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Evaluation of methods for the extraction and purification of DNA of cultured *Lactobacillus* colony isolated from dairy products

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ABSTRACT

Lactic acid bacteria are very significant to human health due to the production of some antimicrobial substances and ability to inhibit pathogenic bacteria. Furthermore, the bacteria are also used as starter culture in the production of various foods. The aim of this study was to evaluate five methods for the extraction and purification of DNA from 6 isolates of *Lactobacillus* colony, isolated from dairy product. The methods were: Modified Pospiech and Neumann, Martin–Platero, Phenol–chloroform, Wizard genomic DNA purification kit and Wizard genomic DNA purification kit with modifications. The results obtained in the study confirmed that extracted genomic DNA using Wizard genomic DNA purification kit with modifications was superior to other methods because it produced a higher DNA yield with the highest purity. Traditional polymerase chain reaction (PCR) was optimized to detect *Lactobacillus* spp. The molecular based culture-dependent methods depend on several factors including best DNA extraction and specific primer set to achieve PCR assay.

INTRODUCTION

In addition to the considerable technological and commercial importance of their role in the manufacturing and preservation of many fermented food products, lactic acid bacteria (LAB) including *Lactobacillus* spp also play an important role in protection of the intestinal and urogenital tracts (Wood, 1992; Gharaei-Fathbad and Eslamifar, 2011). Lactic acid Bacteria occur naturally as indigenous microflora in fermented milk products such as yoghurt (Vernoux et al., 2003).

The genus *Lactobacillus* consists of a genetically and physiologically diverse groups of Gram-positive, rod shaped, catalase negative and non-spore forming bacteria (MacFaddin, 2000). Certain *Lactobacillus* strains are considered important owing to their role in various foods and feed fermentations, production of many important metabolites and owing to their role in the prevention of food spoilage. Furthermore, they play a role in combating intoxication and infection by acting as antagonists against other pathogens through the production of antimicrobials and bacteriocine (Holzapfel et al., 2001; Hirano et al., 2003).

The identification of *Lactobacillus* isolates by phenotypic methods using physiological and biochemical criteria is difficult because it is time-consuming and inaccurate. Classical methods of *Lactobacilli* classification using physiological and biochemical criteria do not always correspond to the actual genetic relativity of the species and are not discriminative enough to distinguish strains of the same species. Methods of identification of Lactobacilli previously based on culture-dependent methods have recently been supplemented with molecular techniques. The development of a molecular culture-independent detection methods such as polymerase chain reaction (PCR) is a simple technique, which quickly amplifies specific sequences of target DNA from indicator organisms. It appears to be invaluable in the case of probiotics, particularly *Lactobacillus* spp (Roy et al., 2000; Ventura and Zink,

2002). This study therefore evaluated several methods in other to identify the most efficient extraction protocol for the production of bacterial DNA used in PCR technology.

MATERIALS AND METHODS

Collection of samples

Yoghurt samples were collected from local markets in the city of Babylon in Iraq; based on their popularities among the consumers. The samples were stored aseptically at 4°C to prevent contamination immediately after collection.

Samples preparation

Ten grams (10 g) of each yoghurt sample were separately diluted in 90 ml of sterile normal saline. Samples were enriched in MRS broth for 24 h at 37°C under anaerobic condition in the presence of 5% CO_2. Samples were then taken and streaked on to the MRS agar plates and were incubated in an anaerobic jar at 37°C for 72 h. After several sub-cultures, the bacterial culture was streaked on MRS agar media. Finally, a single colony of *Lactobacillus* was isolated by observing their colony morphology and specific biochemical tests; and the culture were maintained in MRS broth.

Optimization of DNA extraction

Pospiech and Neumann modified method

Bacteria were cultivated in MRS broth. After 2 days of incubation at 37°C, 1.5 ml of the broth was collected and centrifuged for 5 min at 13,000 rpm. The pellet to be used in the next 4 methods, was re-suspended in 400 µl SET buffer (75 mM NaCl, 25 mM EDTA, 20 mM Tris, pH 7.5). Then 50 µl lysozyme (10 mg/ml) and 20 µl proteinase K (15 mg/ml) were added and incubated at 37°C for 1 h. 50 µl of 10% SDS were added and centrifuged at 13,000 rpm for 10 min). The aqueous layer was re-extracted with isopropanol (1:1) (v/v) and then incubated at -20°C overnight. DNA was centrifuged and washed with 70% ethanol and the supernatant was removed. The purified DNA was re-suspended with TE buffer (Pospiech and Newmann, 1995).

Martin–Platero method

Pellet was suspended in 100 µl of TE buffer (10% w/v sucrose, 25 mM Tris–HCl pH 8.0, mM EDTA, 10 mg/ml, freshly made lysozyme and 40 µg/ml RNase A) and incubated for 30 min at 37°C. The protoplast cells were immediately lysed by adding 600 µl of lysis buffer (100 mM Tris–HCl pH 8.0, 100 mM EDTA, 10 mM NaCl and 1% w/v SDS) and incubated for 15 min at room temperature. The lysates were treated with 10 µl of proteinase K (10 mg/ml) and incubated for 15 min at 37°C. After incubation at 80°C for 5 min and cooling down to room temperature for 5–10 min, 200 µl of sodium acetate (3 M, pH 5.2) were added, chilled on ice for 15 min and centrifuged at 6,000 rpm for 10 min. The supernatant was decanted and 600 µl of isopropanol were added to it to precipitate the DNA. Finally, genomic DNA was dissolved in distilled water and maintained at -20°C for further studies.

Phenol–chloroform method

Pellet was dissolved in 467 µl TE buffer, followed by addition of 30 µl of 10% SDS and 3 µl (20 mg/ml proteinase K). After incubation for 1 h at 37°C, 50 µl phenol: chloroform: isoamyl alcohol (1:25:24) was added and mixed by gentle inversion. Aqueous phase decanted into a new tube and; 0.1 ml of 3 M sodium acetate and 0.6 ml of isopropanol was added to it. The mixture was swirled slowly until DNA precipitated which was spooled with Pasteur pipette. DNA was dried and washed by dipping end of pipette into 1 ml of 70% ethanol for 30 s before dissolving in 150 µl TE buffer (Parayre et al., 2007).

Wizard genomic DNA purification kit

The wizard genomic DNA purification kit (Promega/USA) was used according to the manufacturer's instruction.

Wizard genomic DNA purification kit with modifications

The wizard genomic DNA purification kit (Promega/USA) with several modifications was used as follows: For all DNA preparations, cells were grown in the corresponding medium containing 1.5% glycine to facilitate cell lysis. An overnight culture of 5 ml was pelleted by centrifugation at 14,000 rpm for 3 min at 25°C and re-suspended in 900 µl of 50 mM EDTA. A volume of 120 µl of solution of lysozyme [(40 mg/ml) Sigma Chemical Co.USA] was added to the cell suspension and incubated in a water bath for 1 h at 37°C with occasional mixing. The suspension was centrifuged (14,000 rpm at 25°C for 3 min) and the pellet was gently re-suspended in 900 µl nuclei lysis solution (promega). To induce lysis, the cells were incubated at 80°C for 5 min. After cooling at room temperature, 4 µl of RNase A (50 mg/ml, Sigma/USA) were added and the tubes were inverted 10 times before

the mixture was incubated again for 1 h at 37°C with occasional inversion. For protein precipitation, 300 µl precipitation solution (promega) was added to the mixture and vortexed vigorously for 30 s, and then, the mixture was incubated in ice for 7 min and centrifuged at 14,000 rpm for 15 min). The supernatant was carefully transferred to clean 1.5 ml Eppendorf tube containing 600 µl isopropanol at room temperature and the mixture was gently mixed by inverting the tube followed by centrifugation at 14,000 rpm for 10 min. The pellet was washed with 70% ethanol to remove residual contaminants. Finally, the ethanol was discarded and the pellet containing the genomic DNA was re-suspended in 60 µl DNA rehydration solution (Promega).

Qualitative and quantitative assessment of DNA

Purity and concentration of DNA solutions were measured using Computerized Nano Drop-spectrophotometer. Results of DNA purity and concentration (µg/µl) were recorded and plotted automatically. The Nano Drop-spectrophotometer measures DNA purity and concentration according to the following equations:

$$\text{DNA purity} = \frac{\text{Absorbance at 260 nm}}{\text{Absorbance at 280 nm}}$$

DNA yield (µg) = DNA concentration (µg/µl) × total sample volume (ml)

The quality of the isolated DNA was also evaluated by 1% Agarose gel electrophoresis. A 100 bp plus DNA ladder (SolGent, Korea) was used as a molecular weight standard to compare the intensity and approximate size of the isolated DNA.

Polymerase chain reaction and gel electrophoresis

The primers were synthesized at AccuOligo/Bioneer/ Korea. These were provided in a lyophilized form, which were re-dissolved with TE buffer (pH 8) to a final concentration of 100 pmol/µl and stored at -20°C. The sequence of the primer set Lacto -16S forward - was 5'…..GGA ATC TTC CAC AAT GGA CG…..3' and the primer set Lacto -16S reverse - was 5'…..CGC TTT ACG CCC AAT AAA TCC GG …..3' (Abdulamir et al., 2010); amplifications were carried out in 30 µl volumes containing (10 pmol/µl) of each primer, 2x Taq PCR Pre–Mix (SolGent™ 2x Taq PCR Pre – Mix, SolGent Co., Ltd.), and 200 ng genomic DNA. Amplification was achieved in 40 cycles according to the manufacturer's instruction (SolGent™ 2x Taq PCR Pre – Mix , SolGent Co., Ltd.); using a GTC thermal cycler (Cleaver Scientific, UK). Prior to the first cycle, DNA was denatured at 95°C for 3 min subsequently, and each cycle consisted of denaturation at 95°C for 30 s, followed by annealing at 61°C for 40 s. Elongation was carried out at 72°C and the extensiontime at 1 min subsequently. A final elongation was performed at 72°C for 5 min, and the holding temperature was for 10 s. PCR product was separated by electrophoresis on 1.5% (w/v) agarose gel (sigma/USA) containing ethidium bromide (0.5 µg/ml). 12 µl of each PCR product and 4 µl of 6x loading dye (Sigma/USA) were loaded into Agarose gel and run in 1 × TBE buffer (Sigma /USA). A 100 bp plus DNA ladder was used as a molecular weight standard and a positive control was run together with the PCR products. The PCR products were separated by electrophoresis at a constant voltage of 70 V for 40 min. Then, DNA bands were visualized by ultraviolet (UV) illumination at 300 nm wave-lengths on UV trans illuminator system (Herolab/Germany), and then photographic pictures were taken for each gel (Sambrook and Russell, 2001).

RESULTS AND DISCUSSION

Qualitative and quantitative assessment of DNA

The results obtained revealed that DNA extraction with modified wizard protocol produced the highest DNA purity (between 1.68 and 1.79) and highest DNA yield (between 95.3 and 135.6 ug) when compared with the other four protocols, (Figure 1A). The DNA yield varied significantly depending on the DNA extraction method used. The DNA extracted using the five protocols were observed for degradation by 1% Agarose gel electrophoresis. Also, all DNA extracted by modified wizard protocol produced sharp bands, whereas the bands produced by the other four protocols were not sharp and appeared with smear (Figure 1). High concentrations of lysozyme also worked well as it gave the highest recovery of genomic DNA from all isolates tested. Gram-positive bacteria are much more resistant to cellular lyses due to the high concentration of peptidoglycan within the cell wall. However, the success of DNA purification is dependent on the initial quality of the sample and its preparation (Sambrook and Russell, 2001).

Polymerase chain reaction (PCR) quantification of *Lactobacillus* spp

The purity of DNA extracted from Lactobacilli and other bacterial isolates is a key issue in the sensitivity and usefulness of biological analyses such as PCR. The results of this study indicated that the commercial wizard kit/Promega with several modification was the most successful extraction method for amplification of the target *Lactobacillus* spp 16S r DNA. Figure 2 shows that PCR of six amplicons for *Lactobacillus* spp. with

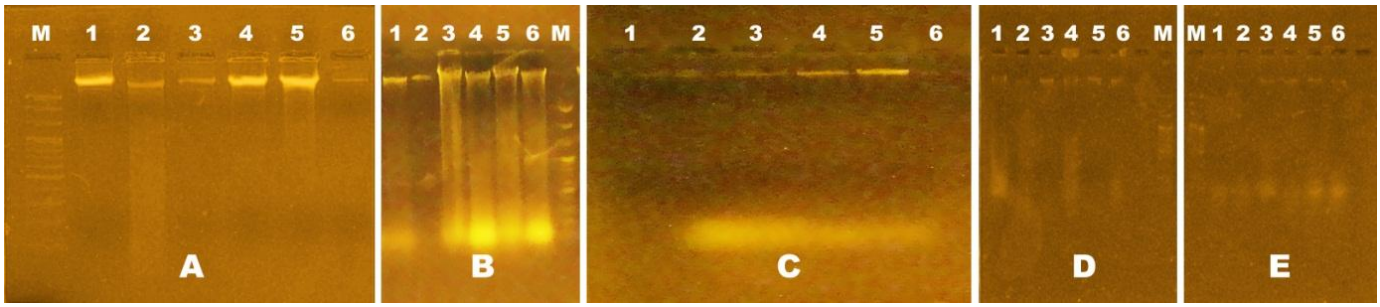

Figure 1. Agarose gel electrophoresis of extracted DNA from 6 isolates of *Lactobacillus* colony. A, wizard genomic DNA purification kit with modifications; B, Martin–Platero method; C, wizard genomic DNA purification kit mehod; D, Pospiech and Neumann modified method; E, phenol–chloroform method. Lane M: 100 bp DNA ladder marker.

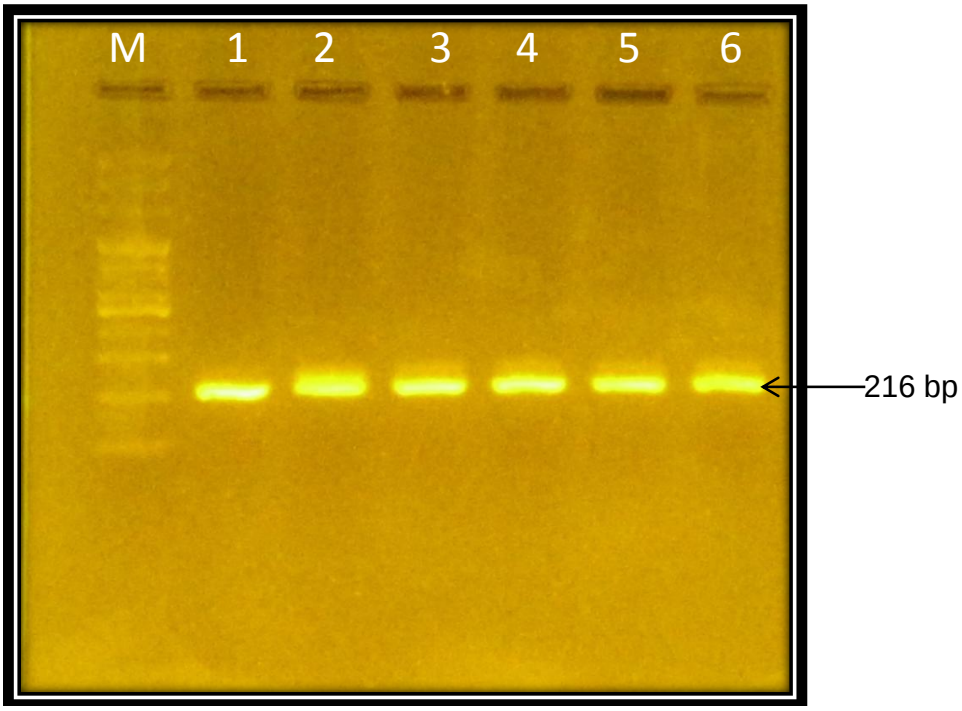

Figure 2. Amplified PCR products from *Lactobacillus* spp with primer set Lacto16S-F + Lacto16S-R. Lane (1-6), PCR products amplified from 6 *Lactobacillus* spp; Lane M, 100 bp markers.

molecular size 216 bp is associated with the calculated size of target gene of *Lactobacillus* spp and shows the single band of PCR. This confirmed that no products of non–specific amplification, like primer dimmers were observed. Successful amplification of target gene of *Lactobacillus* spp was a useful tool in diagnostic of *Lactobacillus* spp, which indicated the primers were specific, the DNA extraction method and the PCR protocol were optimized.

Numerous studies have been done to evaluate microbial DNA extraction methods using various kinds of samples (Solauen et al., 2010; Ariefdjohan et al., 2010; Vanysacker et al., 2010). Efficient and reproducible methods for identification of *Lactobacillus* spp are needed, both for rapid identification and to facilitate the study of the complex microflora in highly mixed environment (Dimitonovo et al., 2008).

Fitzsimons et al. (1999) reported that sometimes it is difficult to identify a microorganism only by using the changes in pH as an indicator of growth in the presence of different sugars because of the various cut-off points used to determine a positive or a negative reaction.

Table 1. Quality of extracted DNA using five different methods.

Isolate	Pospiech and Neumann	Martin–Platero	Phenol–chloroform	Wizard genomic DNA	Modified of wizard
Lac 1	1.05	1.51	1.01	1.11	1.77
Lac 2	1.02	1.33	1.03	1.09	1.71
Lac 3	1.09	1.51	1.22	1.45	1.68
Lac 4	1.13	1.53	1.21	1.50	1.76
Lac 5	1.21	1.52	124	1.52	1.79
Lac 6	1.25	1.53	1.24	1.29	1.68

The identification of microbial species through the use of phenotypic methods can sometimes be uncertain, complicated and time consuming. The use of molecular methods has revolutionized identification by improving the quality and effectiveness of this identification. However, PCR assays need more optimization and standardization before any testing. In this study, the author tried to evaluate several methods to identify the most efficient way associate with the highest purity of DNA extraction since molecular methods like PCR depends on the purity of the samples tested. The isolation and purification of DNA are key steps for most protocols in molecular biology studies and all recombinant DNA techniques. Several DNA extraction methods are widely used to isolate DNA from bacteria including phenol extraction, boiling, salting out and others but they often involve multiple, time consuming steps including the handling of toxic chemicals (Sambrook and Russell, 2001).

The quality of the extracted DNA was assessed by spectroscopy using 260/280 absorbance ratios, where OD 260/280 value from 1.7 to 1.9 was considered as high purity. In this study, only the extracted DNA by wizard kit with several modification produced high purity products (between 1.68 and 1.79), whereas for the other four methods, their purity values were less than 1.53 (Table 1). This may be due to the use of high concentration of lysozyme (40 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. It is conceivable that the use of higher concentrations of Lysozyme resulted in high DNA yield (Rantakokko-Jalava and Jalava, 2002) and gave the highest recovery of genomic DNA from all isolates tested.

The cell walls of Gram-positive bacteria can be efficiently broken by use of peptidoglycan-degrading enzyme, lysozyme (Rantakokko-Jalava and Jalava, 2002). Enzymes that digest peptidoglycan of bacteria are collectively called murein hydrolases. Lysozyme is the best known among muramidases as it binds on bacterial surface and attacks peptidoglycans (Touch et al., 2003). Lysozyme is especially effective in disrupting bacterial cells when used in combination with EDTA (Moore et al., 2004).

Ligozzi and Fontana (2003) have reported that the recovered nucleic acids have an A 260/280 ratio of 1.8–2.0 and are suitable for direct restriction enzyme digestion, and the DNA is also suitable for Southern blot molecular cloning, PCR, extra-long polymerase chain reaction (XL PCR) and other molecular biology and biotechnology applications.

Variation in the efficiency of lysis yield and purity of the DNA can fundamentally affect the success of analytical technique, such as PCR and biases can be introduced in quantitative analysis. Therefore a suitable and selective method with a high extraction efficiency need to be developed that can be used on a routine basis (Shahriar et al., 2011). PCR techniques require the efficient extraction of DNA from any samples, which is problematic due to the presence of PCR inhibitors. However studies assessing the most efficient method for the extraction of bacterial DNA have relied upon conventional PCR methods (Sambrook and Russell, 2001).

In the current study, a PCR assay targeting the 16S r DNA for detecting *Lactobacillus* spp was used to compare the efficiency of five extraction methods for isolation of *Lactobacillus* bacterial DNA from a colony obtained from dairy product. Results obtained show that the wizard kit with several modified is the best among other methods. The requirement for well optimized PCR is the specificity of the used primers. Therefore, specific primer set of *Lactobacillus* spp (Lacto 16s-F and Lacto 16s-R) was subjected to thorough testing. It was found that Lacto 16s-F and Lacto 16s-R is highly specific for *Lactobacillus* spp. The V1–V3 region of 16s rRNA was found to be sufficient variable to provide species–specific patterns in PCR (Doi et al., 2013).

The 16S rRNA sequence is used for phylogenetic studies as it is highly conserved between different species of bacteria. DNA primers (for use in the PCR) can be designed to be highly-conserved sequences on the 16S DNA, which often flank hyper-variable regions that can provide species-specific signature sequences useful for identification. Therefore, 16S DNA gene sequencing has become prevalent in medical microbiology as a rapid and

accurate alternative to phenotypic methods of bacterial identification (Bushell and Burns, 2012).

We have compared the relative efficacy of extraction of bacterial DNA from *Lactobacillus* isolates from a colony obtained from dairy products using five extraction protocols. The PCR amplification of 16S r DNA gene was performed in order to confirm that the quality of the DNA extracted using each method was suitable for downstream biological processes such as PCR. Fragments of approximately 216 bp corresponding to almost full length of 16S r DNA were obtained in different *Lactobacilli* species, as expected Figure 2. 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).

REFERENCES

Abdulamir A. S., Yoke T. S., Nordin N. & Abu Bakar F. (2010). Detection and quantification of probiotic bacteria using optimized DNA extraction, traditional and real-time PCR methods in complex microbial communities. Afr. J. Biotechnol. 9(10):1481-1492.

Ariefdjohan M. W., Savaiano D. V. & Nakatsu C. H. (2010). Comparison of DNA extraction kits for PCR-DGGE analysis of human intestinal microbial communities from fecal specimens. Nutr. J. 9: 23.

Bushell C. & Burns M. (2012). Feasibility study into the use of DNA sequencing for the identification of probiotic bacteria. J. Assoc. Public Analysts 40:28–38.

Dimitonovo S. P., Bakalov B. V., Aleksandrova-Geogieva R. N. & Danova S. T. (2008). Phenotypic and molecular identification of *Lactobacilli* isolated from vaginal secretions. J. Microbiol. Immunol. Infect. 41:469–477.

Doi K., Nishizaki Y., Kimura H., Kitahara M., Fujino Y., Ohmomo T. O. & Ogata S. (2013). Identification of thermo tolerant lactic acid bacteria isolated from silage prepared in the hot and humid climate of southwestern Japan. Springer plus. 2:485.

Fitzsimons N., Cogan T., Condon S. & Beresford T. (1999). Phenotypic and genotypic characterization of non-starter lactic acid bacteria in mature chedder cheese. Appl. Environ. Microbiol. 65:3418–3426.

Gharaei-Fathabad E. & Eslamifar M. (2011). Isolation and applications of one strain of lactobacillus paraplantarum from tea leaves (*Camellia sinensis*). Am. J. Food Technol. 6:429–434.

Hirano J., Yoshida T., Sugiyama T., Koide N., Mori I. & Yokochi T. (2003). The effect of *Lactobacillus rhamnosus* on enterohemorrhagic *E. coli* infection of human intestinal cells *in vitro*. Microbiol. Immunol. 47:405–409.

Holzapfel W. H., Habere P., Geisen R., Bjorkroth J. & Ulrich S. (2001). Taxonomy and important features of probiotic microorganisms in food and nutrition. Am. J. Clin. Nutr. 73:365-373.

Ligozzi M. & Fontana R. (2003). Isolation of total DNA from bacteria and yeast. Afr. J. Biotechnol. 2(8):251–253.

MacFaddin J. F. (2000). Biochemical tests for identification of medical bacteria. 3rd ed. Lippincott Williams and Wilkins, USA.

Martin-Platero A. M., Valdivia E., Maqueda M. & Martinez-Bueno M. (2007). Fast, convenient, and economical method for isolating genomic DNA from lactic acid bacteria using a modification of the protein “salting-out” procedure, Anal. Biochem. 366:102-104.

Moore E., Arnscheidt A., Kruger A., Strompl C. & Mau M. (2004). Simplified protocols for the preparation of genomic DNA from bacterial cultures. Molecular Microbiol Ecology Manual, 2nd edition. 1. 01:3–18.

Parayre S., Falentin H., Madec M. N., Sivieri K., Le Dizes A. S., Sohier D. & Lortal S. (2007). DNA extraction method and optimization of PCR-temporal temperature gel electrophoresis to identify the predominant high and low GC-content bacteria from dairy products. J. Microbiol. Methods. 69:431–441.

Pospiech A. & Neumann B. (1995). Aversatile quick-prep of genomic DNA from Gram-positive bacteria. Trends Genet. 11:217.

Rantakokko-Jalava K. & Jalava J. (2002). Optimal DNA isolation method for detection of bacteria in clinical specimens by broad-range PCR. J. Clin. Microbiol. 40(11):4211–4217.

Roy D., Ward P., Vincent D. & Mondon F. (2000). Molecular identification of potentially probiotic Lactobacilli. Curr. Microbiol. 40:40–46.

Sambrook J. & Russell D. (2001). Molecular cloning: A laboratory Manual, 3rd ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

Shahriar Man., Haque Md. R., Kabir S., Dewan I. & Bhuyian M. A. (2011). Effect of proteinase-k on genomic DNA extraction from Gram-positive strains. S. J. Pharm. Sci. 4(1):53–57.

Solauen A., Nikkila J., Jalanka-Tuovinen J. & Immonen O. (2010). Comparative analysis of fecal DNA extraction methods with phylogenetic microarray: Effective recovery of bacterial and archaeal DNA using mechanical cell lysis. J. Microbiol Methods. 81:127-134.

Touch V., Hayakawa S., Fukada K., Aratani Y. & Sun Y. (2003). Perparation of antimicrobial reduced lysozyme compatible in food-applications. J. Agric. Food Chem. 51:5154-5161.

Vanysacker L., Declerck S. A., Hellemans B. & De Meester L. (2010). Bacterial community analysis of activated sludge: An evaluation of four commonly used DNA extraction methods. Appl. Microbiol. Biotechnol. 88:299-309.

Ventura M. & Zink R. (2002). Specific identification and molecular typing analysis of *Lactobacillus johnsonii* by using PCR-based methods and pulsed-field gel electrophoresis. FEMS Microbiol. Lett. 217:141-154.

Vernoux J. P., Coeuret V. D. Ubernet S., Bernardeau M. & Gueguen M. (2003). Isolation, characterization and identification of *Lactobacilli* focusing mainly on cheeses and other dairy products. INRA, EDP, Sci. 83:269-306.

Wood B. J. B. (1992). The lactic acid bacteria in health and disease. The lactic acid bacteria. Appl. Sci. (1).