Small-Scale Total DNA Extraction from Bacteria and Yeasts for PCR Applications

José Luis Ruiz-Barba*, Antonio Maldonado, and Rufino Jiménez-Díaz

Departamento de Biotecnología de Alimentos, Instituto de la Grasa, Consejo Superior de Investigaciones Científicas (CSIC), 41012 Seville, Spain.

*Corresponding author: Mailing address: Departamento de Biotecnología de Alimentos, Instituto de la Grasa (CSIC), Avda. Padre García Tejero, 4; Aptdo. 1078; 41012 Seville, Spain. Phone: +34 954 69 08 50. Fax: +34 954 69 12 62. E-mail: jlruiz@cica.es.

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The polymerase chain reaction (PCR) has become an essential research and diagnostic tool, being a powerful technique with a vast and increasing range of applications [1,2]. At the basis of PCR is DNA extraction from biological samples, which should render the substrate for the amplification reaction, provided a minimun quality and quantity level is achieved [3,4]. Quite a few rapid methods have been proposed for this purpose in the last years, which essentially depend on the sample and microorganisms to be analysed [5,6]. The simplest case is that of the colony which is directly introduced in the reaction mix: cells are disrupted as temperature raises to 95 ºC for 4 to 5 min in the first denaturation step. This has shown to be a valid method for isolated colonies of Gram-negative bacteria, yeasts and some *Lactobacillus* species [7-9]. However, this method is not valid in many instances, and cell lysis involving lytic enzymes and detergents have been used for recalcitrant microorganisms [10,11]. This is especially the case with Gram-positive bacteria, for which extraction from colonies involves digestion with mutanolysin and cell disruption with Triton X-10 plus boiling, taking about 45 min the whole process [11]. Therefore, DNA extraction and preparation is a time-consuming step which dramatically reduces the speed of the PCR procedure and delays its outcome.

In this study we describe a very simple and rapid method for extraction of both plasmid and chromosomal DNA from colonies or pellets of bacteria and yeasts, which is suitable for subsequent PCR. Our method involves only chloroform as the cell disrupting and extracting agent, contributing also to DNA preservation from inherent and contaminating nucleases.

Bacterial species used as a demonstration included *Lactobacillus plantarum* CECT 748, ATCC 8014, LB6 and LPCO10 [12], *Lactococcus lactis* MG1363, and *Enterococcus faecium* 6T1a, which were cultured in MRS medium (Oxoid, Basingstoke, UK) aerobically at 30 ºC. Yeasts were cultured in YM medium (Difco, Detroit, Mi.) aerobically at 30 ºC.

For DNA extraction, single colonies growing on solid media were removed with a sterile plastic tip and resuspended in 100 μl of sterile deionized water in a microcentrifuge tube. One hundred μl of chloroform-isoamyl alcohol (24:1) were added to the suspensions and, after briefly vortexing for 5 sec, the mixture was centrifuged at 16.000 x*g* for 5 min at 4 ºC. Five to 10 μl of the upper, aqueous phase were used as a source of DNA template for the different PCR applications. The rest of the mixture was stored at 4 ºC until use. When the bacteria or yeasts to be tested were grown in liquid media, pellets obtained after centrifugation of appropriated volumes to render masses equivalent to single colonies were washed with sterile deionized water and treated as above.

Amplification of DNA fragments up to 3 kb was performed in 100-μl reaction mixtures containing 2.5 mM $MgCl_2$, 1× reaction buffer, 100 μM each of the deoxynucleoside triphosphates, 100 pmol of each primer, 5 U of *Taq* DNA polymerase (Promega Co., Madison, Wis.), and 5-10 μl of the source of DNA template as described above. Conditions for amplifications were as described before [13]. For amplification of DNA fragments larger than 3 kb, the Expand Long Template PCR System (Roche Applied Science, Barcelona, Spain) was used according to the manufacturer's instructions. Primers ITS1 and ITS4 for the amplification of the ITS region from yeasts have been previously described [14]. Primers LICIJ1 (5'-GACGACGACAAGATGGGAGCAATCGCAAAA-3'), LICJ2 (5'-GGAACAAGACCCGTTAATGTCTTTTTAGCC-3'), ent7 (5'-ACATTACGTACATTCCTACTAAG-3') and pEF4.1 (5'-CCGGTTTCAGTCGCAGTACGC-3') were designed from the sequence of the 21-kb plasmid pEF1 from *E. faecium* LP6T1a [15]. Primers PlnE-rev (5'-ATGCTACAGTTTGAGAAGTTACA-3'), PlnF-for (5'-CTATCCGTGGATGAATCCTC-3'), and PlnM-for (5'-TAAACAGGTAAAGCAGGTTGG-3') were designed from the sequence of the plantaricins cluster in *L. plantarum* C11 [16]. For RAPD, the method of Rodas et al. [17] was followed using the primer OPL5 (Operon

Biotechnologies, Inc., Alameda, Ca.). All of the PCR and RAPD amplifications were performed in a GeneAmp® PCR System 2400 thermal cycler (Perkin-Elmer Corporation, Norwalk, Conn.). After the reaction, 3 to 10 µl of the products were run in 0.7-1.2% agarose gels, stained with ethidium bromide and visualized under UV light.

When chloroform-extracted samples from bacterial or yeast single colonies were used as the source of DNA template, amplification of DNA fragments from 0.3 up to 12.0 kb was obtained in all cases (Figs. 1 and 2). PCR amplifications of short and long fragments could be observed either from genes encoded by plasmid or chromosomal DNA. For instance, from *E. faecium* 6T1a single colonies, amplification of both short (150 and 300 bp, corresponding to *entI* and *entI-enJ* genes of the enterocin I operon, respectively; Fig. 1, lane 3) and long DNA fragments (12 kb; Fig. 2, lane 4) located in the plasmid pEF1 was achieved. Also, long fragments (up to 8 kb) belonging to the plantaricin *locus* in *L. plantarum* LB6 and CECT748 could be amplified from chromosomal DNA (Fig. 2, lanes 2 and 3). This provides an evidence that the method described here is valid to amplify genes which are either as single copy (chromosomal) or multicopy (plasmid). Furthermore, the fact that fragments as long as 12.0 kb can be indeed amplified indicates that the DNA obtained by this method is reasonably intact. Interestingly, identical PCR amplifications could be observed when the same DNA preparations were used after storage at 4 ºC for periods up to 12 months (data not shown), indicating that DNA preparations were highly stable and suitable for successive PCR amplifications for at least that period of time.

On the other hand, DNA extraction and subsequent PCR amplification of the region spanning the 5.8 rRNA gene and the two ITSs from two different yeast species was achieved (Fig. 1, lanes 4 and 5), indicating that this method is suitable for microorganisms other than bacteria. Actually, similar results were obtained when the method was applied to yeasts from genera such as *Saccharomyces, Issatchenkia, Geotrichum, Zygosaccharomyces, Hanseniaspora* and *Dekkera* (data not shown). Moreover, PCR products obtained were

suitable for direct further digestion with restriction enzimes in order to generate a restriction pattern for identification of the yeasts at the species level (F.N. Arroyo, personal communication).

Finally, the DNA obtained by this method allowed also efficient RAPD amplifications, as it is shown in Figure 1 (lanes 6 and 7) for two different *L. plantarum* strains. Band patterns were virtually identical to those obtained using standard total DNA preparations from the same strains (data not shown). This shows that this technique is also suitable for unspecifically primed PCR.

The amount and quality of the DNA extracted by the method described in this paper warrants accurate and reproducible DNA amplifications by PCR for application in rapid screening and differentiation of microorganisms. Further subcloning, sequencing, and restriction analysis of the amplified fragments could also be eased. In our laboratory, we have been using this method for a few years, mainly in the screening of *E. coli, L. plantarum* and *L. lactis* colonies after transformation with recombinant plasmids, as well as for identification and typing of lactic acid bacteria with specific primers. Also, we have used the method to extract total DNA from pellets obtained after centrifugation of samples from olive fermentation brines, where we have detected specific *L. plantarum* and *L. pentosus* strains by PCR. In addition, this method offers the posibility of performing repetitive PCRs from the same DNA extraction, which is safely kept for a considerable extent of time. This is an advantage over those methods that involve introduction of the colony to be tested directly into the reaction mix, because exactly the same DNA template is used over the time for different purposes. Finally, our results suggest that other molecular applications such as RT-PCR can benefit.

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Legends to the figures

Figure 1.- Agarose gel electrophoresis showing short-fragment PCR amplifications and RAPD products obtained from different bacteria and yeasts. Lanes 1 and 8, 1-kb PLUS DNA Ladder (Life Technology Inc., Gaithersburg, Md.). Lane 2, PCR amplification product from *L. plantarum* CECT 748 with primers PlnE-rev and PlnF-for; lane 3, PCR amplification products from *E. faecium* 6T1a with primers LICIJ1 and LICJ2; lanes 4 and 5, PCR amplification products from *Rhodotorula glutini* and *Candida boidinii*, respectively, using the primers ITS1 and ITS4; lanes 6 and 7, RAPDs from *L. plantarum* ATCC8014 and LPCO10, respectively, using the primer OPL5.

Figure 2.- Agarose gel electrophoresis showing long-fragment PCR amplification products obtained from either chromosomic or plasmidic bacterial DNA. Lanes 1 and 5, 1-kb PLUS DNA Ladder; lane 2, PCR amplification product from *L. plantarum* LB6 with primers PlnM-for and PlnE-rev; lane 3, PCR amplification products from *L. plantarum* 748 with primers PlnM-for and PlnE-rev; lane 4, PCR amplification products from *E. faecium* 6T1a with primers ent7 and pEF4.1.

Figure 1

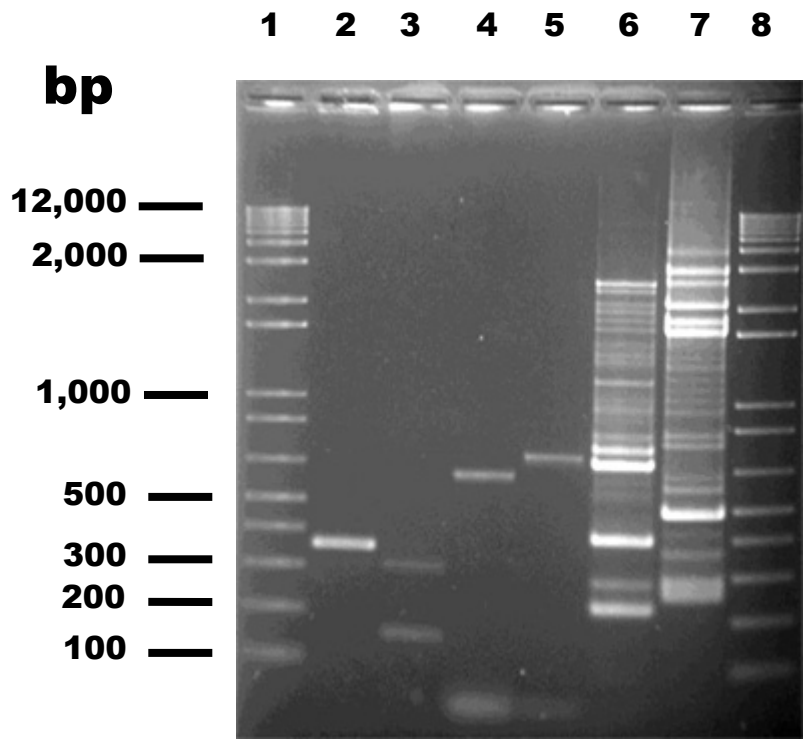

Figure 2