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APPLICATION OF PCR TECHNIQUE IN COMBINATION WITH DNASE TREATMENT FOR DETECTION OF VIABLE *LACTOBACILLUS ACIDOPHILUS* BACTERIA

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ABSTRACT

In this study, we examined whether application of DNase I can serve as differential eliminator of DNAs from dead cells, leaving viable probiotic lactic acid bacteria such as *Lactobacillus acidophilus* to be assessed by polymerase chain reaction (PCR). When dead cells were treated with DNase I, DNA amplification was not completely suppressed. Increasing the concentration of DNase I, up to 66 u/100 μL, and the preparation of dead cells using high temperatures did not seem to make difference in the level of PCR product from the dead bacteria. Assessment of free DNA degradation, when mixed with dead cells, showed that stability of free DNAs or their degradation by DNase I was not affected by presence of the dead cells. In conclusion, we tend to suggest that for using this technique, one should take great deal of caution and that its reliability should be tested for different species independently.

PRACTICAL APPLICATIONS

Lactobacillus acidophilus is one of the most common probiotic bacteria incorporated into food products. To have their health-promoting properties, consumption of high levels of the viable bacteria is recommended. Meanwhile, access to reliable protocols for assessment of viable bacteria remains elusive. Recent studies have focused on providing differential conditions for clearance of DNA material of the dead cells followed by quantification of the viable bacteria by molecular techniques. Despite the previous reports on application of DNase treatment along with PCR assays, for evaluation of viable harmful food bacteria, our data do not support the notion to be applied for detection of *L. acidophilus*.

INTRODUCTION

Monitoring of viable probiotic bacteria in food is one of the main factors for meeting the health benefits of these bacteria (Ashraf and Shah 2011). Traditionally, viability in bacteria is synonymous with the ability to form colonies on solid growth medium and to proliferate in liquid nutrient broths. These traditional, culture-based tests are time-consuming and can work poorly with slow-growing or viable, but noncultivable, organisms (Lahtinen *et al.* 2006; Reimann *et al.* 2010). Moreover, enumeration with differentiating between different species has been shown to illusive and challenging (Lahtinen *et al.* 2006). Thus, faster methods based on molecular techniques, such as polymerase chain reaction (PCR) or real-time PCR, which are extremely sensitive, have been developed to support or replace the traditional techniques. This valuable method, however, suffers from a major drawback of its inability to distinguish the dead cells from the viable ones (Shimizu *et al.* 2009; van Frankenhuyzen *et al.* 2011). This challenge has been the subject of many investigations, and addressed by different approaches such as application of reverse transcriptase

PCR, that targets the mRNAs (Vaitilingom *et al.* 1998). But RNA-based quantifications are difficult because the expression of genes is affected very much by the environmental conditions (Milner *et al.* 2001). Another strategy is combination of DNA-based real-time method with the nucleic acid-binding dyes, including ethidium monoazide bromide (EMA) or propidium monoazide (Rudi *et al.* 2005; Martinon *et al.* 2012). Despite the successful use of EMA, it has been shown that this reagent can effectively penetrate the intact cell membranes of some bacterial species, and bind to DNAs of viable microorganisms (van Frankenhuyzen *et al.* 2011). Many investigators have attempted to detect viable cells of certain food pathogens by utilization of DNase for elimination of the DNA debris from dead cells, leaving DNAs only from viable cells to serve as template for PCR (Nogva *et al.* 2000; Rueckert *et al.* 2005; Do *et al.* 2009). It is based on the fact that cellular membranes are disintegrated after the cells die and their DNAs are subjected to DNase degradation. Over the other DNA-based methods, this technique could provide a less expensive and faster alternative as a working method for accurate detection of the viable probiotic bacteria in food industry. So in the current study, we investigated the influence of the DNase treatment on DNA in living and heat-killed *Lactobacillus acidophilus* cells. This would provide suitable information for application and development of reliable methods for detection of probiotic bacteria in foods and probiotic products.

MATERIALS AND METHODS

Bacterial Strain and DNA Extraction

Lyophilized culture of *L. acidophilus* (ATCC 4356) and *L. delbrueckii subsp. bulgaricus* (ATCC 11842), was obtained from Iranian Research Organization for Science and Technology (Tehran, Iran). The bacteria were activated in de Man Rogosa Sharpe (MRS) broth (Merck-Darmstadt, Germany) at 37C. For extraction of total genomic DNA from Lactobacillus cultures, the harvested overnight cultures were resuspended in 500 μL of TE (10 mM Tris–HCl pH 8.0, 1 mM EDTA) buffer with 15 mg/mL lysozyme and incubated for 1 h at 37C, followed by addition of 6 μL of 20% sodium dodecyl sulfate and 10 μL of proteinase k (20 mg/mL). Incubation was carried out at 50C for 1 h. Genomic DNA was purified using Guanidine Thiocyanate-Silica Gel method (Boom *et al.* 1990).

Primers and PCR

The specific PCR primer set for *L. acidophilus*, previously designed by Tabasco *et al.* (2007) from a region in the 16S rRNA encoding genes, were used in this study. PCR amplification of bacterial DNA with these primers produces a 227 bp product. Detection of *L. bulgaricus* was based on the designed specific oligonucleotide primers by Song *et al.* (2000). The primer set yielded two 450 and 700 bp amplicons. All the PCRs were carried out in a model 2000 (Biometra, Gottingen, Germany) thermocycler. Amplification reaction mixtures (25 μL) contained a DNA sample (2 μL), 2.5 μL 10X PCR buffer, 2.5 mM $MgCl_2$, 200 mM of each dNTPs, 5 pM of each corresponding primers and 1 U of Taq DNA polymerase. PCR conditions for *L. acidophilus* were as follows: one cycle of initial denaturation at 94C for 3 min; 35 cycles at 94C for 30 s, 60C for 20 s and 72C for 20 s; and one cycle as final extension time at 72C for 5 min. For *L. bulgaricus*, these conditions were as follows: the initial denaturation at 95C for 3 min; 29 cycles of 94C for 30 s, 64C for 40 s and 72C for 1 min; and 72C for 10 min as final extension. PCR products were loaded on a 2% agarose gel and subjected to electrophoresis. Gels were then stained with ethidium bromide, and photographed. The images were analyzed with ImageJ 1.38X software (National Institutes of Health, Bethesda, MD) to determine the intensities of bands. All experiments were performed in triplicates.

DNase Treatments

The effect of DNase I (Fermentas, EN0525, Vilnius, Lithuania) treatment on the bacteria was investigated by using approximately 10^6–10^7 cells of viable *L. acidophilus* diluted to this concentration in sterile 0.15% peptone water. The number of the bacteria was determined by phase contrast microscopy (Olympus, BH2, 1000× magnification, London, UK) using a Thoma counting chamber. The cultures were pelleted at 13,000 rpm for 10 min, washed, resuspened in water and transferred into microcentrifuge tubes. Dead cells were prepared by heat treatment of the tubes containing approximately 7.1×10^6 viable cells at 97C for 10 min and 2.1×10^7 viable cells at 121C for 15 min. Growth absence of heat-killed cells was confirmed by growing on MRS broth and plate counting (data not shown). After the heat treatments, the tubes were centrifuged at 13,000 rpm for 10 min, and the supernatants were discarded. The pellets were resuspended in 100 μL of 1X DNase buffer with $MgCl_2$ and then 10 or 66 units of DNase I were added to the mix, followed by a brief vortexing and incubated at 37C for 1 h. Subsequently, the samples were centrifuged at 16,000× g for 10 min, resuspended in 100 μL sterile deionized water and boiled for 10 min to denature the DNase I enzyme. For the PCR analysis, DNAs were then released as described above. In addition, approximately 7.1×10^6 viable cells were subjected to DNase I according to the same method. The DNase-free samples were included as negative controls in each experiment. Possible effects of the enzymatic treatment on the genomic DNA were also tested.

For these purpose, purified DNAs from approximately 9.1×10^6 cells of *L. acidophilus* were treated with and without DNase I. After inactivation of the enzyme, the samples were centrifuged at 16,000× g for 5 min and 5 µL of the supernatant used for PCR analysis.

Stability of Free DNA Incubated with the Dead Cells

Extracted genomic DNA from approximately 9.3×10^5 *L. bulgaricus* was added to a suspension containing 1.1×10^7 dead cells (heated at 97C for 10 min) of *L. acidophilus*. Resulting solutions were subjected to 10 units of DNase I in 1X DNase buffer with $MgCl_2$. After 1 h of incubation at 37C, the cells were pelleted at 16,000× g for 5 min and 50 µL of the supernatant was boiled for 10 min. 5 µL of the supernatant was then used in the PCR assay. We included a control sample with added genomic DNA but without the enzyme treatment and dead cells in the experiment.

Experimental Design

All experiments were performed in three runs with three technical replicates. They were also repeated three times to confirm the reproducibility of the data.

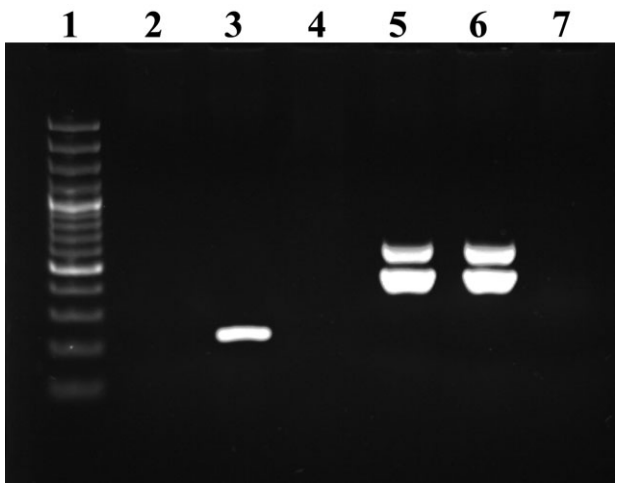

FIG. 1. AGAROSE GEL ELECTROPHORESIS OF PURIFIED DNAS FROM *L. ACIDOPHILUS* AND *L. BULGARICUS* VERSUS DEAD CELLS OF *L. ACIDOPHILUS*, WITH AND WITHOUT DNASE I TREATMENT
Numbers 1–7 represent 100 bp DNA ladder; *L. acidophilus* genomic DNA treated with DNase I; *L. acidophilus* genomic DNA not treated with DNase I; genomic DNA of *L. bulgaricus* mixed with *L. acidophilus* dead cells with DNase I treatment; genomic DNA of *L. bulgaricus* mixed with *L. acidophilus* dead cells without DNase I treatment; genomic DNA of *L. bulgaricus* without dead cells and DNase I treatment; and negative control, respectively.

RESULTS AND DISCUSSION

Effect of DNase I on *L. acidophilus* DNA Degradation

The effect of DNase I treatment on purified DNA from *L. acidophilus* was investigated using PCR and agarose gel electrophoresis. The PCR on extracted DNAs from samples without DNase I treatment, generated products of 227 bp in size (Fig. 1). While the same experiment on the DNAs treated with DNase I showed no PCR amplification, showing the degradation of large quantities of genomic DNA under experimental conditions (10 u of DNase I, 37C, 1 h).

Effect of DNase I on the Stability of DNA in *L. acidophilus* Cells

Dead bacterial cells were prepared by heat treatment. DNAs were extracted from the cells before and after DNase I treatment, to be tested for their resistance to the enzymatic degradation. As indicated in Fig. 2, the integrity of the extracted genomic DNAs, tested by gel electrophoresis, shows not much difference after the enzymatic digestion. For the samples heated at 97C, the DNase I treatment resulted in 8.9% ± 0.07 reductions in the intensity of the amplified target band, compared with that of the control, without DNase I (Fig. 2A). This somehow is an indication that the cellular DNAs are either not released from the bacterial cell walls, even after they are killed by heating, or there might be an inhibitory microenvironment, created after heating the cells that meddle the activity of the enzyme. While these results are in agreement with previous study on DNA degradation by DNase in the heat-killed *Campylobacter jejuni* (Nogva *et al.* 2000), shown by reduction of the PCR products in the reactions, which DNase treated DNAs from dead bacteria was applied as template, they contradict a report by Rueckert *et al.* (2005), where it was shown that contribution of dead cells of *Anoxybacillus flavithermus* in milk powder was removed by the use of 200 Kunitz units per mL of DNase I. The present results also showed that even the DNase at higher concentration of up to 66 u/100 µL did not improve the rate of DNA degradation within the dead cells as this was about 4.4% ± 0.49 (Fig. 2B).

In another work, it was pointed out that treatment of immobilized cells of food pathogens with DNase I, prior to DNA extraction, could efficiently eliminate false positives because of the presence of nonviable cells (Do *et al.* 2009). However, it is important to note that the dead cells had been prepared under the drastic treatment (treated in alcohol freeze chamber then autoclaved at 121C for 15 min), which may be different from the naturally occurring dead bacterial cells. The reason for the different results is still unknown. The technique however seems to work differently

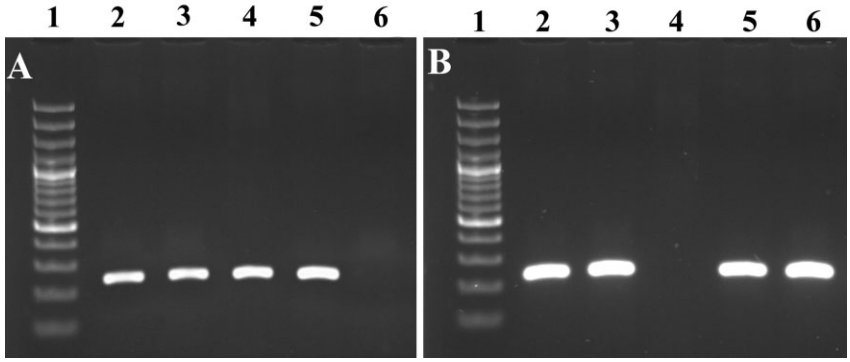

FIG. 2. GEL ELECTROPHORESIS OF PCR PRODUCTS ON DNA TEMPLATES OBTAINED FROM LIVE AND HEAT-KILLED *L. ACIDOPHILUS* BEFORE AND AFTER DNASE I TREATMENT

(A) PCR products on DNAs extracted from approximately 7.1×10^6 viable and heat-killed (at 97C for 10 min) *L. acidophilus* with or without DNase (10 u) treatment. Lane numbers 1–6 represent 100 bp DNA ladder; live cells without DNase I treatment; live cells with DNase I treatment; heat-killed cells with DNase I treatment; heat-killed cells without DNase I treatment; and negative control, respectively. (B) PCR products on DNAs extracted from approximately 2.1×10^7 heat-killed (at 97C for 10 min or 121C for 15) *L. acidophilus* with or without DNase (10 or 66 u) treatment. Lane numbers 1–6 represent 100 bp DNA ladder; heat-killed cells at 97C for 10 min followed by 66 u DNase I treatment; heat-killed cells at 97C for 10 min followed by 10 u DNase I treatment; negative control; heat-killed cells at 121C for 15 min with DNase I treatment; and heat-killed cells at 121C for 15 min without DNase I treatment.

in different bacterial species. Other reason for these discrepancies might be associated with the protocols of dead cell preparation. Bacterial cell structure and different approaches for killing the cells may affect the quality of the cell lysates and therefore the efficiency of the DNA degradation by the externally added DNase. Regarding the results obtained by Simmon *et al.* (2004), it should be taken into account that bacteria are lysed by rapid heating and depressurization in an autoclave. Therefore, it is likely that high-quality results were achieved by a pressure-jacked autoclave, which is not feasible by small units that generate their own pressure in the chamber. Based on two lines of evidences, in current studies, it became obvious that at least in the case of *L. acidophilus*, heating the bacteria with 121C for 15 min followed by DNase I treatment did not seem to be that effective in removal of the unwanted DNAs. First, the heating reduced the amount of template DNAs, as examined by PCR, by only $5\% \pm 0.39$ compared with the untreated samples (Fig. 2B); and second, the microscopic observations indicated that the autoclave treatment did not result in observable cell lysis using the same autoclave condition.

Our results indicated that DNase I treatment had no damaging effect on the DNA present inside the viable cells (Fig. 2A), and the intensities of the observed PCR bands with DNase I exceeded those without enzymatic treatment, by up to $2\% \pm 0.22$. Many studies in this field have demonstrated that DNase I is unable to penetrate the membrane of viable cells and has no effect on the cell viability in their applied concentrations (Fischer 1982; Frankfurt 1983; Nogva *et al.* 2000; Rueckert *et al.* 2005; Do *et al.* 2009).

Effect of DNase I on Free DNA versus Dead Cells of *L. acidophilus*

To test the possible effect of presence of the dead *L. acidophilus* on efficiency of DNA degradation, pure genomic DNA, from *L. bulgaricus*, was subjected to the DNase I treatment in this condition. As shown in Fig. 1, the DNAs seemed to disappear in the lane corresponding to the DNase I treatment of the purified DNA of *L. bulgaricus* in presence of the dead cells, while the untreated pure DNAs show obvious difference in pattern of gel electrophoresis. Furthermore, the PCR products obtained from the untreated samples, with and without dead cells, had similar intensities on the agarose gel, highlighting that these bacteria probably did not secrete thermostable extracellular DNases and the stability of free DNA was not affected by the dead cells. Meanwhile, it has been recently shown that some bacteria produce extracellular thermonucleases (Mann *et al.* 2009; Nijland *et al.* 2010). In general, inability of the external DNases to remove the DNAs within the dead bacteria could be speculated by two notions of (1) a component released from the dead cells, which may irreversibly inhibit the DNase I and (2) the DNase I could not penetrate the cell wall of the dead bacteria because of its high molecular weight, as reported by Soejima *et al.* (2008). However, based on the present results, the former possibility seems unlikely because no PCR products were observed from the DNase-treated DNA of *L. bulgaricus* in current study, adding more weight to the second notion.

To conclude, in contrary to the assumption of using PCR technique for quantification of the viable bacteria, following

successful removal of DNAs from dead cells in the cultures (Rueckert *et al*. 2005; Do *et al*. 2009), we report here that this protocol should be practiced with high level of caution before generalization for different species, and that it could not be applied at least in the case of *L. acidophilus*, based on the results from current study. Our investigation suggests that bacterial cell wall and membrane play a major role in DNA access by the enzyme in dead cells of the bacteria. Because of a recommended level of $\geq 10^6$ viable probiotic cells/g necessary for the corresponding products (Ashraf and Shah 2011), this protocol would not be recommended, as it fails to meet the detection sensitivity in the required range.

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