A comparison of methods for extracting plasmids from a difficult to lyse bacterium:

Lactobacillus casei

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ABSTRACT

There are few practical protocols to extract efficient plasmid DNA from the difficult-to-lyse bacterium, *Lactobacillus casei*. This is related to production of a large amount of exopolysaccharide coat and its special physiological characteristics. In this study, we optimized a protocol to extract efficient plasmid DNA from a recombinant *L. casei* strain. Different extraction methods were evaluated in three classes of conventional, kit-based, and combined protocols. The quantity and quality of the extracted plasmid DNA were determined by spectrophotometry, agarose gel electrophoresis, and PCR. Results revealed that the yield of the extracted plasmids differed for each protocol and conventional protocols showed higher plasmid yields. We suggested an effective, inexpensive protocol to extract plasmid DNA from the recombinant *L. casei* for downstream biological processes.

Keywords: *Lactobacillus casei*; Plasmid; Extraction; PCR

INTRODUCTION

Bacteria belonging to the genus *Lactobacillus* are the members of lactic acid bacteria (LAB), a broadly defined group of the intestinal microflora [1]. These bacteria exist in the intestine of most animals and humans [2]. This phylogenetically diverse genus plays an important role in the food industry and has a significant potential as an oral vaccine delivery vehicle using the plasmids that expresses bioactive compounds at mucosal surfaces [3, 4].

The metabolism of lactose, fermentation of citrate, production of bacteriocin, proteinase activity and resistancy to phage or drugs are some of plasmid associated phenotypes in *Lactobacillus*. Also, some plasmids are traditionally used for genetic engineering in this genus [5-9].

Failure to demonstrate plasmids in *L. casei* could reveal ineffectiveness of the current protocols to extract efficient plasmid DNA from this bacterium [10]. The most important problem is the difficulty of cell lysis for extraction and purification of plasmids [10]. This is due to the huge exopolysaccharide coat of *L. casei* [11]. The important key in cloning molecular assays on *L. casei* is the extraction of efficient and purified plasmids. In the present investigation, different extraction methods were studied and an efficient protocol was optimized to extract plasmid DNA from a recombinant *L. casei* strain.

Materials and methods

Bacterial strains and growth conditions

For precise control of the test, a previously constructed recombinant *L. casei* strain was used [12]. Briefly, epsilon toxin gene of *Clostridium perfringens* was synthesized by Generay Biotechnology Company (China) and cloned between *Bam*HI and *Nae*I restriction sites of pT1NX vector obtained from BCCM/LMBP plasmid collection, University of Ghent, Belgium (<http://bccm.belspo.be/about/lmbp.php>). The modified vector was transformed into *L. casei* ATCC: 393 by a Gene PulserTM apparatus (Bio-Rad Laboratories, Richmond, CA). This strain was anaerobically grown at 37°C for 24 h in deMan, Rogosa and Sharpe (MRS) medium (Himedia, India), supplemented with erythromycin (7.5 µg/ml).

Plasmid DNA extraction

All the DNA manipulations were basically performed according to the standard procedures [13]. In this study, eleven plasmid extraction and purification protocols were tested in three classes: kit-based, conventional and combined protocols (Table 1).

Kit-based protocols

In this class, five protocols were performed (*P1-P5*). For all these protocols, after growing overnight cultures of *L. casei* at 37°C, a volume of 4 ml of each culture was centrifuged for 15 min at 13,000 rpm (16,000 × g) and 4°C. This pellet was used for plasmid extraction in different protocols as described below.

The *P1* protocol was accurately performed based on *PrimePrep*TM plasmid DNA isolation kit (*GeNet* Bio, Korea) according to the manufacturer's instruction. The *P2* protocol was done similar to *P1* with one modification: addition of lysozyme (20 mg/ml) to PR buffer

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(*PrimePrep*[™] kit) for better lysis performance. In the *P3* protocol, the lysis step was carried out according to O'sullivan [9] and other steps were continued based on the kit protocol. In the last kit based protocols (*P4* and *P5*), after preliminary centrifugation, the pellet was washed once with STE buffer (8% sucrose, 50 mM Tris HCL, 1 mM EDTA, pH=8.0) and then resuspended in 200 μ L of the same buffer containing lysozyme (20 mg/ml). The mixture was incubated for 1 h in the *P4* and 2 h in the *P5* protocol at 37°C. Alkaline SDS lysis (3% SDS, 0.2N NaOH) was performed according to O'sullivan et al. (1993) and continued based on the kit protocol [9].

Conventional protocols

In the conventional category, four previously described protocols were performed with some modifications [3, 10, 14]. First, after growing the overnight culture of *L. casei* at 37°C, a volume of 2 ml of culture was inoculated in 10 ml of the fresh MRS broth medium and then incubated at 37°C for two hours in the presence of 10% CO₂. Then, four milliliters of each culture were centrifuged for 5 min at 8000 rpm (6,000 $\times$ g) and 4°C and then, pellets were collected for extraction of plasmids in different protocols (*P6-P9*).

Both the *P6* and *P7* protocols were based on Klaenhammer with some modifications [10]. In the lysis step of the *P6* protocol, the mixture was incubated at 37°C for 1 h, whereas in the same step in the *P7* protocol, the mixture placed in an ice bath for one hour. After the completion of this step, deproteinization was performed with the addition of 50 μ L of 2 M Tris HCl, pH=7, and 70 μ L of 5 M NaCl to each tube, which was then mixed gently and centrifuged at 13000 rpm (16,000 $\times$ g) for 10 min. The supernatant was transferred to a new

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5 tube and the amount of 800 μ L of ice cold ethanol was added, mixed by inversion, and
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7 placed on ice for one hour. This mixture was centrifuged at 12000 rpm ($13,500 \times g$) for 10
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9 min and the collected pellet was washed with 500 μ L of 70 % ethanol. The pellet was dried
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11 and dissolved in 100 μ L of TE buffer (50 mM Tris and 10 mM EDTA, pH=7), and
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13 incubated at 65°C for 30 min. At the end, preparations were incubated overnight at 4°C for
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15 solubilization of the extracted plasmids.
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19 Two other conventional protocols (*P8* and *P9*) were based on Soomro et al. (2007) with
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21 few modifications as follows. In these protocols, we added 100 μ L of lysozyme (40 mg/ml)
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23 solution to suspended pellets and placed them on ice for 1 h and 2 h in the *P8* and *P9*
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25 protocols, respectively. Other steps of these protocols were similar to originated procedure
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35 **Combined protocols**

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37 In this study, two combined protocols, namely *P10* and *P11*, were designed. The *P10*
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39 protocol was a combination of the *P9* and *P1* protocols. Indeed, we performed *P10* as *P9*
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41 until the neutralization step and later steps were done according to the *P1* protocol. In the
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43 *P11* protocol, a combination of the *P7* and *P1* protocols was used. This protocol was
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45 carried out according to *P7* until the purification step and afterwards, it followed the *P1*
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Table 1: Different plasmid extraction and purification protocols from a recombinant *L. casei* tested in three classes of conventional, kit-based, and combined protocols

Steps	Protocols											
	Kit-based					Conventional				Combined		
	P1	P2	P3	P4	P5	P6	P7	P8	P9	P10	P11	
Culture	Overnight culture in MRS broth					Overnight culture in MRS broth						
	No					Addition overnight culture to fresh medium (1:5) Incubation for 2 h at 37°C with 10% CO ₂						
Centrifugation	4 ml at 13000/15 min/ 4°C					4 ml at 8000/5 min/ 4°C						
Washing	No			With STE buffer		No		With STE buffer		No		
Centrifugation	No			13000/15 min/ 4°C		No		8000/5 min/ 4°C		No		
Suspension	Kit	B1 (250 µl)	B1 (250 µl)	STE (200 µl), 20 mg/mlLysozyme		P1 (1000 µl) 15 min on ice		STE (1000 µl)		P1 (1000 µl) 15 min on ice		
P1(75 µl), 30 mg/mlLysozyme						STE (100 µl), 40 mg/mlLysozyme		P1(75 µl), 30 mg/mlLysozyme				
incubation		30 min at 37 °C		1h at 37 °C	2h at 37 °C	1h at 37 °C	1h on ice	1h on ice	2h on ice	1h on ice		
Centrifugation		No					10000/5 Min/RT					
Lysis step		Kit	B2 (400µl)	B3 (400µl)	B3 (200µl)	P1 Lysis solution (500µl)		P2 Lysis solution (500µl)		P1 Lysis solution (500µl)		
Incubation												R/T 5 min
Precipitation		Kit					Tris Hcl 2M (50µl)			Kit	Tris Hcl 2M (50µl)	
							NaCl 5M (70µl)				NaCl 5M (70µl)	
Centrifugation		13000/10 Min/RT					13000/10 Min/RT					
Purification		Kit					Transfer the supernatant to new microtube			Kit		
							Add 800 µl of cold ethanol and Store for 1h on ice					
							12000/10 Min/RT					
washing	Kit					Washing the pellet with 70% ethanol			Kit			
						13000/10 Min/RT						
Elution	70 µl PE buffer of Kit					100 µl TE buffer/ 30 min at 65°C/ overnight at 4°C			70 µl PE buffer of Kit			

Kit: PrimePrep™ Plasmid DNA Isolation Kit (GeNet Bio, Korea)

STE buffer: 8% Sucrose, 50 mM Tris HCL, 1 mM EDTA, pH=8.0

PRL buffer: PR Buffer of Kit; 30 mg/ml Lysozyme

B1 buffer: 25% Sucrose; 30 mg/ml Lysozyme

P1 buffer: 25% Sucrose; 50 mM Tris, pH, 7.5; 5 mM EDTA

B2 buffer: 3% SDS; 0.2 N NaOH

B3 buffer: 1% SDS; 200 mM NaOH

P1 Lysis Solution: 50 mM Tris; 5 mM EDTA; 50 mM Glucose; 3% SDS

P2 Lysis Solution: 50 mM Tris-HCl; 5 mM EDTA; 3% SDS, pH, 12.35

PE buffer: 10 mM Tris-HCl, pH 8.5

TE buffer: 10 mM Tris-HCl; 1 mM EDTA, pH, 8.0

Evaluating the quantity and quality of the extracted plasmids

The presence and integrity of the extracted plasmids were evaluated by agarose gel electrophoresis using the horizontal electrophoresis system (Bio-Rad). The types of band patterns indicate the quality of the extracted plasmids.

Moreover, the extracted plasmids were quantified by spectrophotometry using a BioPhotometer plus (Eppendorf, Germany) at 260 and 280 nm. The quality of plasmid DNA was determined by A_{260}/A_{280} ratio. The system software provided the DNA concentration (ng/ μ l) and automatically calculated the absorption ratio (A_{260}/A_{280}). Further, total plasmid yield was calculated as described by Sambrook [13].

PCR using extracted DNA

In order to check the efficiency and applicability of the extracted plasmid DNA, each DNA was tested by PCR. The extracted plasmids were used as template for the selective amplification of cloned epsilon toxin gene (*etx*) of *C. perfringens*. The selected specific primers (Sinaclon, Iran) were forward 5'TGGGAAGCTTCGATACAAGCA3' and reverse 5'CCACTTACTTGTCCTACTAAC3'. Expected size of the amplified fragment corresponded to 238 bp.

PCR reaction was performed using 5 μ l of the extracted DNA with 25 μ l of ready-to-use PCR master mix 2x (Sinaclon, Iran), 2.5 μ l (20 pmole/ μ l) of each primer, and dH₂O until 50 μ l volume. Amplicons of *etx* were obtained with 35 cycles following an initial denaturation step at 95°C for 10 min. Each cycle involved denaturation at 94°C for 1 min, annealing at 52.5°C for 1 min, synthesis at 72°C for 1 min, and the final extension step at

72°C for 10 min. PCR products were then examined for clarity and intensity. A constant amount of the amplified products (10 µL) was electrophoresed in 1.5 % agarose gel and observed using gel documentation system.

Sequencing

The PCR products were purified using AccuPrep® PCR Purification Kit (Bioneer, Korea) and sequenced for confidence by Macrogen, Korea. The same primers as for the original PCR were used.

Results

Quantity and quality of extracted DNA

In this study eleven plasmid extraction protocols were evaluated. Plasmids were extracted and purified from a recombinant *L. casei* strain and estimated spectrophotometrically. Results revealed that the total amount of extracted plasmids differed for each protocol, as confirmed by the PCR amplification of the cloned gene (Fig. 1).

Fig 1: Amplified PCR products (10 µL of each sample) with the primer set epsilon toxin gene on the extracted plasmid DNA. Lanes 1-11: Amplicons (238 bp) from the eleven plasmid extraction protocols; Lane M: 50 bp DNA markers (Fermentas). Lane C: Negative control (PCR on wild type *L.casei*)

The agarose gel electrophoresis of the extracted plasmids showed better results for the *P6* and *P7* protocols in terms of both concentration and purity. In some of the tested protocols (*P1* and *P11*), it was not possible to visualize the plasmid. In comparison with the conventional protocols, using the kit-based and combined protocols resulted in lower total amount of the extracted plasmids (Table 2).

Table 2: Concentration and quality of the extracted plasmids from *L. casei* by different protocols

Protocols	KIT based protocols					Conventional protocols				Combined Protocols	
	<i>P1</i>	<i>P2</i>	<i>P3</i>	<i>P4</i>	<i>P5</i>	<i>P6</i>	<i>P7</i>	<i>P8</i>	<i>P9</i>	<i>P10</i>	<i>P11</i>
Plasmid Concentration (ng/µl)	18	16	44	42	46	116	73	1041	1160	48	56
Quality of Plasmid (A_{260}/A_{280} ratio)	1.55	2.25	1.90	1.98	2.00	1.69	1.72	2.06	2.01	1.76	2.52

PCR amplification of DNA

All the protocols, except *P1*, provided acceptable plasmids for PCR amplification with the pair of the used primers. In these protocols, the single band of 238 bp of the target cloned gene was amplified and visualized in agarose gel (Fig.1); however, the resolution of amplicons was different for each protocol.

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Sequencing

The results of sequencing showed the validity of the target gene (Fig.2).

Fig. 2: Sequencing analysis for PCR product. Sequencing chromatogram of an exemplary 238 bp amplicon purified by PCR purification kit.

Discussion

The application of reproducible and efficient strategies to extract plasmid DNA is essential in molecular biology analyses [15, 16]. This study evaluated different methods to find the most efficient, economic, and performable way associated with the adequate purity of the extracted plasmid DNA from a recombinant *L. casei* for downstream molecular assays.

Previous studies have been done to evaluate the effectiveness of different plasmid extraction methods from *L. casei* [16-19] but, an efficient and economic method is still required for recombinant *L. casei* strains. Thus, different protocols were tested here.

Formerly, it has been shown that there are some flaws in *Lactobacillus spp* cell lysis to extract plasmids [10]. These inability to extract plasmids prevent further studies of natural

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5 or recombinant plasmids in this genus. For *L. casei*, it is more critical because of inefficient
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7 and time-consuming procedures of cellular lysis due to its specific physiological and
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9 morphological characteristics. This difficulty in plasmid extraction may be associated with
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11 lysozyme insensitivity [11, 18, 20]. To overcome this, the application of a 37°C incubation
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13 step for 1 h can facilitate cell wall digestion by lysozyme [9-11, 14, 20-24]. We tested this
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15 in different protocols and the *P6* protocol showed a better performance. Longer lysozyme
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17 treatment was not recommended because it allows the endogenous nucleases to become
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19 active [23].
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25 Our findings indicated that the application of the conventional protocols resulted in better
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27 extraction of plasmids from recombinant *L. casei* strains than the other tested protocols.
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29 The concentration of the extracted plasmid DNA was significantly higher in the
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31 conventional protocols (range: 73-1160 ng/μl) than in the kit-based procedures (range: 16-
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33 46 ng/μl) or combined protocols (range: 48-56 ng/μl). The highest yield of plasmid DNA
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35 was extracted from the *P9* protocol as a conventional method (1160 ng/μl). This may be
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37 related to lysozyme concentration (40 mg/ml) and incubation period on ice for 2 hours.
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39 Another key issue is the quality of the extracted plasmids from bacterial isolates that affects
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41 the sensitivity and usefulness of biological analyses such as PCR [16]. This study showed
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43 that different protocols resulted in variable sensitivity of PCR detection. Also, the purity of
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45 the extracted plasmid DNA varied from 1.55 to 2.52 in different protocols. In the *P1*
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47 protocol, the lowest quality product was obtained ($A_{260}/A_{280} = 1.55$). Moreover, a low
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49 molecular band was seen in the amplified PCR products of *P6*, *P8*, and *P9* protocols (Fig.
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6 1) which contributed to their A_{260}/A_{280} ratios (Table 2). This may be related to the high
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8 concentration of lysozyme or composition of lysis solution used in these protocols.
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10 In conclusion, the comparison of different extraction protocols showed that the *P6* protocol
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12 could be the best method (compared with other protocols) for the efficient extraction of
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14 plasmid DNA from difficult-to-lyse gram positive bacteria such as recombinant *L. casei*
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16 strains, which resulted in superior downstream performance in PCR. This method could be
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18 used for many purposes such as screening of recombinant colonies after transformation.
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