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Purification of PCR Products Using Bandstab PCR Method: Application to Short 16s-23s Intergenic Spacer Region of *Lactobacillus* Spp

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In view to show a simple DNA purification step from agarose gel, we applied the DNA bandstab technique to the 16S-23S Intergenic Spacer Region (ISR) (a polymorphic region resulting in multiple bands after gel-electrophoresis) of selected *Lactobacillus* spp. The short ISR was successfully extracted by bandstab after numerous unsuccessful attempts of DNA cutting-elution techniques. The purification of band was further confirmed by the distinctive peaks observed in the DNA chromatograms generated after the sequencing procedure.

Key words: Bandstab-PCR; Lactobacillus; 16S-23S intergenic spacer region; Sequencing.

Polymerase Chain Reaction of DNA can lead to multiple products especially when targeted gene is present in multiple copies with slight differences, or when the primers specificity is weak. This situation is really troublesome for most of researchers whose PCR products were destined to sequencing which requires highly pure product. Solutions do exist to purify PCR products such as gel cutting, DNA elution or cloning into vectors, but these techniques are time and money consuming. Moreover, apart from the fact that they are cost expensive, they are also known to have some limitations such as the decrease of DNA concentration (Vogelstein and Gillespie, 1979) rendering the success of sequencing uncertain.

We have taken an example to illustrate a tricky situation faced by researchers who want to identify lactobacilli at genomic level. The use of lactobacilli as probiotic organisms has recently gained lot of attention as well as their potential use as starter for fermentation of dairy products and/or for the development of functional and nutraceutical foods. These organisms can be identified based on their physical and biochemical properties. However, these techniques are not powered enough to discriminate closely related lactobacilli species. Several studies have reported that they can accurately be identified by using molecular techniques, particularly by sequencing the 16S-23S or 23S-5S intergenic spacer region (ISR) (Nakagawa *et al.,* 1994; Tilsala-Timisjarvi and Alatossava, 1997; Berthier and Ehrlich, 1998; Nour, 1998; Tannock, 1999; Moghadam *et al.,* 2010) of genes located on the ribosomal RNA (rRNA) operon. Unfortunately, in Lactic Acid Bacteria (LAB), the number of operons varies from 2 (Klappenbach *et al.,* 2001) to 6 (Tulloch *et al.,* 1997; Klaenhammer *et al.,* 2002), an important factor which makes tricky the use of polymerase chain

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reaction (PCR) amplification when ISR is targeted for strain identification purpose. The reason behind this complication is the presence of multiple copies of operon that will lead inevitably to the occurrence of multiple bands after performing PCR amplification. Consequently, further purification steps are needful before sequencing. Herewith, we describe a simple trick used in diverse others aspects of biotechnology (Bjourson and Cooper, 1992; Drew *et al.*, 1998, Yesilkaya *et al.,* 2003) called Bandstab-PCR in order to optimize the purification and sequencing of the complete lowest band of the 16S-23S ISR PCR products of selected lactobacilli.

MATERIALS AND METHODS

Seven fully phenotyped *Lactobacillus* strains (Codified LAB1 to LAB7) stored in glycerol stock in Laboratory of functional food and nutraceutical (National Dairy Research Institute, India) were selected and re-activated in MRS broth by repeated growth under optimal condition (37°C/ 24-48h). Genomic DNA was isolated as in previous study (Pospiech and Neumann, 1995). The primer set IDL04F (Kwon *et al.,* 2004) as forward primer and 23-10C (Gürtler and Stanisich, 1996) as reverse primer, were used for an expected product sizing at least 800 bp predicted *in silico* using incorporated programs of DNAStar software (version 7, Madison, USA). PCR amplification was done following the conditions: initial denaturation: 94°C for 2 min; 35 cycles (denaturation at 94°C for 30 sec; annealing at 62°C for 30 sec; elongation at 72°C for 30 sec); final elongation: 72°C for 10 min. Electrophoresis was performed in an 0.8% (wt/vol) agarose gel containing 0.5 $\mu g{\bullet}ml^{-1}$ of Ethidium Bromide in 1× Tris-Borate-EDTA buffer (pH 8.0) and the gel was visualized by Gel Documentation System ALPHAIMAGER-HP (Model AITM-26).

We further proceeded to the extraction of the band of interest (lowest band) from these multiple bands by stabbing it with the help of a micropipette tip (10µL). The tip was then briefly dipped onto a PCR tube containing 25 µl of reaction mix and subjected to a PCR amplification using same primers, protocol and program conditions (see above). The bandstab amplified products were visualized on an agarose gel as prepared before. Afterward, these new PCR products were purified using Exonuclease-I and Shrimp Alkaline Phosphatase (Fermentas, Life Sciences) following the standard protocol and processed for DNA sequencing reaction following standard sequencing protocols of Applied Biosystems. The products were then run in the ABI 3730 XL DNA analyzer.

RESULTS AND DISCUSSION

The primer set IDL04F and 23-10C was selected for the specific amplification of the 16S-23S intergenic spacer region of *Lactobacillus* spp. We obtained multiple bands after gel electrophoresis, confirming the presence of multiple copies of the ribosomal operon (Figure

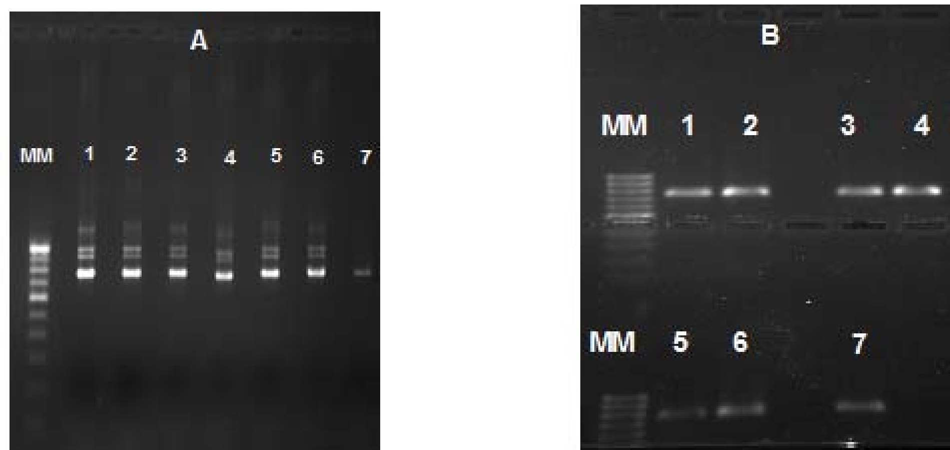

Fig. 1. Agarose gel picture of 16S-23S ISR after normal PCR amplification [A] and after performing bandstab-PCR of the lowest band [B]. Lane 1 to 7: Probiotic LAB1 to LAB7; MM=Molecular Marker 100-1000bp

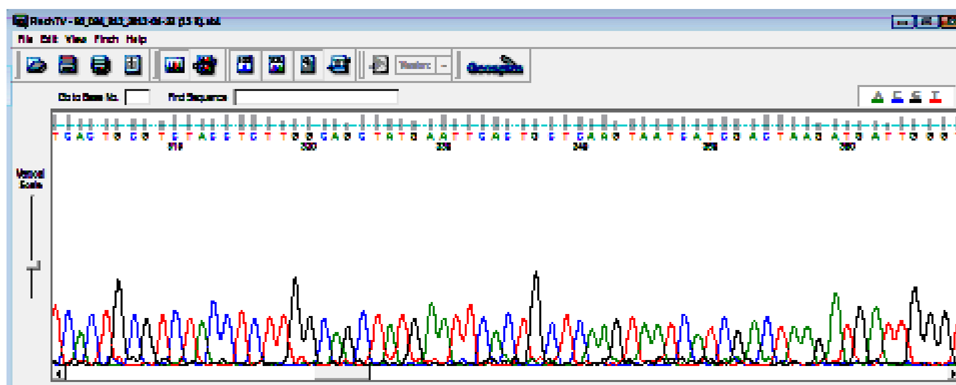

Fig. 2. Chromatogram picture of the ISR sequenced after performing bandstab-PCR method

1A). In fact, it is well known that the number and size of 16S-23S ISR in ribosomal RNA operons vary considerably within the *Lactobacillus* genus (Tulloch *et al.,* 1997; Klappenbach *et al.,* 2001; Klaenhammer *et al.,* 2002). These differences in size are due to the insertion of a tRNA-Ala gene within the medium ISR or tRNA-Ala and tRNA-Ile genes within the long ISR (Kabadjova *et al.,* 2002; Moreira *et al.,* 2005). The lowest band sizing 800bp (figure1A), called "short ISR" generally does not contain a tRNA on its sequence (Moreira *et al.,* 2005). The short ISR was extracted from the gel by stabbing and a second round of PCR was performed. The results revealed the presence of a single unique band (with a good intensity) visible on an agarose gel (Figure 1B) after performing the Bandstab-PCR technique. How important this novel finding is for the improvement of lactobacilli strains identification through the sequencing of the ISR? Taking into account that targeting ISR the molecular identification of lactobacilli strains is more accurate (Nour, 1998; Tannock, 1999; Moghadam *et al.,* 2010), especially when facing closely related species, this trick will make that identification, which was very difficult earlier due to the high variability of this region, easily achievable. The quality of sequence generated from the DNA analyzer after performing stabbing PCR illustrated by the chromatogram (Figure 2) showed an extremely low background level and nucleotides called without ambiguity. This chromatogram clearly highlights the level of purity and the quality of the sequence investigated.

CONCLUSION

The aim of this study was to purify efficiently the full "short" 16S-23S ISR from many bands using a simple/cost effective method and to check the quality of sequence obtained after purification. Based on our finding, it is therefore easily realistic to target the sequencing of the full ISR for the identification of lactobacilli species.

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