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Evaluation and modification of alkaline lysis plasmid preparation method from *Lactobacillus* spp.

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Abstract : Lactic acid bacteria (LAB) has been regarded as a useful microorganism and tried to manipulate plasmid DNA for increasing the usefulness. Although several methods have been developed to isolate plasmid DNA from *Escherichia coli* (*E. coli*), these methods were not sufficient to apply to LAB with exception of O'Sullivan's lysis method. So, we evaluated plasmid DNA extraction from LAB using general *E. coli* preparation methods and tried to improve the extraction yield and DNA purity by modifying O'Sullivan's alkaline lysis method. To improve the extraction yield, salt and carrier were added to precipitant and those were incubated at −70°C. Only incubation at −70°C was the effective method of those modifications. Purity of plasmid DNA was improved by two times of each centrifugation and phenol/chloroform extraction. However, DNA was damaged by twice extraction with phenol/chloroform. Also, exclusion of ethidium bromide showed negative effect to purity. Additionally, it was recommended that improvement of the extraction yield may be due to centrifugation at high speed for more time and to dissolving complete DNA pellet before addition of 7.5 M ammonium acetate. Extraction using this modification produced higher quality of plasmid DNA.

Key words : extraction yield, Lactic acid bacteria (LAB), plasmid DNA, purity

Introduction

Lactic acid bacteria (LAB) has been regarded as a useful microorganism especially played an important role as a starter of fermented food product [8, 15]. Nowadays, LAB was recognized as GRAS (generally recognized as safe) scientifically and used for several purposes such as live vaccine and other clinical practices [11, 13]. To increase the usefulness, it was tried to manipulate plasmid DNA and that was regarded as useful tools [17, 19].

Plasmid DNA has been used in many molecular manipulations of DNA and it was complemented by the development of efficient and rapid methods for isolation. Recently, many procedures have been developed for plasmid DNA preparation [2-4, 6, 7, 14, 16, 18, 20] and many commercial kits are now available. However, these procedures were not sufficient to apply LAB because most of them have been developed based on the isolation of plasmid DNA from *Escherichia coli* (*E. coli*).

A method developed by Anderson *et al.* [1] was useful to isolate plasmid DNA from lactic *Streptococci* and also used in several experiments [5, 10] but it had a limitation in the elimination of chromosomal DNA. To overcome this limitation, O'sullivan and Klaenhammer [12] developed a new method based on alkaline-mediated denaturation of chromosomal DNA and extraction of protein with phenyl/chloroform/isoamylalcohol.

O'Sullivan's method [12] was effective to acquire high quality of plasmid DNA for restriction analysis and cloning experiments. However, it could induce damage of DNA such as the breakage and loss due to extensive break of cell wall. Furthermore, it was difficult to acquire sufficient amount of plasmid as LAB usually had the low-copy number of plasmid DNA. Also, the isolation method from LAB is time-consuming compared with methods for *E. coli*.

In those reasons, we tried to evalaute the possibility

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of plasmid DNA extraction from LAB using general *E. coli* preparation methods and to improve the extraction yield and DNA purity by modifying O'Sullivan's alkaline lysis method.

Materials and Methods

Bacterial strains and growth conditions

Lactobacillus reuteri (*L. reuteri*, KACC 91266p) used in this study was grown anaerobically at 30°C on MRS medium (Difco, USA) without antibiotics. *E. coli* M15 transformed with pQE30-UA (QIAGEN, Germany) vector was grown with vigorously shaking at 37°C on LB medium (Difco, USA) contained 25 μg/ml of kanamycin and 100 μg/ml of ampicillin. Bacterial strains were cultured on large volume of each media and divided into several tubes with the same volume for excluding possible error due to different bacterial growth rates in each culture tubes. Plasmid DNA was extracted from 5 ml of *L. reuteri* and 3 ml of *E. coli*, respectively.

Comparison with plasmid extraction methods

General mini-prep methods were applied to *E. coli* and *L. reuteri* to compare with the efficacy of each method and to confirm the availability of *Lactobacillus* spp. The general methods were selected and used *E. coli* mini-prep, a commercial kit (QIAGEN, Germany), alkaline lysis, and boiling method [16]. Additionally, O'Sullivan's method [12] was compared with those methods. *L. reuteri* was pre-treated with 1mg/ml of lysozyme to lysis peptidoglycan layer on 37°C for 30 min. The extracted plasmid was analyzed on 0.7% agarose gel.

Modification of O'Sullivan's alkaline lysis method

To increase the extraction yield, three general DNA concentration tips were applied to O'Sullivan's alkaline lysis method. During plasmid DNA precipitation, salt concentration was increased at first step by adding sodium acetate (3.0 M, pH 5.2) into sample; glycogen, a carrier, was added as the second step; the samples were kept on ice to decrease temperature as the final step.

To improve plasmid purity, protein clumps were eliminated by two times of each centrifugation and phenol/chloroform treatment. Furthermore, treatment of 0.5 mg/ml of ethidium bromide was excluded during protein elimination step to simplify the procedure.

Analysis of plasmid DNA

The concentration of plasmid DNA was measured with a spectrophotometer (GeneQuant; Amersham-Phamarcia Biotech, Sweden) and the purity of DNA was calculated by measuring ratio of Δ260/Δ230 and Δ260/Δ280 for contamination of buffer and protein, respectively. All experiments were triplicated and the comparisons of yield and purity were carried out using the Statistical Package for the Social Sciences, student version. To determine whether this modified mini-prep was able to result in sufficient plasmid DNA for restriction analysis; two types of plasmid DNA (6 kb and 1.5 kb) were digested with *Hin*dIII and *Sal*I and cloned into pQE30Xa (QIAGEN, Germany) and pBluescript-SK (+) vectors (Invitrogen, USA).

Results

Comparison of plasmid extraction methods

The extraction yields of total plasmid DNA were different depending on the extraction methods. In *E. coli*, the highest yield was the boiling method, and the alkaline lysis method, the O'Sullivan's method, and the commercial kit in order (Fig. 1). Both boiling and alkaline lysis methods showed the breakage of genomic DNA and the severe contamination of RNA despite of the high yield efficacy. O'Sullivan's lysis method also showed higher yield efficacy than commercial kit, but it made some nick of plasmid DNA which resulted in the formation of open circular or linear plasmid DNA (data not shown). The commercial kit showed the lowest concentration but high fidelity of plasmid DNA.

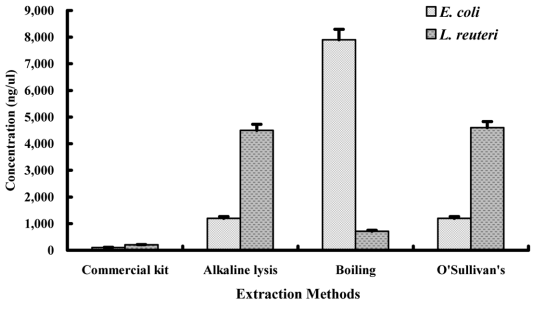

Fig. 1. The concentration of plasmid DNA extracted with several methods from *E. coli* and *L. reuteri*. Plasmid DNA was extracted with a commercial kit, alkaline lysis, boiling and O'Sullivan's lysis method from *E. coli* and *L. reuteri*. The concentration of plasmid DNA was measured using a spectrophotometer.

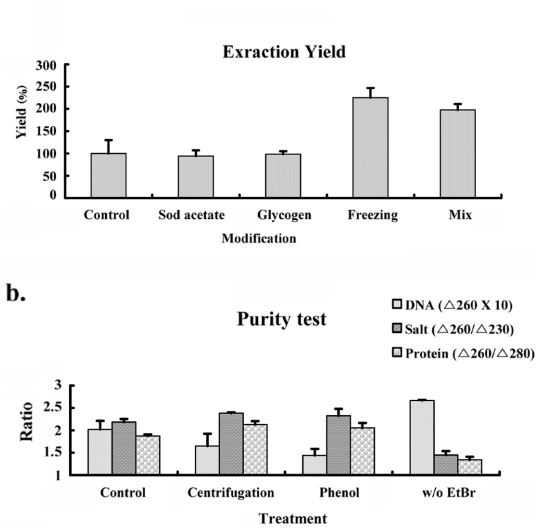

Fig. 2. The efficacy (a) and purity (b) of plasmid DNA extraction by each modified method.

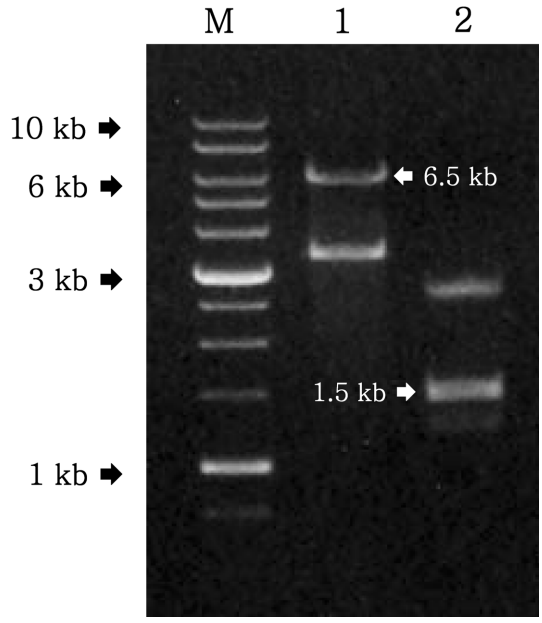

Fig. 3. Confirmation of the quality of plasmid DNA for the enzyme analysis. Two types of plasmid DNA from *L. reuteri* were digested by restriction enzyme (*Hin*dIII and *Sal*I) and cloned into pQE-30Xa and pBluescript-SK (+). M, 1 kb DNA ladder; Lane1, plasmid DNA (6.5 kb) cloned into pQE30Xa; Lane2, plasmid DNA (1.5 kb) cloned into pBluscript-SK (+).

In *L. reuteri*, the O'Sullivan's and alkaline lysis methods showed higher than other methods (Fig. 1). All methods showed the plasmid DNA smeared on agarose gel excepting the O'Sullivan's method. The boiling method degraded plasmid DNA completely. Only the O'Sullivan's method yielded the plasmid DNA of high quantity and quality for further study.

The yield and purity of plasmid DNA extraction

The extraction yield of plasmid DNA was decreased by increasing salt concentration (–5.94%) and by adding carrier (–1.86%). However, freezing the sample improved the extraction yield which was increased twice higher than original method. Similar result was also obtained by incubation on the ice for 10 min (data not shown). The freezing method showed the highest efficacy even though the yield of mixed method showed the high efficacy (97.8%) (Fig. 2a)

The purity of plasmid DNA was increased by twice of centrifugation (DNA, –18%; salt, 9.2%; protein, 13.6%) and phenol treatment (DNA, –28.9%; salt, 6.4%; protein, 9.6%) but the concentration was slightly decreased (Fig. 2b). The salt and protein contaminants were almost eliminated and the extracts reached about a 100% in purity [16]. No ethidium bromide (DNA, 32%; salt, –33.7%; protein, –28.5%) sharply increased the concentration but the purity was prominently decreased (Fig. 2b). On agarose gel, some nicks were appeared on the twice of phenol treatment, but not on the twice of centrifugation. The last method without ethidium bromide showed that most DNA was genomic DNA and the contaminants were not eliminated (data not shown).

Isolation and cloning of plasmid DNA from wild *Lactobacillus* spp.

Two plasmid DNAs from *L. reuteri* were digested by restriction enzyme and cloned into *E. coli* vector systems to evaluate plasmid quality for DNA manipulation. The DNA of 6.5 kb was cloned into pQE-30Xa vector which was restricted with *Sal*I and the 1.5 kb, pBluescript-SK (+) with *Hin*dIII (Fig. 3).

The efficacy of extraction and the purity of plasmid were improved with the modification of the O'Sullivan's method. The quality of extracted plasmid DNA reached the degree of molecular manipulation. Based on the results, the O'Sullivan's method was modified at step 4, 5, 6, and 7 (Fig. 4).

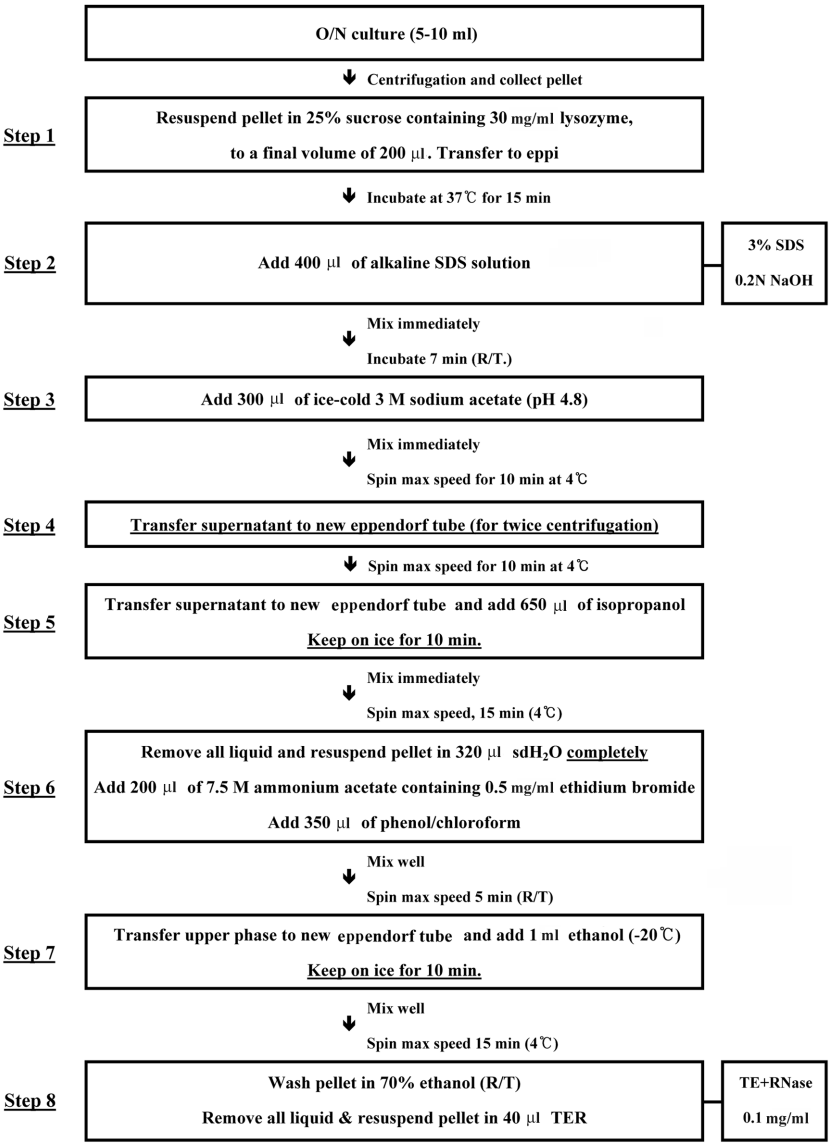

Fig. 4. Out-line of the plasmid DNA mini-prep procedure. The supernatant was re-centrifuged for eliminating cell wall debris in step 4 and precipitation mixture was kept on ice for 10 min to improve precipitation in step 5 and step 7. In addition, DNA pellet should be dissolved completely in sterilized distilled H_2O before the addition of 7.5 M ammonium acetate.

Discussion

Although several plasmid DNA extraction methods have been developed and applied to *E. coli*, only O'Sullivan's method could be applicable to *Lactobacillus* spp. Therefore, general DNA concentration protocols were applied to improve extraction yield and purity of plasmid DNA. Increasing salt concentration and adding carrier reversely decreased extraction yield. Salt and carrier might act as important cofactors in a small quantity of DNA since DNA was enough by themselves to fully precipitate without cofactor. Also, some DNA might be taken with salt and carrier and lost during a washing step. Extraction yield was increased at least twice by decrease of sample temperature and unexpectedly salt or other buffer

contaminants decreased. Therefore, it could be the best method even though the yield was increased by the mixed three methods.

Centrifugation was an effective physical method for separating cell components, genomic DNA and plasmid DNA. Twice of centrifugation were meaningful to produce high quality of plasmid DNA from *Lactobacillus* spp. which cell wall debris were more difficult to be eliminated than *E. coli*, even though the concentration slightly was decreased. Twice of treatment of phenol/chloroform made worse the condition of plasmid DNA than once. The twice of phenol treatment might induce nicks in the plasmid due to the damage of DNA. Exclusion of ethidium bromide made a difficult to take aqueous supernatant by interfering with sticky DNA on the protein layer. However, addition of ethidium bromide made easy to take supernatant and acquire pure plasmid DNA that act as indicators of each layer and may make less sticky middle layer [12].

In addition, PEG treatment was the one of DNA purification methods [9] but extraction with phenol/chloroform and addition of ethidium bromide were enough to adjust the preparation of plasmid DNA from *Lactobacillus* spp. in our study. Furthermore, DNA pellet should be dissolved completely in sterilized distilled water before addition of 7.5 M ammonium acetate, especially maxi- or mini preparation.

To confirm the quality, plasmid DNA from *L. reuteri* was digested and cloned into *E. coli* vectors: pQE30-Xa and pBluescript-SK (+). The result demonstrated that the quality of plasmid DNA was enough to manipulate the plasmid DNA for further study.

Conclusion

In conclusion, it was effective to incubate precipitant on ice or deep freezer for the extraction efficacy and to centrifuge precipitant twice for the purity of plasmid. However, it was not recommended to increase salt concentration, to add carrier, and to treat phenol/chloroform twice for the purpose of this study.

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