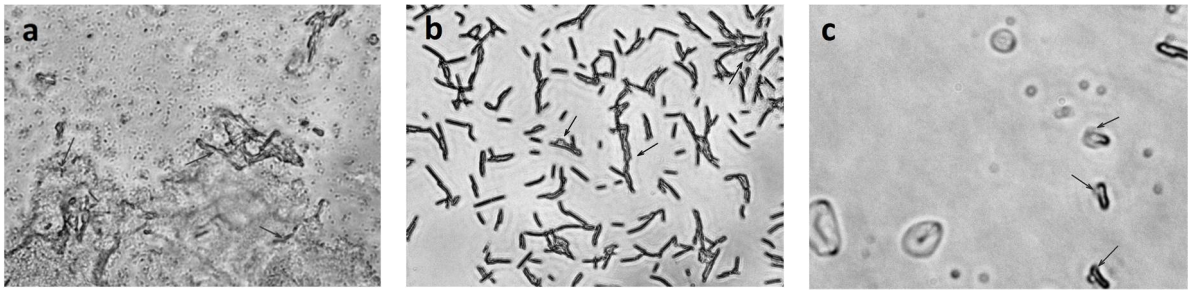

Fig 2. Optical microscopic observation of lysing the cell walls of *L. acidophilus* bacteria. (a) Cell suspension treated with lysozyme, (b) autoclaved cell suspension and, (c) supernatant of the autoclaved cell suspension used for direct PCR.

PCR quality in direct PCR methods

To know how the bacterial cell walls are affected by autoclave, the autoclave-treated bacterial samples were directly amplified and compared with the boiling method. Intensities of gel electrophoresis bands of the PCR amplified products with different numbers of *L. acidophilus* cells represented in Fig. (1). No differences between intensities of bands were visible when about 10^8 CFU/mL of the bacteria was treated (Fig. 1a). However, for 10^4 and 10^3 CFU/mL of the bacteria, clearer differences were observed between the direct boiling (56.6 and 18.6%) and autoclaving (22.8 and 4.9%) PCR methods (Fig. 1b). The data highlighted that the detection limit of the direct autoclaving PCR assay (10^4 CFU/mL) was higher than boiling method (10^3 CFU/mL). Based on the principles of steam sterilization which is used in an autoclave, rapid heating and depressurization can cause to lysing the bacteria. The data suggested that direct autoclaving PCR method may be a suitable technique for colony PCR to identify probiotic lactobacilli, which confirmed previous study by Simmon *et al.* (2004). They have reported that direct autoclaving PCR method was a rapid and cost effective assay with suitable DNA template for downstream PCR applications (Simmon *et al.*, 2004). Considerably, it was found that longer time spent in the autoclave could also damage the DNA, or when the pressure was not reduced quickly, the cell walls could not break. In both cases, the expected bands could not be visualized in direct PCR assay (data was not shown).

Visualization of effects of lysozyme and autoclave treatments on the *L. acidophilus* cell walls

Morphological changes in the bacterial cell walls were visualized through optical microscopy after thermo-mechanical and enzyme treatments. The results of microscopic observation are shown in Fig. (2). However, the lysozyme treatment completely lysed the cells (Fig. 2a), cell walls and membranes of a large amount of autoclaved cells remained intact (Fig. 2b). This data had also been confirmed by the

DNA concentration obtained from extraction method M2A (Table 1). In the autoclaving assay, the concentration of extracted DNAs was very low, which was inversely related to the intact cell walls. The breakage of low numbers of cell walls was well observed in the supernatant of the autoclaved cell suspension (Fig. 2c), which was in accordance with direct PCR results. Because some of the cell walls were broken under the autoclave condition, direct PCR applied to the lysate showed an expected PCR band. The cell adhesion was also observable in the autoclave process. The denaturation and coagulation of proteins under the autoclave conditions seem to be associated with the adherence of the treated bacteria (Yoo, 2018). Although autoclave method was applicable for the identification of gram-positive *lactobacilli*, it cannot be recommended for molecular quantification analysis of bacteria as well as microbial diversity studies. Indeed, this protocol is not sufficient to break down all the cell walls.

Conclusions

In conclusion, this study assessed several DNA extraction methods, including autoclave and lysozyme, with a combination of silica-guanidinethiocyanate assay. DNA concentrations and qualities were also compared with the commercial kit. For *L. acidophilus* bacteria, protocols that included lysozyme produced a higher amount of DNA than the autoclave method. However, this is only one step in the multistep procedure of DNA extraction from gram-positive bacteria with resistance to cell wall degradation, which alters the characteristics of the extracted DNA. After the lysis step, the material used for DNA binding, such as the silica powder or fiberglass columns in the kit, had a powerful effect on the DNA qualities. Characteristics of the method, including lysozyme and silicon particles (M1) were comparable with the used commercial kit (M3). Notably, the autoclaving method was suitable for direct PCR while high numbers of bacteria existed in the sample. Clearly, virtual numbers of the bacteria could be affected by autoclaving process. This method could not completely break down all the bacterial cell walls. Thus, it is recommended not to use autoclaving steps in DNA extraction protocols done to quantify nucleic acids or determine the diversity of the bacterial community. Based on the assessments done, it revealed that the lysozyme-based approach presented here was simple, cost-effective, and comparable with commercial kits for extracting DNA of probiotic *lactobacilli* in downstream PCR applications.

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Author contributions

Monir-Sadat Shakeri: Presenting the research idea and study design, Writing the draft of the manuscript, Data collection, Data analysis, Revising and editing the manuscript, Data analysis and interpretation, Supervising the study, Approval of the final version; **Maryam Sadat Shakeri**: Presenting the research idea and study design, Data analysis and interpretation, Revising and editing the manuscript, Supervising the study, Approval of the final version.

Conflicts of interest

The authors declare that there is no conflict of interest.

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مقایسهٔ روش‌های استخراج DNA جهت شناسایی لاکتوباسیل‌های پروبیوتیکی، باکتری‌های مقاوم به تجزیه

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چکیده

استخراج DNA یک مرحلهٔ مهم در تمام پروتکل‌های مبتنی بر اسید نوکلئیک جهت شناسایی میکروارگانیسم‌ها می‌باشد. باکتری‌های اسید لاکتیک بخش مهمی از جمعیت میکروبی سالم در دستگاه گوارش انسان هستند. این باکتری‌های گرم مثبت چندین لایه پپتیدوگلیکان در دیوارهٔ سلولی دارند که باعث ایجاد مشکلاتی در تجزیهٔ سلول و دستیابی به روش‌های مطمئن برای استخراج DNA می‌گردد. هدف از این مطالعه، ارزیابی فرایندهای استخراج DNA مبتنی بر اتوکلاو و لیزوزیم برای دستیابی به DNAهای ژنومی با کیفیت بالا از باکتری *لاکتوباسیلوس اسیدوفیلوس* بود. همچنین غلظت و کیفیت DNA با کیت تجاری مقایسه شد. نتایج نشان داد براساس کاربرد DNA در فرایندهای پایین دستی، روش‌های مناسب برای استخراج DNA متفاوت خواهند بود. روش‌هایی که دارای تیمار با آنزیم لیزوزیم بودند نسبت به تیمار با اتوکلاو مقادیر بیشتری از DNA تولید کردند. تیمار با آنزیم لیزوزیم در ترکیب با روش سیلیکا-گوانیدین تیوسیانات، پروتکل کارآمد و مقرون‌به‌صرفه برای تجزیهٔ روتین باکتری *لاکتوباسیلوس اسیدوفیلوس* بود. غلظت و کیفیت DNA به‌دست‌آمده در این روش قابل مقایسه با کیت تجاری بود. اما تیمار با اتوکلاو تأثیر کمی بر شکستن دیواره‌های سلولی داشت که منجربه استخراج غلظت پایین DNA گردید. این روش نتوانست به‌طورکامل تمام دیواره‌های سلول باکتری را بشکند. البته تعداد کمی از دیواره‌های سلولی شکسته‌شده در سوپرناتانت سوسپانسیون سلولی اتوکلاو شده به‌طور میکروسکوپی مشاهده شدند. لذا این پروتکل برای انجام آزمایش PCR مستقیم روی نمونه‌های حاوی مقادیر زیادی از لاکتوباسیل‌ها از کیفیت خوبی برخوردار بود. این نتاج بر انتخاب دقیق روش استخراج DNA براساس آنالیزهای پایین دستی محصولات PCR تأکید می‌کند.

واژه‌های کلیدی: اتوکلاو، استخراج DNA، *لاکتوباسیلوس اسیدوفیلوس*، لیزوزیم، PCR مستقیم