Vol.55, n. 2: pp.319-327, March-April 2012
ISSN 1516-8913 Printed in Brazil

BRAZILIAN ARCHIVES OF
BIOLOGY AND TECHNOLOGY
A N I N T E R N A T I O N A L J O U R N A L

# Rapid Yeast DNA Extraction By Boiling And Freeze-Thawing Without Using Chemical Reagents And DNA Purification

**Gildo Almeida da Silva[1*], Taís Letícia Bernardi[1], Patrícia Dayane Carvalho Schaker[1], Morgana Menegotto[1] and Patricia Valente[2]**

*[1]Departamento de Microbiologia; Embrapa Uva e Vinho; 95700-000; Bento Gonçalves - RS - Brasil. [5]Departamento de Microbiologia; Universidade Federal do Rio Grande do Sul; Porto Alegre - RS - Brasil*

## ABSTRACT

*The purpose of this work was to study a rapid yeast DNA extraction by boiling and freeze-thawing processes without using chemical reagents or any purification procedures, to obtain a high grade PCR-product. A specific DNA fragment of the 18S region of* Dekkera bruxellensis *and* Saccharomyces cerevisiae *was chosen. The described boiling and freeze-thawing protocols generated the PCR-grade product preparations and could be used to process many samples. The amplification of the fragments could be observed after 30 and 35 cycles. These processes of extraction without using any kind of chemical reagents, especial water, and purification procedures proved to be efficient, reproducible, simple, fast, and inexpensive.*

**Key words**: Boiling, freeze-thawing, DNA extraction, *Dekkera bruxellensis*, *Saccharomyces cerevisiae*

## INTRODUCTION

The success of DNA amplification by PCR depends on the availability of cellular DNA free of Taq polymerase inhibitors. The extraction and purification protocols are used to disrupt the microorganism cell walls and eliminate the inhibitors interference, respectively. Many current protocols are too laborious and time-consuming for routine diagnostic or identification work. All the protocols described so far, use centrifugation or chemicals in one of the steps for the extraction or purification. Even the so-called rapid and efficient protocols require expensive or toxic reagents (Paterson et al. 1993; Harju et al. 2004; Borman et al. 2006; Cheng and Jiang 2006; Melo et al. 2006), utilize either glass beads (Melo et al. 2006) or enzymatic digestion (Borman et al. 2006; Jin et al. 2004) and specific equipments (Müller et al. 1998; Galán et al. 2006) to disrupt the yeast cell wall. Galán et al. (2006) described six fungal DNA extraction methods for the comparative analysis and in all the extractions, some kind of chemical compound was employed.

A device that allows fast heating/cooling rates, and therefore, giving efficient thermocycling suitable for DNA amplification has been developed (Ke et al. 2004). A novel device involving the cell lysis and DNA amplification performed in a single step have been proposed by Ke et al. (2007). A simple and rapid protocol for the preparation of total genomic DNA from the yeast and mould was proposed by Borman et al. (2006, 2008). Filter paper (Whatman FTA) has been used as a DNA immobilizer matrix. The matrix was subjected to the cycles of microwaving at full power, being

[*]Author for correspondence: gildo@cnpuv.embrapa.br

subsequently washed with Whatman FTA wash reagent, followed by two washes with TE buffer. The FTA filter papers are, in fact, fibrous matrices impregnated with chemicals such as chelators and denaturing compounds that lyse and inactivate the microorganisms (Borman et al. 2006, 2008). Thus, it cannot be considered a completely chemical-free extraction protocol. The heat treatment for the fungal DNA preparation uses glass beads and the sample is heated up to 95 °C for 20 min. The freeze-thawing process performed by Borman et al. (2006) submitted the samples to six cycles of freeze-thawing in liquid nitrogen.

The heat procedure developed by Liguori et al. (2007) used phosphate-buffered saline solution, followed by centrifugation to pellet the DNA which was re-suspended in sterile distilled water and heated to 95 °C for 3 min. In the boiling-freezing protocol for DNA-extraction used by de Baere et al. (2002), the cells were suspended in the distilled water and heated at 95°C for 15 min and submitted to freezing at -70°C. The samples were thawed at room temperature, and centrifuged to remove the cell debris. In the boiling process described by Freschi et al. (2005), the samples were centrifuged, followed by re-suspension in sterile distilled water, heated to 95ºC for 10 min, cooled on ice and centrifuged at 13,000 x g for 3 min. The supernatant was used for PCR assay. Medici et al. (2003) compared the boiling, alkaline lysis, Nucleospin, and Dynabeads DNA Direct System I methods for DNA extraction from *Salmonella enterica*. In the boiling process, the sample was also centrifuged three times and DNase-RNase-free distilled water was used to re-suspend the pellet. The simple boiling methods described by Deak et al. (2000) and by Araújo et al. (2004) used centrifugation to collect the cells, to eliminate the cell debris after the boiling procedure, and to pellet the total precipitated DNA. These last six procedures have been by far simpler and more rapid than those described by Borman et al. (2006, 2008) but these protocols required centrifugation as an essential step and some of them required especial care to handle the samples. The current work describes a reliable, robust, effective and easy-to-perform method of DNA extraction for PCR-based amplification of a specific DNA fragment of the 18S region of *Dekkera bruxellensis* and *Saccharomyces cerevisiae*.

## MATERIALS AND METHODS

### Microorganism

The *Saccharomyces cerevisiae* Embrapa 1vvt/97 and the *Dekkera bruxelensis* NRRL Y-12961 strains were used. The yeasts were maintained in G7 (da Silva and de Almeida 2006) and must agar (da Silva 1996).

### DNA Preparation *Boiling process*

An aliquot of 1000 μL of cell suspension containing $10^7$ cells/mL of *S. cerevisiae* and *D. bruxelensis* was transferred to microtubes and incubated at 90°C in a boiling water-bath for 5 min. The suspension containing DNA was vigorously homogenized by vortex for 10 s and the tube was frozen on ice. The DNA sample was stored at -18 °C.

### *Freeze-thawing process*

An aliquot of 1000 μL of cell suspension containing $10^7$ cells/mL of *S. cerevisiae* and *D. bruxelensis* was transferred to microtubes and incubated at -18°C until ice formation. The samples were allowed to thaw at room temperature and homogenized by vortex for 10 s.

### *DNA extraction process with standard cell lysis buffer*

Aliquots of 1000 μL of cell suspension containing $10^7$ cells/mL of *S. cerevisiae* and *D. bruxelensis* were centrifuged at 10,000 x *g* for 15 minutes (Sorval, USA) and the yeast cell pellets were transferred to microtubes containing 500 μL 50 mM (Invitrogen Life Technologies, USA), EDTA 10 mM (Sigma-Aldrich CO., USA), SDS 2.0 % (w/v) (Labsynth, BR); pH 8,0). The mixture was incubated at 60°C for 1 h in a water-bath. An equal volume (500 μL) of buffer-saturated phenol (Sigma Chemical CO., USA): chloroform (Reagen, BR) (1:1) was added to the DNA solution. The mixture was vigorously mixed by vortex, and centrifuged for phase separation in a Sorval centrifuge (Sorval, USA) at 10,000 x *g* for 15 minutes. The upper aqueous layer (containing the DNA) was carefully transferred to a clean tube, avoiding the phenol interface and equal volume of chloroform was added. The mixture was vigorously mixed by vortex and centrifuged at 10,000 x *g* for 15 min. The chloroform extraction was repeated. The upper aqueous layer was

transferred to a clean centrifuge tube and equal volume of cold isopropanol (Chimie Test, BR) was added to the DNA sample. The mixture was vigorously mixed by vortex, then the tube was allowed to stand in an ice-water bath for 30 minutes and centrifuged at 10,000 x *g* for 20 min. To recover the precipitated DNA, the samples were centrifuged and the supernatant was discarded. The pellets were washed with ice-cold 70% ethanol, and centrifuged at 10,000 x *g* for 15 min. The resulting DNA pellet was air dried and dissolved in 100 µL of TE (Tris-HCl 10 mM pH 7.6, EDTA 1 mM pH 8.0). The DNA sample was stored at -18 °C.

**PCR procedure**

Primers YeastF and YeastR (submitted to Regional Patent Office) targeting the conserved region of 18S rDNA were used to amplify a 375-bp segment. PCR amplification was performed at final volume of 25 µL. One microlitre of each DNA sample from boiling, freeze-thawing and chemical extraction protocols was added to the PCR master mixture, which consisted of buffer Taq DNA polymerase enzyme 1x (Invitrogen Corporation, USA), 1.5U Platinum® Taq DNA polymerase (Invitrogen Corporation, USA), 100 µmol each dNTP (Invitrogen Corporation, USA), 0.8 pmol/µL (0.8µM) of each primer (Integrated DNA Technologies Inc., USA), 2.5mM $MgCl_2$. Amplification consisted of an initial denaturation step at 94°C for 5 min, followed by 25, 30, or 35 cycles comprising a denaturation step at 94°C for 30 s, an annealing step at 68°C for 45 s, and an extension step at 72°C for 30 s, followed by a final extension at 72°C for 5 min. A PTC-100 Peltier thermal cycler (Bio-Rad Life Sciences, USA) was used.

**Precipitation with isopropanol**

A cell suspension in water containing $10^7$ cells/mL of *Sacch. cerevisiae* and *D. bruxellensis* was prepared and an aliquot of 1000 µL was transferred to microtubes. After the boiling and freeze-thawing processes, the cell debris were separated by centrifugation at 10,000 x g for 15 min and re-suspended in 100 µL TE buffer. The supernatant was precipitated with cold isopropanol and again centrifuged at 10,000 x *g* for 15 min. The resulting DNA sediment obtained was air-dried and re-suspended in 100 µL TE buffer. The DNA present in the supernatant and the cell debris were submitted to 25 and 30-cycle PCR for amplification separately.

**Retention of DNA in the cell**

After 25 and 30-cycle PCR of the cell debris, the products of amplification were centrifuged at 10,000 x g for 15 min. The supernatant and debris were again submitted to electrophoresis separately.

**Reduction of primers concentration**

The concentration of each primer was reduced from 0.8 pmol/µL (0.8 µM) to 0.4 pmol/µL (0.4 µM) (Integrated DNA Technologies Inc., USA). The concentration of all the other reagents was the same as described before. An aliquot of 1.0 µL of debris was used as template DNA and submitted to 25 and 30-cycle PCR.

**Electrophoresis**

Aliquots of 5.0 µL of PCR-amplified products and the 100-bp DNA Ladder (100 to 1500-bp) (Invitrogen Life Technologies, USA) were loaded onto 1.5% agarose electrophoresis gels (Sigma Chemical, USA). The run was performed with 1 x Tris-acetate- EDTA (TAE) buffer, containing 40 mM Tris-HCl (Invitrogen Life Technologies, USA), pH 8.3, 20 mM acetic acid (Merck, Germany) and 1 mM EDTA (Sigma-Aldrich CO., USA), at 90 V/cm for 45 min. The gels were stained with GelRed (TM Biotium, Inc. California, USA). The stained gels were photographed using UV transilluminator Eagle Eye II Video Imaging System (Stratagene, California, USA).

## RESULTS AND DISCUSSION

The protocols for PCR require extraction and purification of DNA from the yeast cells. The extraction procedures of yeast cells are either time-consuming or generate poor yields compared to the methods of DNA extraction from the animal cells or viruses (Galán et al. 2006). The extraction using standard cell lysis buffer appeared to be more efficient than both boiling and freeze-thawing processes, since the band corresponding to the 375-bp fragment of the 18S region of *S. cerevisiae* and *D. bruxellensis* could be clearly visualized after 25 PCR cycles (Fig. 1A), meaning that this extraction process was genus-independent. The bands corresponding to the boiling and freeze-thawing processes, although

very weak, could also be visualized. The low yield of PCR products in the boiling and freeze-thawing extraction procedures indicated low concentration of DNA in comparison to the standard cell lysis procedure. In fact, when the number of PCR cycles was increased from 25 to 30, the bands from all the three extraction processes were clearly detected (Fig. 1B). Since the PCR products could be better revealed with the freeze-thawing procedure than with the boiling process (Fig. 1B), it was reasonable to conclude that the former technique allowed a better exposition of the 18S region to the Taq polymerase than the later procedure. As the number of PCR cycles was increased to 35, the 375-bp PCR fragment was clearly observed in all the three extraction procedures with approximately the same resolution (Fig. 1C), meaning that the enzymatic reaction was in more limiting substrate conditions when the physical procedures were used than when the standard cell lysis buffer was employed. This was probably due to the step of DNA precipitation with cold isopropanol described in the chemical protocol. It should be stressed that the entire extraction by standard cell lysis buffer, boiling and freeze-thawing processes took approximately 4-5 h, 30 min, and 1h, respectively. The cellular debris remaining in the samples did not interfere with PCR amplification, which meant that the centrifugation step commonly used to remove this sediment was not required. Similar results were obtained by Harju et al. (2004).

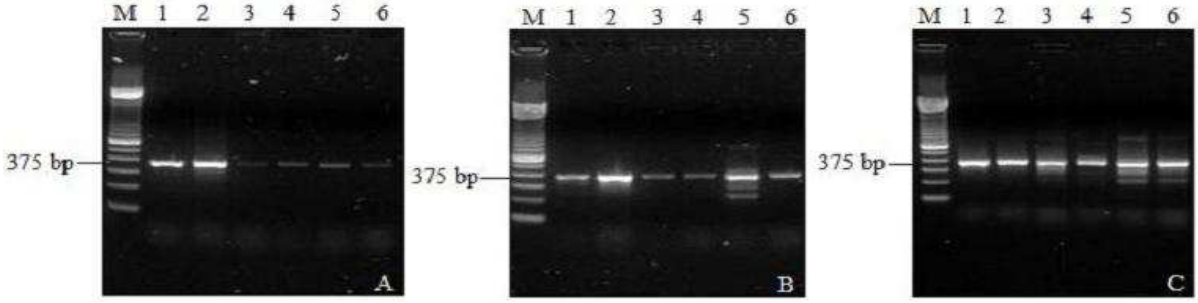


**Figure 1 -** Amplification of a 375-bp specific DNA fragment of the 18S region of *Dekkera* and *Saccharomyces*: M- 100-bp DNA Ladder; 1- *Sacch. cerevisiae* - standard cell lysis buffer; 2- *D. bruxellensis* - standard cell lysis buffer; 3- *Sacch. cerevisiae* - boiling process; 4- *D. bruxellensis* - boiling process; 5 – *Sacch. cerevisiae* - freeze-thawing process; 6- *D. bruxellensis* - freeze-thawing process; A- 25 cycles; B- 30 cycles; C- 35 cycles.

From Figure 1-B, the boiling assay appeared to disrupt the cell wall less efficiently than the freeze-thawing procedure. It was possible that during the former procedure the target DNA remained into the cell and the later provided a higher target DNA leakage from the cell interior. To check this hypothesis, after the boiling and freeze-thawing processes, the cell debris were precipitated with cold isopropanol. After 25-cycle PCR, the bands could be better visualized in both boiling and freeze-thawing processes after 25-cycle PCR (Fig. 2A), showing better resolution for both yeast species in the freeze-thawing process than with the mixture cell debris/supernatant (Fig. 1A). The DNA precipitation with cold isopropanol could help to explain the advantage of the traditional procedure over the physical processes. The concentration of DNA was at threshold conditions in the supernatants of both yeast species since the 375-bp fragments were more efficiently observed when the PCR cycles increased to the number of 30 (Fig. 2B). In these two physical processes, a significant amount of the target DNA was still retained in the cell debris as shown in the Figures 2C and 2D. This showed that the reactions could be initiated directly from cell debris and that the amplification system had access to the fragment of the 18S region contained in this cell debris. Hirano et al. (1997) showed the results of PCR amplification of $\rho^+$ and $\rho^0$ DNA and of DNA from the supernatant and the pelleted cell debris after 10 min of boiling of the buffy coat, using the primers corresponding to the three COX genes. They noted that the yield of supernatant amplified PCR products by using the DNA obtained after 10

min of boiling was surprisingly low. In contrast, the yield of pelleted cell-debris amplified PCR products was high and the yield of supernatant amplified PCR products was greater only when the cells were boiled for at least 20 min.

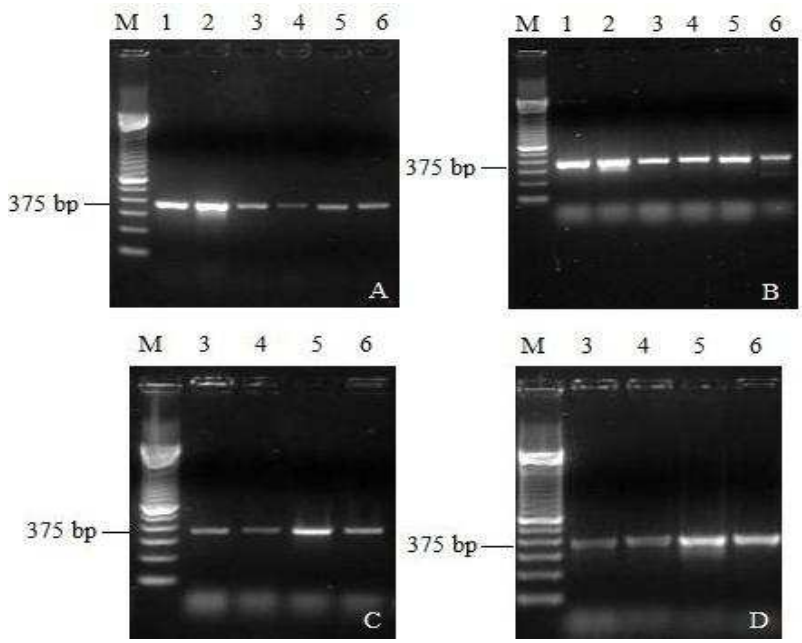


**Figure 2 -** Amplification of a 375-bp specific DNA fragment of the 18S region of *Dekkera* and *Saccharomyces* only present in the cell-free supernatants after extraction process: M- 100-bp DNA Ladder; 1 - *Sacch. cerevisiae* - standard cell lysis buffer; 2- *D. bruxellensis* - standard cell lysis buffer; 3- *Sacch. cerevisiae* - boiling process; 4- *D. bruxellensis* - boiling process; 5 - *Sacch. cerevisiae* - freeze-thawing process; 6- *D. bruxellensis* - freeze-thawing process: A- 25-cycle PCR-cell-free supernatants; B- 30-cycle PCR - cell-free supernatants; C-25-cycle cell debris; D- 30-cycle cell debris.

Based on the assumption that the amplification efficiency was constant from the very first cycle to all over the number of thermal cycles, the DNA amplification number depended upon the initial number of target molecules and the number of thermocycles. Considering an efficiency of 100%, a number of DNA target molecules of 1, a 25-cycle PCR would give a DNA amplification number of $6.71 \times 10^7$. An increase of five more cycles would generate a total of DNA amplification number of $2.15 \times 10^9$.

The cell debris was submitted to PCR for amplification. The 375-bp fragment was detected with a 25-cycle PCR (Fig. 2C) with similar intensity as that previously showed from the supernatants (Fig. 2A). If the thermal cycles were exactly the same, the similar intensity of the bands could be due to a similar amount of target DNA in cell debris and in the supernatant. This confirmed the hypothesis of trapping of the target DNA by the cell debris. The amplification of the 375-bp fragment containing the cell-debris with 30-cycle did not improve the resolution of the bands (Fig. 2D). The results also showed substantial advantage of freeze-thawing treatment over boiling-based system irrespective of whether *D. bruxellensis* or *S.cerevisiae* was used. As a rule, the bands obtained from DNA of all the three extraction processes were detected after 30-cycle PCR (Fig. 2B).

The retention of amplified DNA in the yeast celldebris submitted to physical processes was also examined. Both cell debris and supernatant were separately used. The cell debris corresponded to $6x10^4$ cells and the components of the supernatant were resultant of the same amount of cells. Once again, the 25-cycle PCR amplification (Fig. 3A) proved to be enough to amplify the target DNA containing cell-debris free supernatants of *S. cerevisiae* and *D. bruxellensis* submitted to boiling

and freeze-thawing processes. This showed that the great majority of the target DNA was in the supernatant or the amplified DNA moved to the supernatant after amplification. Little improvement was obtained with 30-cycle PCR (Fig. 3B). Weaker amplification was obtained with the cell debris (Fig. 3C) when compared with 25-cycle PCR of supernatant (Fig. 3A). The 375-bp bands from cell debris of both the yeasts could be clearly detected with 30-cycle PCR amplification (Fig. 3D) irrespective of the two extraction methods used. It seemed that cell debris subjected either to boiling or freeze-thawing processes had a fewer amount of target DNA for amplification than the supernatant. As the cell debris was the unique source of DNA, this provided the evidence that an important amount of DNA went out the cells either during the PCR cycles or during the centrifugation process. It seemed reasonable to suppose that, before centrifugation, the cellular debris retained significant amount of amplified DNA and did not interfere with subsequent PCR amplification. In conclusion, the physical procedures of yeast DNA extraction were reliable and reproducible preparative methods to amplify the DNA without centrifugation and purification steps. The use of cell debris as shown in Figures 2C and 2D should be more useful for PCR amplification than the supernatant resultant of boiling or freeze-thawing. It would be much easier to get rid of inhibitors tied up to the cell debris than of inhibitors present in the liquid phase.

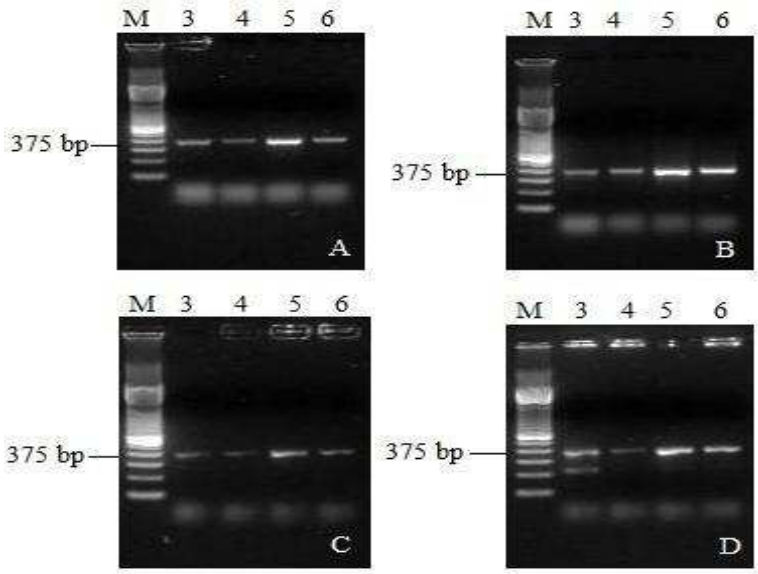


**Figure 3 -** Amplification of a 375-bp specific DNA fragment of the 18S region of *Dekkera* and *Saccharomyces*: M- 100-bp DNA Ladder; 3- *Sacch. cerevisiae* - boiling process; 4- *D. bruxellensis* - boiling process; 5 - *Sacch. cerevisiae* - freeze-thawing process; 6- *D. bruxellensis* - freeze-thawing process; A- 25-cycle PCR of cell-debris free supernatants from a cell suspension of $10^7$ cells/mL after PCR amplification; B- 30-cycle PCR of cell-debris free supernatants from a cell suspension of $10^7$ cells/mL after PCR amplification; C- 25-cycle PCR of cell-debris from a cell suspension of $10^7$ cells/mL after PCR amplification; D- 30-cycle PCR of cell-debris from a cell suspension of $10^7$ cells/mL after PCR amplification.

According to Pruvost and Geigl (2004), small number of initial target-DNA molecules gave less reliable PCR products. Moreover, small amount of target DNA increased the risk of contamination from anything that could come into contact with the DNA solution. It should be stressed that an environment, such as wine, in which the cells of *Saccharomyces* or *Dekkera* and several other yeasts were present, was not sterile and could not be sterilized. To ensure the cleaner PCR product and lower background, a lower concentration of primer (0.4 μM) than the one used so far (0.8 μM) was tested. Despite the typical PCR primer concentrations range from 0.1 to 1 μM, in general, the optimal primer concentrations were from 0.1 to 0.5 μM (Innis and Gelfand 1990) and this range of

primer concentration has been used with a relative frequency (Maneu et al. 2000; Andrighetto et al. 2000, 2004; Freschi et al. 2005; Galán et al. 2006; Liguori et al. 2007). The usual dNTP concentration between 20µM and 200 µM of each of the four dNTPs gave optimal balance among the yield, specificity and fidelity (Innis and Gelfand 1990). The recommended amount of Taq polymerase is approximately 1 unit for a 25µL reaction. As described in Material and Methods, the unique PCR reaction component was above the optimum concentration range stated by Innis and Gelfand (1990). Its concentration was decreased from 0.8 µ to 0.4 µM (Fig. 4A and Fig 4B). The results could be compared with primers concentration of 0.8 µM depicted in Figures 2C and 2D. The fundamental difference between both the primer concentrations was in 25-cycle PCR with boiling procedure. With 0.4 µM, the bands corresponding to DNA from the boiling process for both the yeasts were weaker than that with 0.8 µM. These two bands were better resolved with 30-cycle PCR. Once again, the freeze-thawing extraction was demonstrated to be a better protocol for PCR amplification than the boiling procedure. The freezing-thawing could alter the expression of genes and trigger responses to cell damage process.

Sharma et al. (2006) have provided evidence of structural community changes and expression dynamics of denitrifiers as a result of freeze-thawing stress. The expression levels of periplasmic nitrate reductase gene (*nap*A) and cytochrome cd1 nitrite reductase (*nir*S) were higher just after thawing began, followed by a decrease. If there were several degrees of damage provoked by freeze-thawing procedure, changes in this process could better expose the components of PCR reaction to the target DNA and improve the detection.

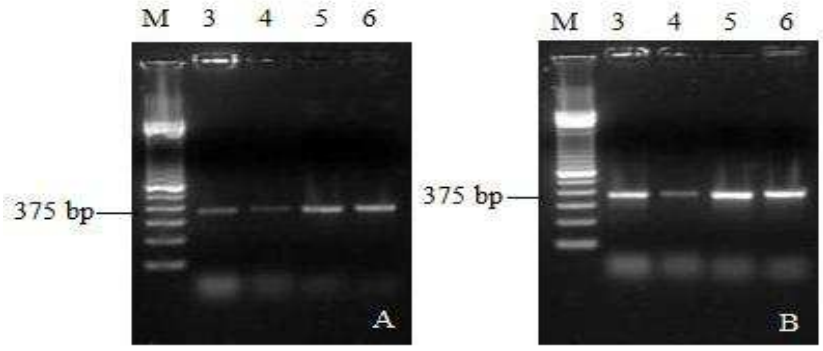


**Figure 4 -** Amplification of a 375-bp specific DNA fragment of the 18S region of *Dekkera* and *Saccharomyces* with 0.4 mM of each primer: M- 100-bp DNA Ladder; 3- *Sacch. cerevisiae* - boiling process; 4- *D. bruxellensis* - boiling process; 5 - *Sacch. cerevisiae* - freeze-thawing process; 6- *D. bruxellensis*– freeze-thawing process; A- 25-cycle PCR of cell-debris from a cell suspension of 107 cells/mL; B- 30-cycle PCR of cell-debris from a cell suspension of $10^7$ cells/mL.

Park et al. (1997) observed that the cell damage was related to both freezing duration and growth phase. High resistance was found during the lag phase and low resistance was observed during the log phase. Decrease in cell viability was observed at temperatures between 0 and -5°C and the cell mortality in this temperature range depended on the cooling rate (Dumont et al. 2006). The DNA extraction and the PCR amplification could be improved by choosing both, the best yeast cell growth phase and a proper freezing rate to increase the physical cell damage. Although cooling to very low temperatures is applied to preserve the living cells and their biological activity, loss of cell viability is suggested to be caused by the membrane damage (Moussa et al. 2008). At supercooled state, according to Moussa et al. (2008), the membrane permeability is increased, there is an uncontrolled mass transfer to and from the cells, the cells suffer an abrupt shrinkage, are exposed to cold osmotic shock and the cell membrane becomes disrupted. These facts play an

important role in yeast cell death. The results suggested that freeze-thawing treatment was more injurious to the membrane of the yeast cells than the boiling treatment and could explain the advantage of this procedure over the boiling process for PCR amplification. The present protocols could be used for routine diagnostic practice.

## ACKNOWLEDGEMENT

The authors thank Capes, CNPq, and Embrapa for financial support.

## REFERENCES

Andrighetto C, Marcazzan G, Lombardi A Use of RAPD-PCR and TTGE for the evaluation of biodiversity of whey cultures for grana padano cheese. Lett Appl Microbiol, 2004, 38(5):400–405. doi: 10.1111/j.1472-765X.2004.01504.x

Andrighetto C, Psomas E, Tzanetakis N, Suzzi G, Lombardi A Randomly amplified polymorphic DNA (RAPD) PCR for the identification of yeasts isolated from dairy products. Lett Appl Microbiol, 2000, 30(1):5–9. doi: 10.1046/j.1472-765x.2000.00589.x

Araújo WL, de Angelis DA, Azevedo JL Direct RAPD evaluation of bacteria without conventional DNA extraction. Braz Arch Biol Technol, 2004, 47:375–380.

Borman AM, Linton CJ, Miles SJ, Campbell CK, Johnson EM, Ultrarapid preparation of total genomic DNA from isolates of yeast and mould using Whatman FTA filter paper technology - a reusable DNA archiving system. Med Mycol, 2006, 44(5): 389–398. doi: 10.1080/13693780600564613

Borman AM, Linton CJ, Miles SJ, Johnson EM, Molecular identification of pathogenic fungi. J Antimicrob Chemother, 61 Suppl, 2008, 1:i7–12. doi:10.1093/jac/dkm425

Cheng HR, Jiang N, Extremely rapid extraction of DNA from bacteria and yeasts. Biotechnol Lett, 2006, 28(1): 55–59. doi: 10.1007/s10529-005-4688-z

da Silva GA, The occurrence of killer, sensitive, and neutral yeasts in Brazilian Riesling Italico grape must and the effect of neutral strains on killing behaviour. Appl Microbiol Biotechnol, (1996). 46(2): 112–121. doi: 10.1007/s002530050791

da Silva GA, de Almeida EA, Production of yellow-green fluorescent pigment by *Pseudomonas fluorescens*. Braz Arch Biol Technol, 2006, 49(3): 411–419.

de Baere T, Claeys G, Swinne D, Verschraegen G, Muylaert A, Massonet C, Vaneechoutte M, Identification of cultured isolates of clinically important yeast species using fluorescent fragment length analysis of the amplified internally transcribed rRNA spacer 2 region (ITS2). BMC Microbiol, 2002, 2:21. doi: 10.1186/1471-2180-2-21

Deak T, Chen J, Beuchat LR , Molecular characterization of *Yarrowia lipolytica* and *Candida zeylanoides* isolated from poultry. Appl Environ Microbiol, 2000, 66(10): 4340–4344.

Dumont F, Marechal PA, Gervais P, Involvement of two specific causes of cell mortality in freezethaw cycles with freezing to -196 degrees C. Appl Environ Microbiol, 2006, 72(2): 1330–1335.

Freschi CR, Carvalho LFOS, de Oliveira CJB Comparison of DNA-extraction methods and selective enrichment broths on the detection of *Salmonella typhimurium* in swine feces by polymerase chain reaction (PCR). Braz J Microbiol, 2005, 36: 363–367. doi: 10.1590/S1517-83822005000400011

Galán A, Veses V, Murgui A, Casanova M, Martínez JP, Rapid PCR-based test for identifying *Candida albicans* by using primers derived from the pH-regulated KER1 gene. FEMS Yeast Res, 2006, 6(7): 1094–1100. doi: 10.1111/j.1567-1364.2006.00114.x

Harju S, Fedosyuk H, Peterson KR Rapid isolation of yeast genomic DNA: Bust n' Grab. BMC Biotechnol, 2004, 4:8. doi: 10.1186/1472-6750-4-8

Hirano M, Shtilbans A, Mayeux R, Davidson MM, di Mauro S, Knowles JA, Schon EA, Apparent mtDNA heteroplasmy in Alzheimer's disease patients and in normals due to PCR amplification of nucleus embedded mtDNA pseudogenes. Proc Natl Acad Sci USA, 1997, 94(26):14894–14899.

Innis MA, Gelfand DH, Optimization of PCRs. In: Innis MA (ed) PCR protocols. A guide to methods and applications. Academic Press, Inc. Harcourt Brasce Jovanovich, Academic Press, Limited, 24-28 Oval Road, London NW1 7DX. 1990

Jin J, Lee YK, Wickes BL, Simple chemical extraction method for DNA isolation from *Aspergillus fumigatus* and other *Aspergillus* species. J Clin Microbiol, 2004, 42(9): 4293–4296. doi: 10.1128/JCM.42.9.4293-4296.2004

Ke C, Kelleher AM, Mathewson A, Sheehan M, Design and fabrication of a silicon microreactor for DNA amplification. Conf Proc IEEE Eng Med Biol Soc, 2004, 3:1964–1967.

Ke C, Kelleher AM, Sheehan M, Mathewson A, Single step cell lysis/PCR detection of *Escherichia coli* in an independently controllable silicon microreactor. Sensors and Actuators B: Chemical, 2007, 120:538–544. doi:10.1016/j.snb.2006.03.019

Liguori G, Lucariello A, Colella G, Luca AD, Marinelli P, Rapid identification of *Candida* species in oral rinse solutions by PCR. J Clin Pathol, 2007, 60(9): 1035–1039. doi:10.1136/jcp.2006.040782

Maneu V, Martínez JP, Gozalbo D, Identification of *Candida albicans* clinical isolates by PCR amplification of an efb1 gene fragment containing an intron-interrupted open reading frame. Med Mycol, 2000, 38(2): 123–126.

Medici DD, Croci L, Delibato E, Pasquale SD, Filetici E, Toti L, Evaluation of DNA extraction methods for use in combination with SYBR green I real-time PCR to detect *Salmonella enterica* serotype enteritidis in poultry. Appl Environ Microbiol, 2003, 69(6): 3456–3461. doi: 10.1128/AEM.69.6.3456-3461.2003

Melo SCO, Pungartnik C, Cascardo JCM, Brendel M, Rapid and efficient protocol for DNA extraction and molecular identification of the basidiomycete *Crinipellis perniciosa*. *Genet Mol Res*, 2006, 5(4): 851–855.

Moussa M, Dumont F, Perrier-Cornet JM, Gervais P, Cell inactivation and membrane damage after long-term treatments at sub-zero temperature in the supercooled and frozen states. *Biotechnol Bioeng*, 2008, 101(6): 1245–1255. doi: 10.1002/bit.21981

Müller FM, Werner KE, Kasai M, Francesconi A, Chanock SJ, Walsh TJ Rapid extraction of genomic DNA from medically important yeasts and filamentous fungi by high-speed cell disruption. *J Clin Microbiol*, 1998, 36(6): 1625–1629.

Park JI, Grant CM, Attfield PV, Dawes IW, The freeze-thaw stress response of the yeast *Saccharomyces cerevisiae* is growth phase specific and is controlled by nutritional state via the ras-cyclic amp signal transduction pathway. *Appl Environ Microbiol*, 1997, 63(10): 3818–3824.

Paterson AH, Brubaker CL, Wendel JF, A rapid method for extraction of cotton (*Gossypium* spp.) genomic DNA suitable for RFLP or PCR analysis. Plant Mol Biol Rep, 1993, 11(2):122–127.

Pruvost M, Geigl EM Real-time quantitative PCR to assess the authenticity of ancient DNA amplification. *J Archaeol Sci*, 2004, 31: 1191–1197. doi:10.1016/j.jas.2002.05.002

Sharma S, Szele Z, Schilling R, Munch JC, Schloter M, Influence of freeze-thaw stress on the structure and function of microbial communities and denitrifying populations in soil. *Appl Environ Microbiol*, 2006, 72(3): 2148–2154. doi:10.1128/AEM.72.3.2148-2154.2006

Received: June 23, 2010;
Revised: December 15, 2010;
Accepted: January 17, 2012

PÁGINA
EM
BRANCO