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Optimization of DNA Extraction of *Lactobacillus spp* for Identification by *tuf* B gene –Based Polymerase Chain Reaction

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Abstract
Lactobacilli are of considerable technological and commercial importance because of their role in the manufacturing and preservation of many fermented food products. The aim of this study was to optimize and evaluate three methods of DNA extraction and purification of DNA from *Lactobacillus* spp to be used for the amplification of 584-bp region of the *tuf* gene by polymerase chain reaction. The methods were: Phenol–chloroform extraction, boiling, and Wizard genomic DNA purification kit with modifications. Results demonstrated that extracted genomic DNA using Wizard genomic DNA purification kit with modifications for DNA extraction provided was higher yield of DNA with the highest purity than the other extraction methods. Purity was documented by gel electrophoresis. The quality of the genomic DNA isolated by this method was verified by polymerase chain reaction targeting the *tuf* gene.
Keywords: *Lactobacillus*, DNA extraction, identification, *tuf* gene, PCR.

1. Introduction

The genus *Lactobacillus* consists of a genetically and physiologically diverse group of Gram positive , rod shaped ,catalase negative , non-spore forming bacteria (MacFaddin,2000), Due to their fermentative properties, Lactobacilli are widely used during gastrointestinal disorders as dietary supplement, and by food manufacturers and processors (Agnew and Hillier, 1995).*Lactobacillus* spp produce a variety of antibacterial compounds such as organic acids, diacetyl, hydrogen peroxide, reuterin and bacteriocin or bactericidal proteins during lactic fermentations (Hirano et al.,2003). Bacteriocins have been fund to be effective in controlling bacterial infections and their extensive use in combinations as natural food bio- preservatives and health care products has been reported (Cleveland et al., 2001).

The identification of *Lactobacillus* isolates by phenotypic methods is difficult because it requires in several cases, determination of bacterial properties beyond those of the common fermentation tests(MacFaddin,2000). The development of a molecular culture- independent detection methods such as PCR is a simple technique that quickly amplifies specific sequences of target DNA from indicator organisms appears to be invaluable in the case of probiotics particularly *Lactobacillus spp*.(Roy *et al* ., 2000 ;Ventura and Zink , 2002). The *tuf* gene has been used as a target gene for phylogenetic studies (Ludwig et al., 1993). This gene encodes the elongation factor Tu, involved in protein biosynthesis, which facilitates the elongation of polypeptides from the ribosome and aminoacyl-tRNA during translation. It is universally distributed

and in most Gram-positive bacteria only one *tuf* gene per genome has been found (Sela et al.,1989), thus it is ideally suited for phylogenetic studies.

The aim of this study was to optimize and evaluate three methods of DNA extraction and purification to be used for the amplification of 584-bp region of the *tuf* gene of *Lactobacillus* spp by PCR assay.

2. Materials and Methods

Bacterial Strains and Growth Conditions: Six *Lactobacillus* strains were grown in MRS broth at 37°C for 24 hours , and were characterized as previously described (Abdulla et al., 2013).

Optimization of DNA extraction

Phenol–chloroform method

This is the protocol reported by Kalia et al.,(1999) and is described here with several modifications.

Briefly, the cell Pellet was re-suspended in 900 µl TNE buffer, cells were collected by centrifugation at (15.000 rpm for 5 min).The pellet of each sample was collected and re-suspended in 800 µl ice-cold 70% ethanol, mixed thoroughly, and placed on ice for 20 min. The suspension was centrifuged at 12000 rpm for 5 min and the pellet was re-suspended in 480 µl of SET buffer and 4 µl of RNase A(10 mg/ml).The tubes were then kept at -20°C for 20 min, then immediately transferred to a water bath at 68 °C for 10 min. Subsequently, 1 vol phenol: chloroform: isoamyl alcohol at (1:25:24) were added and mixed by gentle inversion. The mixture was centrifuged at 5000 rpm for 10 min and the upper phase was collected and placed into a new tube. Finally, 0.1 ml of 3M sodium acetate and equal volume of absolute ethanol at -20°C was added to each tube and mixed gently prior to centrifugation at 5000 rpm for 10 min. The pellet was washed with 70%(v/v) ethanol. Finally, the pellet was re-suspended in 100 µl TE buffer .

Boiling method

One ml sterile distilled water was added to the pellet, after vortexing the samples were boiled at 100°C for 20 min by placing in a boiling water the tubes .The suspension were cooled immediately to - 20°C for 20 min and centrifuged at 13,000 rpm for 5 min and the supernatants were kept under freezing until used (Keegan et al.,2005).

Wizard genomic DNA purification kit with modifications

The wizard genomic DNA purification kit (Promega/USA) was used with several modifications for extracting chromosomal DNA of all strains isolates from the overnight cultures as follows: stock cultures were streaked onto MRS agar and a single colony was used to inoculate 3 ml MRS broth. Following overnight incubation, cells were collected by centrifugation at 12,000 rpm for 5 min at 25°C.The cell pellet was re-suspended in 750 µl of 50 mM EDTA. A volume of 100 µl of solution of lysozyme (50 mg/ml / Sigma /USA) were added to the cell suspension and incubated overnight at 37°C with gentle mixing. Subsequently 100 µl of proteinase k(10 mg/ml Sigma /USA) were added and tubes were incubated for 30 min at 55°C with gentle mixing. The suspension was centrifuged at 12,000 rpm at 25°C for 5 min and the pellet was gently re-suspended in 950 µl nuclei lysis solution . Then 6 µl of RNase A(50 mg/ml, Sigma/USA) was added to the lysate, which was then incubated for 45 min at 37°C with gentle inversion. For protein precipitation, 300 µl precipitation solution was added to the lysate mixture and vortexed at medium speed for 20 s. The lysate was centrifuged at 12,000 rpm for 20 min and the supernatant was transferred to a clean 1.5 ml Eppendorf tube. One additional centrifugation step at 12.000 rpm for 10 min was performed to remove any residual protein. To precipitated DNA, 600 µl of isopropanol, at room temperature were added. The samples were centrifuged at 12.000 rpm for 20 min, then pellet was washed with 70% ethanol before air drying for 10 min. Finally, the pellet was re-suspended in 50µl DNA rehydration solution .

Evaluation of Quantity and Purity of Extracted DNA:

The extracted DNA samples were quantified using aNanoDrop spectrophotometer .The **260/280 nm absorbance ratio** and **DNA yield (µg) = DNA concentration (µg/µl) × total sample volume (ml)** were used to measures DNA purity and concentration as described by Sambrook and Rusell,(2001). The quality of the isolated DNA was also evaluated by 1.5% Agarose gel electrophoresis. A 100 bp plus DNA ladder (Bioneer, Korea) was used as a molecular weight marker to estimate the approximate size of the isolated DNA.

PCR amplification

PCR amplifications mixture of 30 µl was performed using 5 µl of purified DNA solution with the , 2x Taq PCR Pre – Mix (SolGent™ 2x Taq PCR Pre – Mix , SolGent Co.,Ltd.)and 2.5 µl (10 pmole /µl) of each primer. After pre-incubation at 95°C for 3 min, amplifications were carried out in a GTC thermal cycler (Cleaver Scientific, UK). for 35 cycles, each with 30 s denaturation at 95°C, 40 s annealing at 61 ˚C and 1 min extension at 72 ˚C. The final elongation step at 72 ˚C was for 5 min and the holding temperature was 10 sec .The tuf primers (*tufB* gene) (AccuOligo/Bioneer/ Korea) were the Forward 5....′ATGGACGGTGCGATCTTAGTT.. 3 and Reverse 5.....′ACTTGACCACGAACAACTTGTTCA.....3′.Expected size of the amplified fragment corresponds to 584bp. primers described by De et al.(,2010).

Agarose gel electrophoresis:

The amplified PCR products were separated by gel electrophoresis using 1.5%(w/v) agarose gels (sigma/USA) prepared in TBE buffer (Sigma /USA). Gels have been run at a constant voltage of 70 V for one hour. DNA fragments have been visualized with uv transilluminator and photographed as described (Sambrook and Russell, 2001).

3. Results

Evaluation of Quantity and Purity of Extracted DNA:

The results of this study demonstrated that compared to Phenol–chloroform and boiling methods, DNA extraction with modified wizard protocol produced highest DNA yield (between 96.0 and 140.0 ug) that was of the highest DNA purity (between 1.70 and 1.82) when compared with the other two protocols, purity of the DNA extracted by Phenol–chloroform and boiling methods were less than 1.19 (Table 1 & 2). The extracted DNA was free from protein a contamination and could be used in downstream applications such as PCR .

The DNA extracted using the three protocols were observed for degradation by 1.5% Agarose gel electrophoresis. Also, all DNA extracted by modified wizard protocol produced sharp bands, whereas the bands produced by the other two protocols were less sharp and appeared with smear (Figure 1).

PCR Amplification of DNA from Lactobacillus spp

In order to check the efficiency and reliability of the extraction methods, the PCR to amplify *tuf* gene was performed. PCR products were examined for clarity and intensity. Results of this study indicated that the modified commercial wizard kit/Promega was the most successful extraction method for DNA for PCR amplification of the target *tuf* gene of *Lactobacillus* spp. Figure 2 shows the PCR products (amplicons) of six *Lactobacillus* spp were in the form of single band of molecular size of 584 bp that is in agreement with the

calculated size of target *tuf* gene of *Lactobacillus* spp .

4. Discussion

Identification of *Lactobacillus* isolates by phenotypic methods are difficult as it requires the determination of several biochemical bacterial properties (MacFaddin,2000) .The development of a molecular culture-independent detection methods such as PCR is a simple technique that quickly amplifies specific sequences of target DNA from indicator organisms and has been widely used for the identification of major spp of microorganisms including *Lactobacillus* spp(Roy *et al* ., 2000 ;Ventura and Zink , 2002). The *tuf* gene has been used as a target gene for phylogenetic studies (Ludwig et al., 1993). This gene which encodes the elongation factor Tu, involved in protein biosynthesis, which facilitates the elongation of polypeptides from the ribosome and aminoacyl-tRNA during translation. It is universally distributed and in most Gram-positive bacteria only one *tuf* gene per genome has been found (Sela et al.,1989), thus it is ideally suited for phylogenetic studies. Purified DNA materials are important prerequisite for the effectiveness of PCR method.

In this study three different DNA extraction methods;(Phenol–chloroform, boiling, and Wizard genomic DNA purification kit with modifications) were assessed in order to determine the best method to extract DNA of 6 Lacobacillus spp. Results of the indicated that using the modified Wizard genomic DNA purification kit resulted in the highest yield of pure DNA materials in comparison to the boiling and Phenol–chloroform methods for DNA extraction.The DNA sample produced by the modified Wizard genomic DNA purification kit was very suitable for PCR as it resulted in a very sharp and dense amplicones for the tuf gene when compared to the PCR products resulted from using DNA samples extracted by the other two methods.

It is conceivable that the superior purity of the DNA obtained using the modified Wizard genomic DNA purification kit to DNA material resulted from using boiling and Phenol–chloroform methods for DNA extraction can be attributed to the use of high concentration of lysozyme (50 mg /ml) and to additional step of protein precipitation, RNase (50 mg/ml), which may have resulted in the removal of contaminants and increased the purity of the extracted DNA(Ausbel *et al.*, 1998).The components of the modified Wizard genomic DNA purification kit include lysozyme, RNase A and EDTA,and a detergent. RNase A serves to decompose residual RNA which is a frequent contaminant of the extracted DNA sample. Additionally, the inclusion of lysis buffer step in this method may enhance the extraction of DNA of higher purity than the other methods (Sambrook and Russell, 2001).Furthermore, Lysozyme digests cell well components of gram-positive bacteria. Zymolase and murienase aid in protoplast production from yeast cell. Whereas, Proteinase K cleaves glycoprotiens and inactivates RNase and DNase in 0.5 to 1% SDS solution. Heat is also applied to enhance lysis (Ausbel *et al.*, 1998).

Ethanol precipitates the DNA and RNA while isopropanol selectively precipitates DNA leaving RNA and polysaccharides in the solution. The cell wall of Lactobacilli consists of peptidoglycan, which is decorated by teichoic acids, surface proteins, and anionic and neutral polysaccharides therefore, it is much more resistant to cellular lysis (Delcour et al.,1999). Many protocols have been developed for extraction of bacterial genomic DNA However, only few of them provide optimal DNA isolation from widespread types of bacteria. Extracting DNA from Gram-positive and Gram-negative bacteria is an essential preliminary step in species identification, using techniques such as PCR, restriction digestion, pulsed-field gel electrophoresis (PFGE), and optical mapping(Sambrook and Rusell,2001).

The presence of proteins in DNA isolates also can interfere with PCR amplification process, especially if the protein is DNase that can decompose DNA(Sambrook and Russell, 2001).

The use of appropriate DNA extraction methods is critical for successful PCR studies on clinical samples and it is recommended that the DNA extraction techniques should be carefully selected for each specimen type (Yang et al .,2008). Hendolin, et al.(2000) extracted DNA in middle ear effusions containing *Haemophilus. influennzae, Streptococcus penumoniae*, *Alloiococus otitidis* and *Moraxella catarrhalis* using a traditional phenol/ethanol extraction method and extraction kit (QIAamp DNA mini kit, Qiagen) with the addition of treatment with SDS solution (sodium dodecyl sulfate-NaOH-chaotropic salt), and performed multiplex PCR analyses. It is interesting to note that, although the same sample was used, phenol/ethanol extraction resulted in significantly high PCR positive ratio of Gram-negative bacteria (*H. influennzae, M. catarrhalis*), while extraction using the extraction kit resulted in a significantly high detection rate of Gram-positive bacteria (*S. penumoniae, A. otitidis*). The cell wall structure of Gram positive organism is more critical than the Gram negative cell wall structure because of the peptidoglycan layer.

Application of this protocol for the preparative isolation of genomic DNA from Gram-positive bacteria was efficient and suitable for down-stream applications, as the PCR and sequencing results were well in accord with those obtained by the routine method (Rantakokko-Jalava and Jalava, 2002). The results indicated that molecular size of *tuf* gene was approximately 584bp as demonstrated in fig (2). A PCR assay targeting the *tuf* gene for detecting *Lactobacillus* spp was used to compare the efficiency of three extraction methods . The degree of sequence conservation in the *tuf* region of *Lactobacillus* species reflects the evolutionary distance separating

these different species. Bioinformatic analysis suggested a highly conserved DNA module among the *Lactobacillus* strains investigated here, consisting of the *tuf*, *tig*, *clp*, and GTP binding protein genes(Ventura et al.,2003). Comparison of the similarity values of the nucleotide sequences indicated that the *tuf* gene is slightly less conserved that the16S rRNA gene in *Lactobacilli*(Chavagnat et al., 2002).
The use of *tuf* genes in LAB species as an alternative or complement to the 16S rRNA marker mainly supports the phylogenetic relationships that are revealed by the
16S rRNA-based determination of bacterial phylogeny but also provides more detail that can be used to distinguish closely related species and that can be helpful for inferring phylogeny in closely related species (e.g., *B. animalis-B. lactis*, *B. longum-*
B. infantis, and *L. johnsonii-L. gasseri*).**(** Ventura et al.,2003**).** The large similarities between tuf-aa and 16S rRNA trees suggest that the tuf gene evolves generally like the16S rRNA gene in Lactobacilli(Collins et al.,1991).

5. Conclusion

Results of this study suggest that extracted DNA obtained using the Wizard genomic DNA purification kit with modification is superior to the other two described methods and could directly used for quality assessment with downstream application such as PCR for the determination of the *Lactobacillus* isolates at the species level.

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Table 1:Quality of chromosomal DNA (260/280) of Lactobacillus strains.

NO. of strains	Phenol–chloroform	Boiling method	Modified of wizard
1	1.03	1.10	1.80
2	1.05	1.02	1.70
3	1.28	1.02	1.73
4	0.20	1.01	1.70
5	1.33	1.12	1.73
6	1.22	1.19	1.82

Table 2:Yield of chromosomal DNA (ng/ µl).

NO. of strains	Phenol–chloroform	Boiling method	Modified of wizard
1	45.0	40.0	140.0
2	33.0	20.0	96.0
3	49.0	16.0	125.0
4	15.0	32.0	120.0
5	51.0	49.0	118.0
6	15.0	55.0	130.0

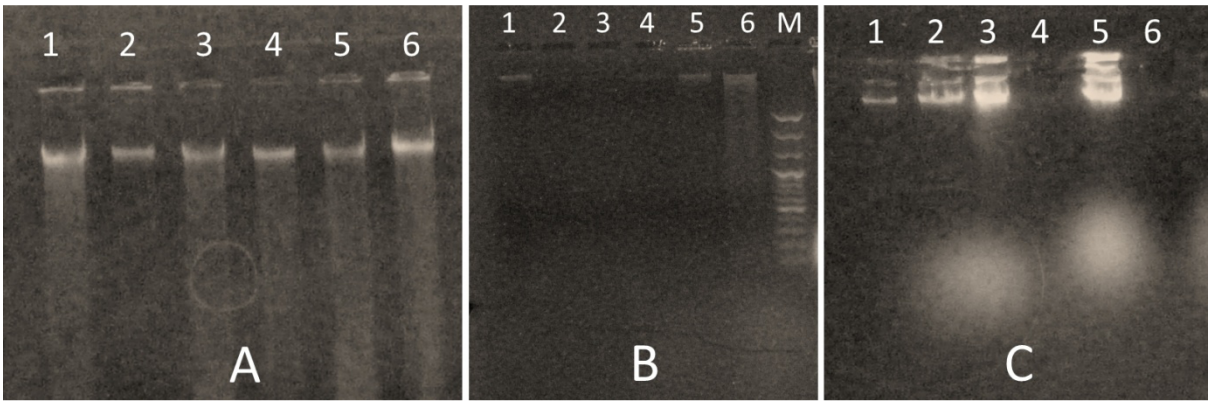

Figure 1. Agarose gel electrophoresis of extracted DNA from 6 *Lactobacillus spp* (1-6) with three methods (A-C): A. modified wizard genomic DNA purification kit , B. Boiling and C. phenol–chloroform method .

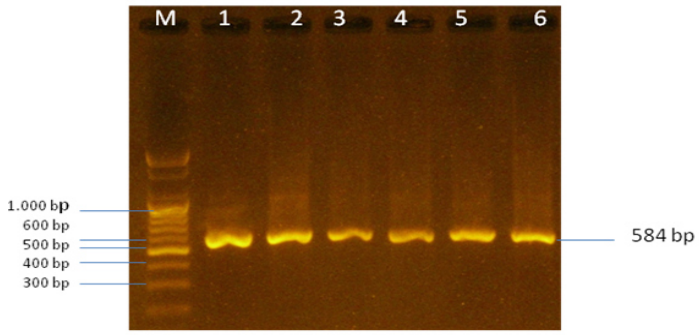

Figure 2. Amplified PCR products from *Lactobacillus spp* with primer set *tuf B* gene. Lane (1-6) PCR products amplified from 6 *Lactobacillus spp*. Lane M: 100 bp markers.

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