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Short communication

Identification of fungi from dairy products by means of 18S rRNA analysis

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Abstract

The role of fungi as cause of spoilage of dairy products, such as cheese and yoghurt, has been clearly demonstrated. Despite of this, there is still a lack in rapid methods for the identification of food-associated fungi. In the course of the present work, molecular taxonomical techniques were developed and used to identify yeasts involved in the spoilage of yoghurt and moulds responsible for spoilage of vacuum-packaged hard cheese. Three methods for DNA extraction and purification were evaluated and the fungus-specific primers TR1 and TR2 were used to amplify a 581-bp fragment within the gene, coding for the small ribosomal subunit (18S rRNA) of fungi. The 18S rRNA sequence analysis of fungi isolated from yoghurt and packaged cheese allowed to identify yeast belonging to *Zygosaccharomyces microellipsoides* and moulds belonging to *Penicillium chrysogenum* and *Cladosporium cladosporoides*. © 2001 Elsevier Science B.V. All rights reserved.

Keywords: Fungi; 18S rRNA; Cheese; Yoghurt

1. Introduction

Fungi are among the leading cause of spoilage of dairy products: yeasts carried by fruits are responsible for sweetened fruit yoghurt spoilage, inducing the alteration of the sensorial properties of this fermented product (Spillmann and Geiges, 1983). Moreover, growth of moulds is a defect which occurs sporadically on surface of hard cheese and packaged cheese (Hocking and Faedo, 1992).

In recent years much interest was devoted, and major progress have been made towards the prevention of spoilage caused by moulds. In this regard, it has been useful that international agreements on taxonomy and analytical methods for food-borne moulds have led to the conclusion that a very limited fungi species are responsible for the spoilage of a particular food (Filtenborg et al., 1996). However, the identification of fungi associated with the spoilage of dairy products has been hampered by the lack of a rapid system for the identification of these microorganisms.

Over recent years, the study of small subunits of ribosomal RNA has revolutionised the classification of microorganisms, both bacteria and fungi. These techniques, based on the PCR amplification of genes coding for rRNAs and sequence comparison, offer a new tool for the identification and study of food-associated fungi (James et al., 1994). Rapid identification of filamentous fungi was reached using two specific PCR primers sets (Bock et al., 1994; Pedersen et al., 1997; Turenne et al., 1999).

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In the course of the present work, molecular taxonomical techniques were developed and used to identify yeasts involved in the spoilage of yoghurt and moulds responsible for spoilage of vacuum-packaged hard cheese, by amplification and sequencing of the 18S rRNA gene.

2. Materials and methods

2.1. Isolation and identification of fungi associated with the spoilage of yoghurt and packaged Grana cheese

The cultures studied in this work were isolated from spoiled fruit yoghurt and from vacuum-packaged Grana cheese, as follows: samples (1 g) were homogenized in 1% Na-citrate, serially diluted in peptone water and plated onto Rose–Bengal chloramphenicol agar (Oxoid, Basingstoke, UK). Randomly selected colonies were streaked twice onto Wort Agar Plates (Oxoid) at 30 °C and pure colonies were used for further work.

2.2. DNA extraction

Three methods were applied in order to obtain chromosomal DNA extraction and purification from fungal cultures. Six colonies of yeasts and six of moulds were collected.

2.2.1. Extraction 1

Genomic DNA was directly isolated from the single colonies grown on the agar plates, resuspended in 10 μl of distilled water; 2 μl were added with 13 μl of resin Gene Releaser (Bioventures, TN, USA) into a PCR tube, mixed for 10–30 s and overlaid with 50 μl of mineral oil. The Cell lysis was achieved directly in the amplification tube by a microwave oven treatment, at 700 W for 10 min.

2.2.2. Extraction 2

The colonies grown on agar plates were recovered from the surface and placed into 1 ml of sterilised water and frozen in liquid nitrogen. The cells were mechanically disrupted using an ultra-turrax T25 apparatus (IKA-LABORTECHNIK, Janke and Kunkel, Staufen, D) in 1.5 ml tubes. The DNA was purified from the cell debris by Wizard Minipreps columns (Promega, Madison, USA), following the manufacturer's instruction.

2.2.3. Extraction 3

The cells were recovered and disrupted as described above in Extraction 2. The extraction and purification of DNA were performed as reported from Dellaporta (1994) and modified as follows: after the addition of potassium acetate (5 M) to precipitate proteins and polysaccharides, the supernatant was filtered on paper filter Whatman no. 4 (Maidstone, UK) and one volume of isopropanol was added to the tube and mixed. To recover the DNA, centrifugation at $12{,}000 \times g$ at 4 °C was performed, the supernatant discarded and the DNA dissolved by 70 μl Tris–HCl 50 mM pH 8, EDTA 100 mM pH 8. After phenol, chloroform and isoamyl alcohol (25:24:1) purification, the upper phase was transferred in a fresh tube and added with 1/10 volume of sodium acetate 3 M and 0.7 volume of isopropanol. After a brief centrifugation at $12{,}000 \times g$ for 30 s, the supernatant was carefully removed and the pellet of the DNA was dissolved in 40 μl of Tris–HCl 50 mM pH 8, EDTA 100 mM, pH 8.

2.3. PCR amplification and sequencing of 18S rDNA

The fungus-specific primers TR1 5′-GTTTCTAGGACCGCCGTA-3′ (position 834) and TR2 5′-CTCAAACTTCCATCGACTTG-3′ (position 1415) (Bock et al. 1994) (primers purchased Primm—s.r.l. Milan, I) were used to amplify a 581-bp fragment within the gene coding for the small ribosomal subunit (18S rRNA) of fungi. Amplification was performed in a Thermal Cycler Gene Amp PCR System 9700 (PE Applied Biosystems, Norwalk, USA). Two thermal amplification cycles were used to amplify the fungal 18S rDNA. The parameter of cycle 1 was as follows: an initial denaturation step of 96 °C for 10 min, followed by 30 cycles of 96 °C for 1 min, annealing at 46 °C for 1 min and extension at 72 °C for 2 min, with a final extension step of 72 °C for 5 min. Cycle 2 was composed by a denaturation step of 95 °C for 3 min followed by 35 cycles of 95 °C for 1 min (denaturation), 53 °C for 1 min (annealing), 72 °C for 2 min (extension) and final extension of 72 °C for 3 min. The amplification products were verified by electrophoresis in 0.8% w/v agarose gel (LE

agarose, Rockland, USA) and DNA stained by ethidium bromide.

The sequences of 18S rDNA were obtained using an ABI PRISM™ dye terminator cycle sequencing kit (PE Biosystem) and the primer TR2. The reaction products were analysed using an Applied Biosystem 373A automated DNA sequencer (Perkin Elmer).

2.4. Data analysis

The software package of the University of Wisconsin Genetic Group was used for the analysis and comparison of DNA sequences comparing the 18S rRNA sequences of fungal isolates, with the sequences present in the small subunit database (SSU-Prok) of Ribosomal Database Project (Maidak et al., 2000).

3. Results and discussion

Since the aim of this work was the application of molecular taxonomy techniques for the identification of food-associated fungi, the first step was the development of a fast and reliable technique for isolation of DNA from yeast and mould cultures. To verify the efficiency of the applied method, the extracted DNA was separated by agarose gel electrophoresis (Fig. 1). Both methods 2 and 3 gave good results in terms of quantity and purity. Moreover, a reliable reproducibility was achieved by methods 2 and 3, while use of protocol 1 did not give a satisfying DNA purification, as shown in Fig. 1, either from yeasts or moulds.

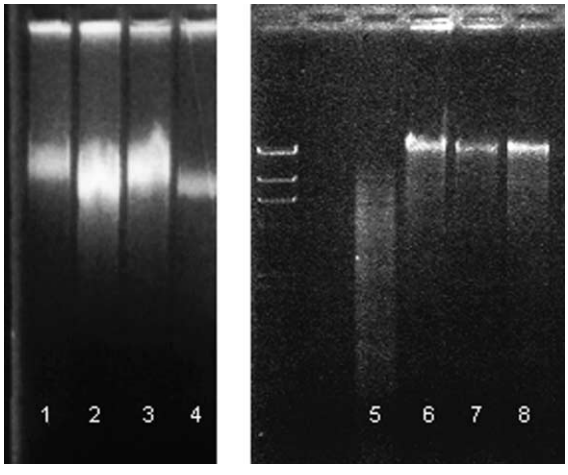

Fig. 1. Lanes 1, 2, 3, 4 and 6, 7, 8 show DNA purified using extractions 2 and 3, respectively; lane 5 shows DNA purified using extraction 1 (for details of extraction methods, see Materials and methods).

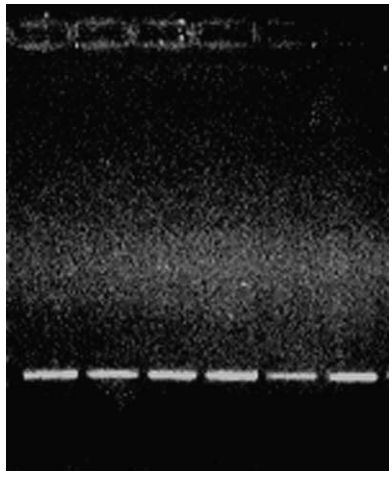

Fig. 2. 18S rDNA obtained using TR1 and TR2 primers and cycle 2 to amplify the template DNA purified by extraction 2 (for details on extraction 2 and cycle 2, see Materials and methods).

To evaluate the influence of the PCR method on the amplification of 18S rDNA, the samples from extraction 1, 2 and 3 were subjected to two different amplification cycles 1 and 2. Both cycles 1 and 2 gave amplified products. The use in cycle 2 of a higher annealing temperature (53 °C) increased the specificity of 18S rDNA amplification, reducing the non-specific reaction products generated in cycle 1 as reported in Fig. 2.

The six strains of yeasts and moulds, respectively, were identified by means of a sequence analysis of at least 400 bp of the region 1000–1410 of the 18S rRNA gene. The results obtained by the analysis of the partial sequence of the six yeast strains, isolated from spoiled fruit yoghurt, showed the same sequence, suggesting that they belong to the same species. The isolates were identified as *Zygosaccharomyces microellipsoides* species (Maidak et al., 2000).

The sequence analysis of the six mould strains isolated from spoiled vacuum-packaged Grana cheese revealed that two different species were present; two belonging to *Penicillium chrysogenum* and four to *Cladosporium cladosporoides* (Maidak et al., 2000).

The identification and classification of fungus have traditionally been based on morphological, physiological, and biochemical traits. In recent years, different molecular biology techniques have been developed for yeast and mould identification. In the present study, a molecular approach has been chosen to study the spoilage fungi in dairy products.

Z. microellipsoides is an important food spoilage yeast and its presence in non-pasteurised fruits and fruit concentrates, purees, syrups and juices (Andrews et al., 1997) is a possible source of spoilage when these products are used as ingredients in milk products, as the yoghurt analysed in the present study.

Moreover, *P. chrysogenum* and *C. cladosporoides* are components of the surface microbiota of cheese during the final stages of cheese ripening and are responsible for "thread mould" defect that occurs sporadically in vacuum-packaged cheese (Hocking and Faedo, 1992).

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